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Expression of Neutrophil Gelatinase-Associated Lipocalin in Calcium-Induced Keratinocyte Differentiation

In a previous search for the differentially expressed genes in keratinocyte differentiation, we identified neutrophil gelatinase-associated lipocalin (NGAL) as a calcium-induced gene. In this study, we further verified the expression of NGAL in cultured keratinocytes as well as in several skin diseases. Reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and ELISA clearly showed that NGAL expression was markedly increased in calcium-induced keratinocyte differentiation in vitro. However, in our previous report, NGAL expression was not detected in normal skin tissue except for hair follicle by in situ hybridization and immunohistochemistry, indicating the difference of cell status between in vitro and in vitro conditions. Interestingly, NGAL expression was highly increased in psoriasis-like inflammatory disorders (lichen planus and pityriasis rubura pilaris) and skin cancers (keratoacanthoma and squamous cell carcinoma), implying that NGAL may be related with the epidermal hyperplasia. Collectively, these results reveal the potential importance of NGAL in the maintenance of skin homeostasis.

Key Words : *LCN2 Protein, Human; Keratinocytes*

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INTRODUCTION

Lipocalins are small extracellular proteins that share a common cup-shaped structure consisting of an eight-stranded, antiparallel, continuously hydrogen-bonded β-barrel (1, 2). They provide the binding site for a range of small hydrophobic molecules as well as soluble macromolecules. Eventually, the lipocalin proteins affect the transport of a variety of small lipophilic molecules, functioning as the homeostatic regulators, immunomodulators, retinol transporters, and prostaglandin producers (3-5). Neutrophil gelatinase-associated lipocalin (NGAL) is one of the lipocalin family proteins, which was originally identified as a 25-kDa protein covalently associated with the 92-kDa neurophil gelatinase (matrix metalloproteinase-9 [MMP-9]) (6, 7). NGAL is capable of protecting MMP-9 from degradation, thereby preserving MMP-9 enzymatic activity (8). However, NGAL can also be purified in both a monomeric and dimeric form without gelatinase, and is mainly exocytosed from neutrophils in forms not complexed with MMP-9, indicating that both NGAL and gelatinase exist predominantly in forms not associated with each other (6, 9). Evidence shows that NGAL binds to a variety of bacterial ferric siderophores that transport iron to bacteria. By taking the iron away from bacteria, NGAL acts as a potent bacteriostatic agent under iron-limited conditions (10). Furthermore, NGAL delivers iron to kidney cells where it activates or represses iron-responsive genes, thereby playing an important role in mesenchymal-epithelial transition during development of the mammalian nephron (11).

The epidermis is a stratified tissue that is composed of progenitor cells in the basal layer, differentiating keratinocytes in the suprabasal layers, and dead, fully-differentiated cells on the surface. These phenotypically and functionally different cells are formed by the well-programmed gene expression changes during the terminal differentiation process (12-14). Although a number of genes required for keratinocyte differentiation have been already discovered, it is likely that many important molecules remain undisclosed. In an effort to identify the genes related to keratinocyte differentiation, we previously performed suppression subtractive hybridization (SSH) using a calcium-induced keratinocyte differentiation model. Among the genes obtained, NGAL showed marked induction by calcium treatment in cultured keratinocytes (15). Thus, in this study, we attempted to further verify the expression of NGAL in cultured keratinocytes as well as in several skin diseases.

MATERIALS AND METHODS

Skin samples

All skin samples were obtained with given written informed consent of donors, in accordance with the ethical committee approval process of Chungnam National University Hospital. The study was conducted according to Declaration of Helsinki Principles.

Cell culture

Skin specimens were briefly sterilized in 70% ethanol, minced, and then treated with dispase for overnight at 4℃. The epidermis was separated and placed in a solution containing 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Gibco BRL, Rockville, MD, U.S.A.) for 15 min at 37℃. After vigorous pipetting, cells were pelleted and resuspended in keratinocyte-serum free medium (K-SFM) supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Gibco BRL). Cells were grown in collagen-coated dishes at 37℃, 5% CO_2 atmosphere. At the third passage, cells were switched to the same medium containing 1.2 mM calcium and cultured for 1, 3, 7, and 14 day.

