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Neuronatin is related to keratinocyte differentiation by up-regulating involucrin

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ABSTRACT

Background: Neuronatin (Nnat), which is a neuronal developmental and differentiation molecule, is expressed in the endoplasmic reticulum of non-neuronal cells and is involved in insulin secretion from pancreatic β-cells by plausibly modulating their intracellular calcium concentration. However, the role of Nnat in keratinocyte differentiation remains unclear.
Objective: To unveil a possible integration of Nnat in controlling the keratinocyte differentiation markers such as involucrin, cytokeratin1, filaggrin, loricrin and S100A7.
Methods: Immunohistological staining was done using psoriasis, chronic eczema, lichen planus and normal skin. Immunofluorescence staining, Western blotting and semi-quantitative real-time PCR were performed for detecting Nnat, involucrin, cytokeratin1, filaggrin, loricrin and S100A7 using human keratinocytes with or without Nnat gene transfection. Small interference RNA was applied to knockdown the Nnat gene expression.
Results: Nnat existed in normal human epidermis and cultured keratinocytes. In the hyperplastic epidermis of psoriasis, chronic eczema and lichen planus, over-expression of Nnat was evident along with involucrin and cytokeratin1 expression. Coordinate up-regulation of Nnat and involucrin, but not cytokeratin1, was demonstrated in cultured keratinocytes under differentiation stimuli such as extracellular calcium elevation, exposure to phorbol myristate acetate, and increased cell density. Transfection of small intereference RNA for Nnat decreased the mRNA levels of Nnat and involucrin, but not of cytokeratin1. Furthermore, a gene transfection assay showed increased involucrin expression in the Nnat-transfected keratinocytes than in mock-transfected counterparts, without any appreciable influence on cytokeratin1, filaggrin, loricrin and S100A7 expression.
Conclusion: These data indicate that Nnat is related to keratinocyte differentiation by up-regulating involucrin expression.

1. Introduction

Neuronatin (Nnat) was first identified as a gene that is predominantly expressed in the neonatal rat brain. The expression of Nnat becomes prominent during late gestation in the central and peripheral nervous systems, primarily in post-mitotic and differentiating neuroepithelial cells [1]. In the brains of adult rats, Nnat expression is markedly down-regulated [2,3], which suggests its involvement in neuronal cell development and differentiation.

In addition to the neuronal expression of Nnat, it is also expressed in non-neuronal tissues such as the pituitary glands, lung, adrenal glands, uterus, skeletal muscles, ovaries, and pancreas [4,5]. Nnat is expressed in pancreatic β-cells and is possibly involved in ion-channel transport or channel modulation [6,7]. Nnat and insulin are co-localized in the cytoplasm of pancreatic β-cells, and knockdown of Nnat dramatically decreases insulin secretion after glucose challenge [6,7]. Additional data from pancreatic cells and 3T3-L1 cells showed that Nnat resides in the

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endoplasmic reticulum and modulates intracellular Ca^{2+} stores [5–7].

Interestingly, our previous immunohistological studies have showed that human keratinocytes as well as cutaneous nerves are target tissues for Nnat expression [8,9]. Keratinocytes are the major cell type of the multilayered stratified squamous epidermis that covers the body surface. To establish the epidermal structure and skin barrier, keratinocytes undergo fine-tuned differentiation from basal to spinous and granular layers, thereby resulting in the formation of a cornified layer. The accumulation of involucrin (Inv) and cytokeratin1 (CK1) is a well-characterized hallmark of epidermal differentiation [10,11]. Although the expression of Nnat is evident in keratinocytes that are located in the basal to spinous layers of the epidermis, sweat glands, and sebaceous glands [10], the role of Nnat in epidermal differentiation has not been clarified. This study, to our knowledge, is the first to elucidate that (1) cultured keratinocytes indeed express Nnat, (2) Nnat is over-expressed in the acanthotic epidermis of psoriasis, chronic eczema and lichen planus, and (3) Nnat is related to keratinocyte differentiation by up-regulating Inv expression.

2. Materials and methods

2.1. Reagents and antibodies

Phorbol myristate acetate (PMA), calcium, and anti-Inv mouse IgG antibody (SY5) were purchased from Sigma–Aldrich Chemical (St. Louis, MO). Anti-Nnat rabbit IgG antibody and anti-CK1 mouse IgG antibody (AE1) were purchased from Abcam (Cambridge, MA). Anti-GAPDH rabbit IgG antibody (FL-335), normal rabbit IgG, and normal mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

Normal human epidermal keratinocytes (NHEKs), which were obtained from Clonetics-BioWhittaker (San Diego, CA), were grown in culture dishes at 37 °C in a 5% CO_2 atmosphere. The NHEKs were cultured in serum-free keratinocyte growth medium (Lonza, Walkersville, MD) that was supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine. The culture medium was replaced every 2 d. At near confluence (70–90%), the cells were disaggregated with 0.25% trypsin/0.01% ethylenediamine tetra-acetic acid and passaged. Second-to-fourth-passage NHEKs were used in all of the experiments.

