Proteolytic processing of human growth hormone by multiple tissue kallikreins and regulation by the serine protease inhibitor Kazal-Type5 (SPINK5) protein

Nahoko Komatsu a,b,c, Kiyofumi Saijoh d, Norio Otsuki e, Tadaaki Kishi a, Iacovos P. Micheal a,b, Christina V. Obiezu a,b, Carla A. Borgono a,b, Kazuhiko Takehara c, Arumugam Jayakumar f, Hua Kang Wu f, Gary L. Clayman f, Eleftherios P. Diamandis a,b,⁎

a Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5
b Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada M5G 1L5
c Department of Dermatology, Graduate School of Medical Science, School of Medicine, Kanazawa University, Kanazawa 920-8641, Japan
d Department of Hygiene, Graduate School of Medical Science, School of Medicine, Kanazawa University, Kanazawa 920-8641, Japan
e Department of Dermatology, Maizuru Kyosai Hospital, Maizuru, Kyoto 625-0036, Japan
f Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030-4095

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Abstract

Background: Human growth hormone (hGH) is naturally present in numerous isoforms, some of which arise from proteolytic processing in both the pituitary and periphery. The nature of the enzymes that proteolytically cleave hGH and the regulation of this process are not fully understood. Our objective is to examine if members of a newly discovered human tissue kallikrein family (KLKs) are expressed in the pituitary and if these enzymes can cleave hGH in-vitro.

Methods: Expression of 12 of the KLKs (KLKs 4-15) and serine protease inhibitor Kazal-type 5 (SPINK5) genes and their proteins in the pituitary was examined by RT-PCR and immunohistochemistry. Recombinant hGH was digested by various recombinant KLKs and fragments were characterized by N-terminal sequencing. SPINK5 recombinant fragments were used for inhibition of KLK activities.

Results: We here describe for the first time expression of numerous KLKs (KLKs 5–8, 10–14) and SPINK5 in the pituitary. KLK6 and SPINK5 appeared to be localized to hGH-producing cells. KLKs 4–6, 8, 13 and 14 were able to cleave hGH, yielding various isoforms, in vitro. Inhibitor SPINK5 fragments were able to suppress activity of KLKs 4, 5 and 14 in vitro. Based on these data, we propose a model for the proteolytic processing of hGH in the pituitary and the regulation of this system by SPINK5 inhibitory domains. We speculate that loss of SPINK5 inhibitory domains, as in the case of Netherton syndrome, may lead to proteolytic over-processing of hGH and to growth retardation.

Conclusion: We conclude that many KLKs and SPINK5 are expressed in the pituitary. This serine protease-inhibitor system is likely to participate in the regulated proteolytic processing of hGH in the pituitary, leading to generation of hGH fragments. Our data suggest that KLKs 5, 6 and 14 might be involved in this process.

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1. Introduction

Human growth hormone (hGH) is known to exist as a heterogeneous population of molecules in the human body [1]. In the periphery, 70–75% of hGH exists as the classic, 22 kDa, 191-amino acid single-chain form (single-hGH). Single-hGH can be post-translationally processed by proteolytic cleavage
not only in the pituitary but also in plasma [2,3]. Putative cleavage sites for numerous proteases, such as thrombin, plasmin, collagenase, subtilisin and chymotrypsin-like serine proteases are located around residues 134–150 [1,4,5]. These modifications transform single-hGH to a 2-chain form, linked by a disulfide bond [6], significantly slowing its rate of metabolism in the circulation [7]. Besides the 22 kDa form, the translated product of a splice variant and fragments of 17 (or 16), 12 and 5 kDa have also been detected both in the pituitary and the periphery [8]. Upon reduction of the disulfide bond in the 2-chain form, the generated 16 kDa N-terminal hGH fragment inhibits endothelial cell proliferation and angiogenesis in the early stage chick chorioallantoic membrane assay [9]. The proportion of circulating non-22 kDa hGH isoforms, especially 2-chain form, increases in some children with growth retardation, without a decrease in total hGH levels [10]. These reports suggest that hGH not only requires proteolytic cleavage to exert its full biological activity [2], but also that hGH isoforms are potential endogenous regulators of physiological and pathological processes such as angiogenesis [11]. However, hGH isoforms are not distinguishable from single-hGH or from each other by routine assays of hGH levels in plasma or other biological fluids [8].

Human tissue kallikreins are secreted serine proteases, encoded by a group of 15 genes that are tandemly located on chromosome 19q.13.4 [12–14]. With the new nomenclature [15], the genes are designated KLK1 through KLK15 and the encoded proteins KLK1 through KLK15. By using RT-PCR, immunohistochemistry and ELISA assays, previous studies have shown that these enzymes are expressed in diverse tissues, mainly steroid hormone-regulated tissues, including the pituitary [12,16–21]. One member of this family, KLK3, better known as prostate-specific antigen (PSA), is the primary biomarker for diagnosis and monitoring of prostate cancer [22]. Many other kallikreins have already been shown to be useful biomarkers for pituitary tumors and ovarian, breast, prostate, testicular and other cancers [12–14,23–26]. Despite the wealth of literature on kallikrein over-expression in many different types of cancers, the function and regulation of these enzymes in normal and cancerous tissues are largely unknown. A recent review describes possible links between kallikreins and receptor, hormone or cytokine processing, angiogenesis, metastasis, cell growth, etc. [13].