Reverse transcription-polymerase chain reaction (RT-PCR)

Two micrograms of total RNAs were reverse transcribed with M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.). Aliquots of RT mixture were subject to PCR cycles with specific primer set for NGAL (5'-CAATTCCAGGGGAAGTGGTA and 5'-GCCTGAGGGCACATGTTTAT) as follows: 94℃ for 30 sec, 58℃ for 30 sec, 72℃ for 1 min for 30 cycles.

Western blot analysis

The cell and tissue extracts were prepared using Pro-prep protein extraction solution (Intron, Daejeon, Korea). Protein samples were run on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred onto nitrocellulose membrane and incubated with anti-human NGAL polyclonal antibody. For an internal control, anti-actin antibody (Sigma, St Louis, MO, U.S.A.) was used. Blots were then incubated with peroxidase-conjugated secondary antibody and developed by enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.).

Enzyme-linked immunosorbent assay (ELISA)

The level of NGAL in conditioned medium was quantified according to the method previously reported (16). Cells received fresh medium daily, and NGAL secreted into the medium was detected at the indicated time points. Measurements were repeated at least three times, with independent cell batches obtained from three different donors.

Zymography

The gelatinase activity was assayed according to a minor modification of the method reported previously (8). Samples of conditioned medium (40 μL) were run on 10% SDS-polyacrylamide gel containing 0.1% gelatin (Sigma), under non-reducing condition. Following electrophoresis, SDS was removed by washing with 2.5% Triton X-100 for 90 min at room temperature. Substrate digestion was carried out by incubating the gel in 50 mM Tris, pH 7.6, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, for 24 hr at 37℃. The gel was visualized by staining with 0.25% Coomassie Blue solution (Sigma).

Immnohistochemistry

Paraffin sections of skin specimens were dewaxed, rehydrated, then washed three times with phosphate-buffered saline (PBS). After treatment with proteinase K for 5 min at 37℃, sections were treated with H_2O_2 for 10 min at room temperature, blocked in 0.1% Tween-20, 1% bovine serum albumin in PBS for 20 min, and followed by reaction with anti-NGAL antibody for 1 hr. Sections were incubated sequentially with peroxidase-conjugated secondary antibody and visualized with Chemmate envision detection kit (Dako, Carpinteria, CA, U.S.A.). The list of skin diseases determined in this study are as follows; lichen planus (LP), pityriasis rubura pilaris (PRP), pityriasis rosea (PR), keratoacanthoma (KA), squamous cell carcinoma (SCC), and basal cell carcinoma (BCC). At least three samples of each disease were analyzed, with similar results. All SCCs tested in this study were in the state of well-differentiated.

Statistical analysis

Data were analyzed statistically using Student's t-test or one-way analysis of variance followed by Fisher's least significant difference test for a *post hoc* comparison. The statistical significance was set at $p<0.01$.

RESULTS

We previously identified NGAL as a calcium-induced gene in cultured keratinocytes by the SSH technique (15). In this study, we attempted to further verify whether the expression of NGAL was related to the calcium-induced keratinocyte differentiation in vitro. For this end, cultured normal human epidermal keratinocytes (NHEK) were treated with 1.2 mM calcium for the indicated time points, then the expression of NGAL was investigated using RT-PCR and Western blot analysis. As shown in Fig. 1A, NGAL mRNA was highly

increased at 7 and 14 day after treatment with calcium of keratinocytes. Western blot analysis also showed that NGAL protein was remarkably increased by calcium treatment in a time-dependent manner (Fig. 1B). Since NGAL is a well-known secretory protein, we determined the secreted NGAL by a specific ELISA method developed by Kjeldsen et al. (16). Consistent with the Western blot data, the amount of NGAL in conditioned medium was highly increased at the late time points after calcium treatment (Fig. 2A). Next, we determined the MMP-9 activity in conditioned medium, as the previous report indicated that over-expression of NGAL in human breast carcinoma cells leads to the protection of MMP-9 thereby increasing its activity in conditioned medium (8). However, the MMP-9 activity in our system did not show significant changes (Fig. 2B), suggesting that the increased NGAL is not associated with the MMP-9 activity in calcium-treated keratinocytes. This result is consistent with the data by Kjeldsen et al. (9), which showed that NGAL can be also purified

