Regulation of serine protease inhibitor Kazal type-5 (SPINK5) gene expression in the keratinocytes

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Abstract

Objectives Serine protease inhibitor Kazal type-5 (SPINK5) plays a crucial role in deciding the timing of desquamation of the skin. Its gene expression is limited at the very surface of the stratum granulosum (SG), whereas expression of kallikreins (KLKs) encoding proteases is usually found throughout the stratum spinosum (SS) and SG.

Methods In order to explore the difference in expression regulation of these proteases/inhibitor, the function of SPINK5 promoter was examined using Luciferase assay.

Results Luciferase assay targeting the SPINK5 promoters (nucleotide -676/-532 and -318/-146 from the major transcription start site) showed high intensity in NHEK human keratinocyte. These two sites had neither common cis elements nor GATA3 element but electrophoretic mobility shift assay showed similar retardation bands. Moreover, DNA footprinting did not display specific protected bands. Thus, we could not identify cis-element(s) that controlled these elements. Differentiation induced by high Ca2+ medium failed to alter their luciferase activities. Transfection of GATA3 expressing vector significantly but slightly increased them and that of vector expressing its dominant negative form decreased.

Conclusions Although GATA3 is reportedly important for inhibition of proliferation and induction of differentiation of keratinocytes, its effect on SPINK5 expression was indirect and GATA3 alone was insufficient for final differentiation of keratinocytes where full SPINK5 expression was observed.
Introduction

To maintain a steady number of the stratum corneum (SC) layers is an important factor for the solid skin barrier function. In the SC adjacent to the stratum granulosum (SG), corneocytes are tightly bound to each other through the corneodesmosomes consisted of extracellular corneodesmosin, transmembrane desmoglein-1 and desmocollin-1, and cytoplasmic desmosomal plaque proteins (plakoglobin) [1, 2]. These firm contacts reduce toward the surface and finally disappear on the surface where desquamation of the corneocytes occurs [3]. This reduction is due to corneodesmosome degradation caused by serine protease activities supplied by the tissue kallikrein gene (KLK) family proteins consisting of fifteen members known as KLK1 to KLK15 [4]. Their activity is regulated by serine protease inhibitor Kazal type-5 (SPINK5) [5, 6] in order to match the shedding schedule to the proliferation rate of keratinocytes [7, 8]. This turnover is very steady and maintained regardless of age [9, 10] on behalf of serine protease-SPINK5 balance. In fact, the loss of function mutation of SPINK5 gene causes hyperdesquamation and loss of the SC known as Netherton syndrome [11-13].

Thus, KLK and SPINK5 proteins co-localize and interact each other at the SC [14]. Nonetheless, expression of KLKs is usually found throughout the stratum spinosum (SS) and SG [5] and, on the other hand, expression of SPINK5 is limited at the very surface of SG [12]. For the ubiquitous expression of KLKs, GATA3 plays a very important role [15], whereas the major regulators of local expression of SPINK5 are yet known. Limited expression of SPINK5 plays a crucial role on avoidance of premature-and/or hyper-desquamation. In order to clarify the basis of such limited expression of
SPINK5, the function of its promoter was explored in the present study.

**Materials and methods**

**Keratinocyte culture**

Normal human keratinocyte (NHEK; Kurabo, Osaka, Japan, several different single donors) was inoculated at a concentration of 2,500 cells/cm\(^2\) in culture media, HuMedia-KG2 (Kurabo) supplemented with insulin 10 µg/ml, human epidermal growth factor 0.1 ng/ml, hydrocortisone 0.5 µg/ml, bovine pituitary extract (BPE) 0.4% v/v, gentamycin 50 µg/ml and amphotericin B 50 ng/ml. The culture was maintained at 37°C in a 95% O2/5% CO2 humidified chamber. The cells were subcultured twice in the media of which calcium concentration was as low as 0.15 mM in order to avoid differentiation. In vivo differentiation was induced as described elsewhere [16]. Briefly, at the second passage when it became 40-60% confluent, the media was changed to normal Ca\(^{2+}\) medium (0.15 mM Ca\(^{2+}\); low Ca\(^{2+}\)) or high Ca\(^{2+}\) medium (1.5 mM Ca\(^{2+}\)) in the absence of BPE and cultured for 2 days.