SPINK5 (also known as LEKTI for its protein product) is a gene encoding a 125 kDa secretory serine protease inhibitor pre-proprotein, containing 15 potential inhibitory domains [27,28]. SPINK5 protein is thought to be cleaved by furin to yield at least 14 independently working serine protease inhibitory domains [29,30]. Since SPINK5 protein and many tissue kallikreins co-localize in the skin (in lamellar bodies of the uppermost epidermis and the pilosebaceous units of normal human skin tissue) [20,31–34], it has been hypothesized that these proteins may be part of a proteolytic enzyme-inhibitor system that controls skin desquamation and shedding [29,34–38]. Indeed, recent evidence suggests that Netherton syndrome patients, who lack SPINK5 inhibitory domains due to gene mutations, suffer from severe erythroderma, hair shaft defects and other atopic features [28,39,40]. These features are attributed to elevated stratum corneum trypsin-like activity which leads to over-desquamation and severe skin permeability barrier dysfunction in Netherton syndrome patients [29] and SPINK5-deficient mice, mimicking Netherton syndrome [41].

One consistent feature of Netherton syndrome patients is growth retardation [39,40]. Based on this and the other findings mentioned above, we hypothesized that the tissue kallikrein-
SPINK5 protein proteolytic cascade pathway, which operates in the skin, may also function similarly in the pituitary, controlling hGH and other hormone processing in this tissue. The objectives of this study were (a) to examine kallikrein and SPINK5 gene and protein expression in the human pituitary, (b) to investigate if serine proteases of the kallikrein family can proteolytically digest hGH and characterize the cleavage sites, (c) to examine if this proteolysis can be inhibited by SPINK5 protein fragments and (d) to develop a model for hGH processing by multiple kallikrein enzymes.

2. Materials and methods

2.1. Reverse transcription-polymerase chain reaction

Normal human pituitary cDNA was purchased from Clontech (Palo Alto, CA). One nanogram of cDNA was used as template for PCR amplification. The primers and PCR conditions for (mRNAs) KLK4 (amplification from exon 2 to 5), KLK5 (exon 2 to 5), KLK6 (exon 2 to 5), KLK7 (exon 2 to 5), KLK8 [non-coding region (upstream of exon 1) to exon 5], KLK9 [exon 1 to non-coding region (downstream of exon 5)], KLK11 (exon 3 to 4), KLK14 (exon 3 to 5), SPINK5, and GAPDH mRNAs were described elsewhere[28]. Our experiments have been performed under the following conditions: 1) we followed the experimental procedures described in detail in Ref.[42]. 2) All PCR products presented in Fig. 1 have been fully confirmed by DNA sequencing. 3) PCR products for which we could not confirm the DNA sequence were not included in our data. In addition, the following primers were used for amplification of KLK10; Forward (F) 5'-GGAAACAAGCCACTGTGGGC -3' (on exon 2), Reverse (R) 5'-GAG-GATGACCTTGGAGGGTCTC -3' (on exon 5), annealing temperature at 60 °C, product size 468 bp, KLK12; F 5'-TTGACCAACAGTGGTGCGC -3' (on exon 2), R 5'-GTGTAGACTCCAGGATGCCA -3' (on exon 5), 61 °C, 542 bp, KLK15; F 5'-CTACGAGCACTTTCTGGGGTC -3' (on exon 3), R 5'-GACACCCAGCCTTTGTTGTGT -3' (on exon 5), 65 °C, 459 bp. Forty amplification cycles were used for all (mRNA) KLKs and SPINK5 mRNA and 26 cycles for GAPDH mRNA.

2.2. Immunohistochemistry

Anti-KLK6 rabbit polyclonal antibody (Ab) was developed in-house, using full-length recombinant protein as immunogen. No cross-reactivity of this Ab against all other kallikreins (KLKs 3–15)[20], and (KLKs 1–2) (data not shown) was observed by Western blotting. Since the specificity of Abs towards other KLKs has not as yet been determined, immunohistochemistry was restricted to