2.3. Immunofluorescence and confocal laser-scanning microscopy analysis

NHEKs that were cultured on slides were washed with PBS, fixed with acetone for 10 min, and blocked by using 10% BSA in PBS for 30 min. Samples were incubated with primary rabbit anti-Nnat (1:50), anti-Inv (1:1000), and/or anti-CK1 (1:200) antibody in PBS overnight at 4 °C. The slides were washed with PBS before incubation with anti-rabbit (Alexa Fluor 546) or anti-mouse (Alexa Fluor 488) (Molecular Probes, Eugene, OR) secondary antibody for 1.5 h at room temperature. The slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). All of the samples were analyzed by using a D-Eclipse confocal laser-scanning microscope (Nikon, Tokyo, Japan).

2.4. Immunohistochemical analysis

All formalin-fixed and paraffin-embedded tissues (10 psoriasis, 10 chronic eczema, 10 lichen planus and 10 normal skin) were obtained from the archives of the Department of Dermatology of Kyushu University Hospital, Japan. Several patients were associated with metabolic syndromes; 2 hypertension, 1 diabetes and 1 coronary heart disease in psoriasis; 1 hypertension, 1 diabetes and 1 coronary heart disease in chronic eczema; and 1 hypertension in lichen planus.

The paraffin-embedded tissues were evaluated via hematoxylin and eosin staining. The paraffin-embedded tissue blocks were cut into 4-μm thick tissue sections. The sections were de-paraffinized by using xylene for 10 min, rehydrated through a graded ethanol series, and then blocked for endogenous peroxidase activity in 0.3% H_2O_2 in methanol for 30 min. Antibody-binding epitopes were retrieved by pressure-cooking the tissue sections in 10 mM sodium citrate buffer at pH 7.0 (Yatoron, Tokyo, Japan) for 10 min, and nonspecific binding was blocked by using 10% goat serum. The sections were then incubated with anti-Nnat, anti-Inv or anti-CK1 (Zymed Lab Inc., San Francisco, CA) antibody overnight at 4 °C. Immunodetection was carried out by employing the avidin–biotin horseradish peroxidase method by using 3,3′-diaminobenzidine as the chromogen followed by light counterstaining with hematoxylin. Washes with Tris-buffered saline or phosphate-buffered saline were performed between each step according to the manufacturer's protocols. Appropriate positive and negative controls were included in each assay.

2.5. Reverse transcription-PCR and semi-quantitative real-time PCR analysis

Total RNA was extracted by using an RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription was performed by using a PrimeScript RT-PCR kit (Takara Bio, Shiga, Japan). Amplification was started at 95 °C for 10 s as the first step, followed by 35 cycles of PCR at 95 °C for 5 s, and at 60 °C for 20 s. The PCR products were electrophoresed through a 1.5% agarose gel and densitometric analysis was performed by using ImageJ software (NIH). ImageJ is a public domain, Java-based image processing program that was developed at the National Institutes of Health (NIH; Bethesda, MD). The primers from Takara Bio were as follows:

hNNAT-FW: 5′-CTCGGCTGAACTGCTCATCATC-3′
hNNAT-RV: 5′-TTCTCGCAATGGGCTGTGTC-3′
hGAPDH-FW: 5′-GCACCGTCAAGGCTGAGAAC-3′
hGAPDH-RV: 5′-TGGTGAAGACGCCAGTGGA-3′
hINV-FW: 5′-CTGCCTCAGCCTTACTGTGA-3′
hINV-RV: 5′-TGGGTATTGACTGGAGGAGG-3′
hCK1-FW: 5′-ACAAGAAGTCACTATCAACCAGAG-3′
hCK1-RV: 5′-GCTCCAGGAACCTCACCTTG-3′
hFILAGGRIN-FW: 5′-TTTCGGCAAATCCTGAAGAATCC-3′
hFILAGGRIN-RV: 5′-ACTGTGCTTTCTGTGCTTGTG-3′
hLORICRIN-FW: 5′-GCACCGATGGGCTTAGAG-3′
hLORICRIN-RV: 5′-AGAAACCAAAGAGGCTAAACAG-3′
hS100A7-FW: 5′-ATGTCTCCCAGCAAGGACAG-3′
hS100A7-RV: 5′-TGCTGACGATGAAGGAG-3′

2.6. Western blotting analysis

NHEKs, treated with or without PMA or different concentrations of Ca^{2+} for 48 h, were incubated with lysis buffer (Complete Lysis-M, Roche Applied Science, Indianapolis, IN). The lysate protein concentration was measured by using a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were dissolved in NuPage LDS Sample Buffer (Invitrogen) and 10% NuPage Sample Reducing Agent (Invitrogen). The lysates were boiled at 70 °C for 10 min and then loaded and run on 4–12% NuPage Bis-Tris Gels (Invitrogen) at 200 V for 40 min. The proteins