**Reporter vectors**

The proximal promoter of the human SPINK5 gene [nucleotide -1 to -1141 from the major transcriptional start site (Accession No; Chromosome 5 - NC_000005.9)] was PCR amplified using Sac I and Nhe I tailed primers, respectively -1141 and -1 (Table 1),
from the genome of NHEK as a template. Evolutional conservation search between 
human and Rhesus macaque (rheMac2) using ECR browser (http://ecrbrowser.
dcode.org) displayed that -1 to -1214 was a putative promoter enhancer. Unfortunately,
the primer search program (DNasis-Mac v3.6) selected a primer start with -1141 instead
of -1214 was suitable for PCR amplification. After digestion with Sac I and Nhe I, the
amplified products were initially cloned into pGL4.10[luc2] (Promega, Madison, WI,
USA), designated as -1141/-1. To generate clones with different 5’ terminal length, PCR
was performed using different 5’ primers for deletion and the 3’ primer to conserve
pGL4 sequence (Table 1). After digestion with Sac I, the products were re-ligated to
obtain deletion clones. All cloned promoters in the reporter construct were sequenced to
confirm their sequence was conserved after cloning.

Other vectors used

pGL-954/+40; among reporter vectors containing KLK1 promoter, the highest
luciferase activity possessed [15], pcDNA3.1 (Invitrogen, Carlsbad, CA, USA),
pcGATA3; the coding region of GATA3 [the open reading frame with 119 bp upstream
to the start codon and 33 bp downstream from the stop codon] was cloned into
pcDNA3.1, and pcGATA3mut; to obtain dominant negative mutant of pcGATA3, its
sequence corresponding to 305KRR was changed to AAA [15].

SPINK5 promoter activity in NHEK cells
NHEK cells cultured on 24-well plates were transfected with 0.75 μg of reporter vectors pGL4.10[luc2] cloned with different fragments from the proximal promoter of the human SPINK5 using Tfx™-20 (Promega, Madison, WI, USA). After 48 hours, the cells were lysed using a luciferase reporter assay system (Promega, Madison, WI, USA) and the firefly luciferase activities were measured with a Lumat LB9507 (Berthold Technologies, Tokyo, Japan). All experiments were performed at least three times for each reporter plasmid and the relative luciferase activity was calculated. Results were presented as the mean ± SD. In order to confirm that the putative sequence was really active, -676/-532 or -318/-146 was PCR amplified using a set of primers (Table 1), used for electrophoretic mobility shift assay (EMSA) and cloned into pGL4.10[luc2] for the luciferase assay.

Search for putative cis elements

In order to find putative cis elements, sequence of -1141/-1 was subjected to TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html), ALGGEN PROMO [17, 18] and PhysBinder (http://bioit.dmbr.ugent.be/physbinder/ [19]).

Electrophoretic mobility shift assay (EMSA)