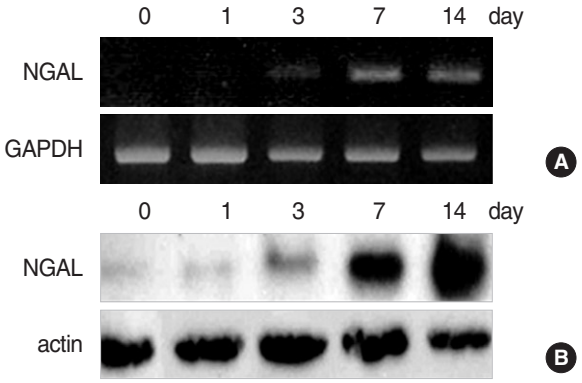

Fig. 1. Expression of NGAL in normal human epidermal keratinocytes cultured in vitro. (A) RT-PCR analysis. Cells were differentiated by addition of 1.2 mM calcium for the indicated time points. Two micrograms of total RNAs were reverse transcribed with M-MLV reverse transcriptase and used for PCR amplification. GAPDH was used as an internal control. (B) Immunoblot analysis. Cellular proteins (20 μg per lane) were separated on duplicate 15% polyacrylamide gels, and transferred onto nitrocellulose membranes. Each membrane was reacted with anti-NGAL antibody, and anti-actin antibody as a loading control, respectively.

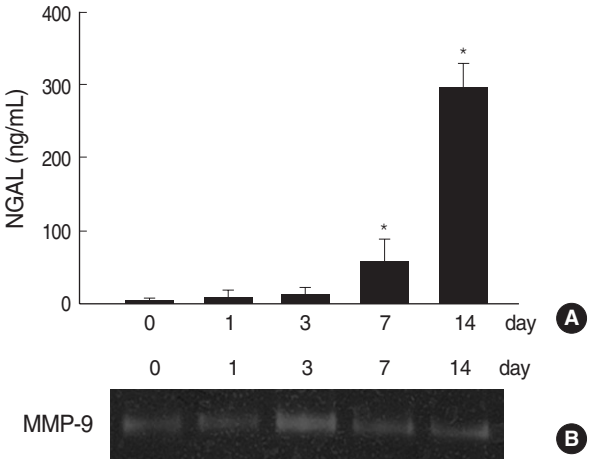

Fig. 2. (A) Cells received fresh medium daily and conditioned medium were prepared. The amount of NGAL was measured by a specific ELISA method. The results are shown as mean values ±SEM of triplicate measurements (*p<0.01 vs. control). (B) MMP-9 activity in conditioned medium. Samples of conditioned medium were run on 10% SDS-polyacrylamide gel containing 0.1% gelatin, under a non-reducing condition. After substrate digestion, the MMP-9 activity was visualized by Coomassie staining. The bands migrated to a location corresponding to a molecular mass of 92-kDa.