According to the results of luciferase assay, -676/-532, and -318/-146 supposed to
contain activating element, were PCR amplified using primers listed in Table 1. Nuclear protein was obtained from 1×10^6 NHEK cells cultured with KG2, or 0.15 and 1.5 mM Ca^{2+} media without BPE, based on the protocol [20]. Briefly, after washed with ice cold PBS (Ca^{2+} and Mg^{2+} free), cells were resuspended with 5 volumes of ice-cold cell homogenization buffer (10 mM HEPES-KOH, pH7.8, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 1× proteinase inhibitor cocktail (Nakalai tesque, Kyoto, Japan) to stand on ice for 10 min, and collected by centrifuging at 250 g for 10 min. The cell pellet was resuspended in 3 volumes of ice-cold cell homogenization buffer containing 0.05% (v/v) Nonidet P-40, and homogenized on ice with 20 strokes of a tight-fitting Dounce homogenizer. The nuclei were collected by centrifugation at 250 g for 10 min at 4 °C. After removing the supernatant, the nuclei pellet was resuspended with 20 ml cell suspension buffer (40 mM HEPES-KOH, pH 7.8, 0.4 M KCl, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 1× proteinase inhibitor cocktail). NaCl was added to a final concentration of 300 mM, and the resuspended nuclei was incubated on ice for 30 min then centrifuged at 15,093 g at 4 °C for 15 min. The supernatant containing nuclear protein was divided into aliquots and stored at -80 °C until use. Protein concentration was determined by the Bradford method on Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA). After 100 ng of each DNA were mixed with 6 μg of nuclear protein in a final volume of 20 μl binding buffer containing 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 3.5 mM DTT, 0.25% Tween 20, and 0.025 μg salmon sperm DNA and allowed to stand for 25 min at room temperature, electrophoretic mobility shift assay (EMSA) was performed on 1% agarose/ethidium
bromide gel. Poly(dI-dC) as DNA and BSA as protein were served as negative control.

In order to clarify the location that nuclear protein was attached, DNA footprinting using DNase I protection was performed on pGLSP-676/-532 and pGLSP-318/-146 that showed high luciferase activities. pGL-954/+40 was served as positive control. These DNAs were bound with nuclear protein as described above. To this mixture, 1 U RQ1 RNase-free DNase I (Promega) and supplied DNase I buffer were added and allowed to stand at room temperature for 1 minute. The digested DNAs were phenol/chloroform extracted and ethanol precipitated. Footprinting ladder was PCR synthesized using PCR primer, RVprimer3 by BigDye (Applied Biosystems, CA). The sequence was analyzed by ABI Prism 3100 (Applied Biosystems).

In order to clarify statistical significance among luciferase activities, one-way ANOVA with Tukey's HSD test as post hoc test was used.
Results

To examine the location of putative activating and/or inhibitory cis-elements, reporter vectors were transfected into NHEK cells and luciferase assay was performed. Transcriptional activity was almost null when pGLSP-1141/-1 and pGLSP-1076/-1 were transfected to NHEK (Fig.1). When a clone deleted -1141 to -932, i.e. pGLSP-932/-1, was transfected, the significantly higher intensity than pGLSP-1141/-1 and pGLSP-1076/-1 was observed. Moreover, transfection of a clone deleted -1141 to -676, i.e. pGLSP-676/-1, and a clone deleted -1141 to -318, i.e., pGLSP-318/-1 gave the significantly higher intensity than other clones. The intensities of these two clones did not show significant difference. The intensity of pGL-954/+40 containing KLK1 promoter was more than three times higher than that of pGLSP-318/-1.

Effect of high Ca$^{2+}$ medium on transcriptional activity of SPINK5 putative promoter

Intensities of pGLSP-676/-1 and pGLSP-318/-1 were significantly higher than that of pGLSP-932/-1, and those of pGLSP-318/-1 and pGLSP-318/-146 were almost the same (Fig.2). Thus, pGLSP-676/-1, pGLSP-318/-1 and pGLSP-318/-146 were subjected to examine the effects of high Ca$^{2+}$ medium. Removal of BPE from Humedia-KG showed no significant differences in the intensities of pGLSP-676/-532, pGLSP-318/-1 and pGLSP-318/-146 (Fig.2). High Ca$^{2+}$ media-cultured NHEK also did not show any differences in their intensities.
TFSEARCH, ALGGEN PROMO and PhysBinder to search putative cis elements

Sequence of -1141/-1 was subjected to TFSEARCH, ALGGEN PROMO and PhysBinder for searching putative cis elements. Among elements that hit by these programs, those did not exist in -1141/-933, -860/-677, -554/-319, -169/-1 were listed (Table 2). Excluding SRY, no common cis-elements existed in the activating regions -932/-837, -676/-532 and -318/-146. A point mutation on -206 G to A reportedly produce GATA3 binding site [21], but our -206 was G. Thus, no GATA3 binding site was extracted in any of the activating regions.