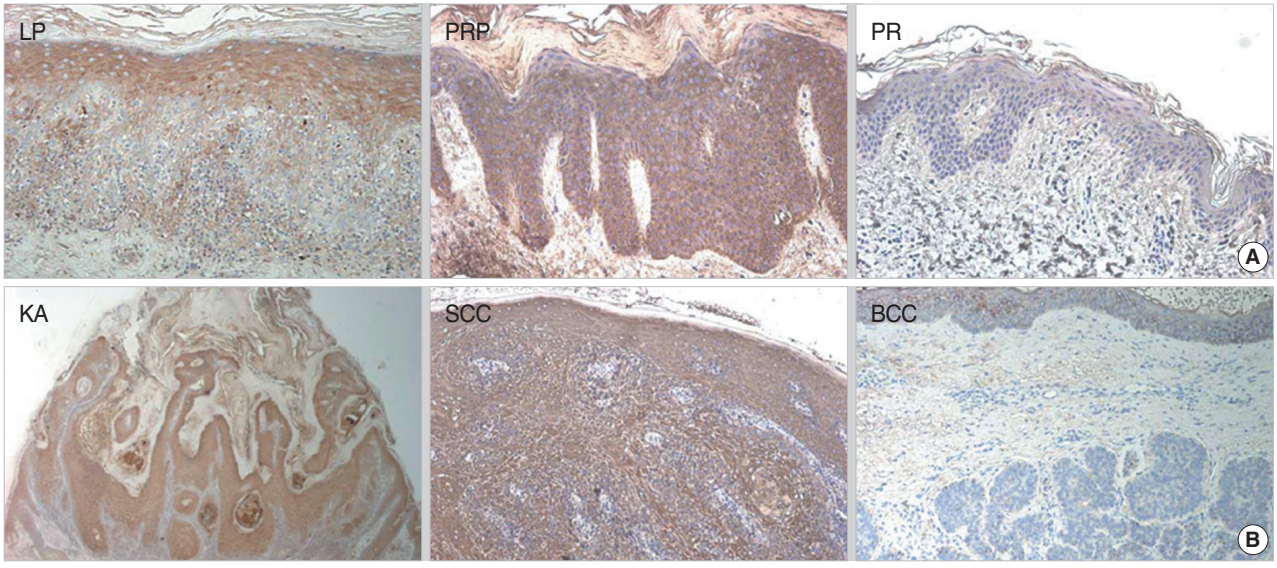

Fig. 3. Immunohistochemistry analysis of NGAL expression in several skin diseases. Paraffin-embedded tissue sections of skin specimens were stained with anti-NGAL antibody. (A) Inflammatory disorders. LP, lichen planus; PRP, pityriasis rubura pilaris; PR, pityriasis rosea. (B) Skin cancers. KA, keratoacanthoma; SCC, squamous cell carcinoma; BCC, basal cell carcinoma.

in both a monomeric and dimeric form without gelatinase and is mainly exocytosed from neutrophils in forms not complexed with MMP-9.

In this study, we attempted to further investigate the expression of NGAL in several skin diseases. These include psoriasis-like inflammatory disorders (LP, PRP, and PR) and skin cancers (KA, SCC, and BCC). As shown in Fig. 3A, NGAL expression was detected at the upper granular layer of LP and all layers of PRP, but not in PR. These results suggest that NGAL expression reflects the different status of inflammation, and may be related with the epidermal hyperplasia. In addition, NGAL expression was highly increased in KA and SCC, but not in BCC (Fig. 3B), suggesting that the onset of keratinocyte differentiation may be one trigger for high induction of NGAL expression.

DISCUSSION

In this study, we showed that NGAL expression is highly increased in calcium-induced keratinocyte differentiation in vitro, and in several skin diseases such as psoriasis-like inflammatory disorders (LP and PRP) and skin cancers (KA and SCC). A previous report indicates that NGAL expression is related with the dysregulated keratinocyte differentiation. For example, strong induction of NGAL in the epidermis was seen in psoriasis, PRP, and SCC (17), well consistent with our results. In addition, NGAL is highly expressed in well-differentiated SCC of the lung, implicating that NGAL is linked to either keratin synthesis or terminal differentiation (18). However, in our previous study, NGAL expression was not detected in atopic dermatitis, another important skin disease characterized by disturbed terminal differentiation, suggesting that increased NGAL is not tightly associated with dysregulated epithelial differentiation, but rather relates with the different status of abnormal keratinocytes disease by disease (19).

Although NGAL was originally identified from human neutrophils, it is now clear that NGAL is expressed in a variety of normal and pathological human tissues including stomach, liver, kidney, colon, and skin (5, 18, 20, 21). However, the role of NGAL in several pathologic statuses largely remains to be elucidated. Considering the potential of NGAL-binding to siderophores, thereby facilitating the iron delivery into cells (11), it could not be ruled out the possibility that NGAL-mediated iron delivery may have direct impact on keratinocyte proliferation and hyperplasia. It has been known that most cells acquire iron by receptor-mediated endocytosis of iron-loaded transferring (22). Interestingly, transferrin receptor expression is correlated with the proliferation rate, being increased by growth stimulation or decreased by induction of terminal differentiation (23). Given that NGAL exerts its role as an iron transporter in the epidermis, one possible explanation for high expression in several hyperplastic and/or hyperproliferative diseases is that it may supplement iron to the upper layer of stratified epithelium. That is, keratinocytes would require more iron delivered by transporters during the hyperproliferation, since the quantity of iron delivered by transferrin is probably insufficient to support cell proliferation. The precise relationship between iron supply and NGAL expression in epidermis, however, remains to be elucidated.

In summary, we showed that NGAL expression was increased in calcium-induced keratinocyte differentiation, and in several hyperplastic and/or hyperproliferative skin diseases, suggesting that NGAL may have a role in the maintenance of skin homeostasis.

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