Electrophoretic mobility shift assay (EMSA)

-676/-532 and -318/-146 supposed to contain activating element were PCR amplified using primers listed in Table 1 and subjected to EMSA (Fig.3). Mobility of both fragments was delayed by all of nuclear extracts from normal HuMedia-KG-, low Ca²⁺ medium-, and high Ca²⁺ medium-cultured NHEK. Location of retarded bands was almost identical regardless of -676/-532 or -318/-146 and regardless of culture media.

Effect of pcGATA3, and pcGATA3mut on SPINK5 promoter

Co-transfection of pcGATA3 with pGLSP-676/-532, -318/-146 and -318/-1 increased the intensities significantly (Fig.4). However, their intensities were still less than 1/3 of
that of pGL-954/+40. On the other hand, pcGATA3mut co-transfection decreased the
activities significantly in any of the plasmids.

DNA foot printing

When pGLSP-676/-532 and pGLSP-318/-146 were subjected to DNA footprinting, no
specific sequence was protected (pGLSP-676/-532; data not shown and
pGLSP-318/-146, Fig.5). However, in case of pGL-954/+40, protected band was found
at -941/-937 where STAT4, c-Ets-1, c-Ets-2, Elk-1, Pax-5 and p53 could be putative
transcription factors. Thus, we could not specify cis-elements for pGLSP-676/-532 and
pGLSP-318/-146 and EMSA using oligo-DNA was currently unavailable.

Discussion

It was surprising that pGLSP-676/-1 and pGLSP-318/-1 displayed high luciferase
activities in NHEK cells. NHEK cells were cultured in culture media containing
0.15mM Ca\(^{2+}\) to avoid differentiation. Expression of SPINK5 is limited at the very
surface of SG, namely SPINK5 expression is limited at the very last step of
differentiation just before denucleation [5]. Thus, in NHEK cells without induction of
differentiation, no luciferase activity was expected in any of reporter constructs.
However, their intensities were only less than 1/3 of that of pGL-954/+40 containing
KLK1 promoter. Expression of KLK1 is observed throughout the SS and SG [5]. It is
not surprising that, independent of 0.15 mM Ca\(^{2+}\), KLK1 promoter appeared to have an
activity in NHEK cells with very high intensity. Therefore, some NHEK seemed to
differentiate to the final step in this medium even when Ca\textsuperscript{2+} concentration was
maintained at 0.15mM and growth factors like BPE were included. Growth factors are
considered to suppress keratinocyte differentiation [22, 23]. However, neither removal
of growth factors alone (low Ca\textsuperscript{2+} medium) nor removal of growth factors and an
increase in Ca\textsuperscript{2+} concentration (high Ca\textsuperscript{2+} medium) altered the intensity of luciferase and
the movability of EMSA bands, indicating that such stimulation is not suitable for the
final differentiation. On the other hand, high cell density reportedly promotes
keratinocyte differentiation [23]. It is conceivable that several colonial clones may
differentiate further.

TFSEARCH, ALGGEN PROMO and PhysBinder did not find common transcription
factors in the activating regions -932/-837, -676/-532 and -318/-146. Only SRY
(Sex-determining region Y) was common to -932/-837 and -318/-146. However, SRY
was encoded on Y chromosome and regulated sex differentiation [24], indicating that
this transcription factor was not responsible for skin function. Judging from the results
of EMSA, the size of transcription factor binding to -676/-532 and -318/-146 was
almost the same. It is possible that the same transcription factor which could not be
detected by these programs occupied -932/-837 and -318/-146. Otherwise, the results of
DNA footprinting might indicate that more than one protein occupied these sequences.

An epidemiological research with case-control design in Chinese Han population
indicated that -206 G>A mutation produced GATA3 binding site in SPINK5 promoter
and that the G allele was significantly more common among patients with asthma than
among the controls [21]. Existence of GATA3 site might directly increase SPINK5 expression. However, our -206 was G, hence no GATA3 binding site was extracted in neither -676/-532 nor -318/-146. Therefore, GATA3 unlikely affected directly on these sites. Although intensity was still less than 1/3 of pGL-954/+40, induction of GATA3 in NHEK increased their luciferase activity. On the other hand, transfection of dominant negative GATA3mut suppressed it. The effect of GATA3 was limited and indirect. This may be via transcription factor(s) downstream to GATA3. For example, GATA3 reportedly react with Smads [25] but none of TFSEARCH, ALGGEN PROMO, and PhysBinder found any Smads site in -676/-532 and -318/-146. It seems necessary to explore transcription factors downstream to GATA3 that could regulate these sites. However, unfortunately, we could not yet identify the transcription factor(s) that regulated SPINK5 expression in keratinocytes. Otherwise, inhibition of proliferation and induction of differentiation of keratinocytes by GATA3 [26] might trigger the next step of differentiation of keratinocytes and SPINK5 expression that was regulated by transcription factor(s)/cis-element(s) different from GATA3 system. That is, GATA3 is a very important factor for the differentiation of keratinocytes, but GATA3 itself seems to be insufficient for the final differentiation and/or SPINK5 expression.

It is very difficult to obtain sufficient amount of nuclear protein from human skin where SPINK5 expresses. It seems also very difficult to induce final differentiation to keratinocyte culture. High Ca²⁺ stimulation in the present study was insufficient for the final differentiation. Further examination on expression regulation mechanism of -676/-532 and -318/-146 will supply important information related to a key regulator of
SPINK5 expression and final differentiation of keratinocytes.

Conflict of interest

We acknowledge that we have no conflict of interest.

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Figure legends

Fig. 1. Relative luciferase activities of reporter plasmids containing different lengths of putative SPINK5 promoter in NHEK. Each column represents the mean of three independent experiments, each done in triplicate; bars, ± SD. Inset; Significant differences in the intensity between the groups (P < 0.05 One-way ANOVA and post hoc test using Tukey's HSD test). When pGL-954/+40 was transfected, the intensity was higher than 450x10³ RLU.

Fig. 2. Effect of high Ca²⁺ medium on relative luciferase activities of SPINK5 putative promoters. pGLSP-676/-532, pGLSP-318/-1 and pGLSP-318/-146 were transfected to normal HuMedia-KG2-, low Ca²⁺ medium-, and high Ca²⁺ medium-cultured NHEK. Each column represents the mean of three independent experiments, each done in triplicate; bars, ± SD. No significant difference was observed in any of pGLSP-932/-1, pGLSP-676/-1 and pGLSP-318/-1 among different culture media (One-way ANOVA and post hoc test using Tukey's HSD test).

Fig. 3. EMSA for fragments -676/-532 and -318/-146. In order to avoid non specific binding, poly(dI-dC) was utilized. Aside from nuclear proteins, bovine serum albumin (BSA) was served as negative control.
Fig. 4. Effects of co-transfection of pcGATA3 and pcGATA3mut with pGLSP-676/-532, pGLSP-318/-1 and pGLSP-318/-146. Each column represents the mean of three independent experiments, each done in triplicate; bars, ± SD. *Significant difference (One-way ANOVA and post hoc test using Tukey's HSD test).

Fig. 5. Sequence ladder of pGSP-318/-146 (Upper) and pGL-954/+40 (Lower; positive control) after digestion by DNaseI along with nuclear protein. Sequence ladder was made by the primer, RV3 (supplied for pGL4.10[luc2]). Underline; protected sequence where TFSEARCH, ALGGEN PROMO, and PhysBinder selected STAT4, c-Ets-1, c-Ets-2, Elk-1, Pax-5 and p53 as putative cis-elements.