SPINK55 and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were described elsewhere [28]. Our experiments have been performed under the following conditions: 1) we followed the experimental procedures described in detail in Ref. [42]. 2) All PCR products presented in Fig. 1 have been fully confirmed by DNA sequencing. 3) PCR products for which we could not confirm the DNA sequence were not included in our data. In addition, the following primers were used for amplification of KLK10; Forward (F) 5'-GGAAACAAGCCACTGTGGGC -3' (on exon 2), Reverse (R) 5'-GAG-GATGACCTTGGAGGGTCTC -3' (on exon 5), annealing temperature at 60 °C, product size 468 bp, KLK12; F 5'-TTGACCAACAGTGGTGCGC -3' (on exon 2), R 5'-GTGTAGACTCCAGGATGCCA -3' (on exon 5), 61 °C, 542 bp, KLK15; F 5'-CTACGAGCACTTTCTGGGGTC -3' (on exon 3), R 5'-GACACCCAGCCTTTGTTGTGT -3' (on exon 5), 65 °C, 459 bp. Forty amplification cycles were used for all (mRNA) KLKs and SPINK5 mRNA and 26 cycles for GAPDH mRNA.
KLK6 only. Anti-SPINK5 protein mouse monoclonal antibody (clone 1C11G6) was developed as described elsewhere[43]. Formalin-fixed paraffin-embedded human pituitaries were sectioned. Anti-hGH Ab was purchased (Pituitary hormones and antisera center, Harbor-UCLA Medical Center, Torrance, CA). Staining procedures included deparaffinization in xylene and rehydration through graded ethanols. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 20 min, and the sections were pretreated with 10 mmol/l citrate buffer (pH 6.0) at 95 °C for 10 min. The primary Abs were diluted in 0.1% bovine serum albumin/phosphate-buffered saline. The normal serum blocking reagent, secondary biotinylated Ab and peroxidase-labeled streptavidin complex were purchased from ID Labs (Ontario, Canada). Samples were stained with 3,3’-diaminobenzidine substrate (DAB substrate kit for peroxidase, Vector Laboratories, Inc., Burlingame, CA) for 5 min. The sections were counterstained with hematoxylin for 1 min. Non-immune rabbit or mouse serum was substituted as the primary antibody for negative control staining.

2.3. hGH proteolytic processing by KLKs and inhibition by SPINK5 protein fragments

KLKs [20], SPINK5 protein fragment 6–9 (sp6) [44], and SPINK5 protein fragment 9–12 (sp9) [38] were recombinantly expressed in insect cells and purified. Recombinant hGH (HUMATROPE®) was purchased from Eli Lilly Canada Inc. Each protein was diluted in 0.01% phosphosaline buffer (PBS) solution. Equal volumes of samples in 0.01% PBS containing hGH, KLKs and/or sp6/9 were incubated for 24 h at 37 °C or 4 °C, then samples were separated on 4–12% Bis–Tris gels (Invitrogen) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were either stained with Simply Blue™ SafeStain (Invitrogen) or electrotransferred to PVDF transfer membranes (Hybond™-P, Amersham Pharmacia Biotech, Buckinghamshire, England) at 30 V for 90 min for N-terminal amino acid sequencing. N-terminal amino acid sequencing was performed by the University of Victoria Proteomics Center (British Columbia, Canada).

3. Results

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine the expression of 12 (mRNA)KLKs and SPINK5 mRNAs in normal human pituitary (Fig. 1). mRNAs for KLKs 1–3 were previously identified in the pituitary [16]. We detected classic forms of (mRNA)KLKs 5–7, 10–12, and 14, as well as SPINK5 mRNA. (mRNA)KLK8 splice variant type 4 [45] and (mRNA)KLK13 type 3 [42] were also detected. These splice variant forms are predicted to encode for truncated proteins devoid of enzymatic activity [46]. These data may not necessarily represent expression in disease states since the ratio of splice forms may change in certain diseases [46–48].

Growth hormone-producing cells were identified by immunohistochemistry. These cells seem to be randomly distributed and have a small round nucleus and compact cytoplasm (Fig. 2a). In contrast, non-hGH producing cells exhibit an irregular (flattened) nuclear shape and a wider cytoplasm. Non-hGH producing cells more frequently displayed a “lobular-like” formation in which the nucleus was found in the outer periphery (Fig. 2a). Staining with specific antibodies against KLK6 and SPINK5 proteins revealed cells similar to those stained with hGH antibodies. Thus, hGH hormone-producing cells seem to...
produce KLK6 and SPINK5 proteins as well (Fig. 2b, c). In addition, the intense KLK and SPINK5 protein staining in the endothelial cells of pituitary small veins (Fig. 2d, e) suggests that the released pituitary hormones could undergo proteolytic processing during passage through pituitary veins, prior to their appearance in the peripheral circulation.

To examine whether KLKs can cleave hGH in-vitro, the recombinant 22 kDa hGH single-chain form was incubated individually with each KLK or trypsin (Fig. 3). Approximately 12 and 5 kDa fragments were produced by trypsin (Fig. 3 lane 2). Judging from the size of the 2 fragments and by amino acid sequencing, they were identified as N-terminal Phe1–Arg134 (••) and C-terminal Thr135–Phe191 (•) fragments (Fig. 3). Arg134/Thr135 is known to be a site also cleaved by thrombin and plasmin[5].

All KLKs examined here possess trypsin-like activity[12]. Thus, excluding KLK14, each KLK produced 12 kDa Phe1–Arg134 (••) and 5 kDa Thr135–Phe191 (•) fragments (Fig. 3a–e lane 3). In addition to identical cleavage of hGH by trypsin and KLK5 (0.2μg) (Fig. 3b lane 3), 0.5 μg of KLK5 cleaved also at Arg8/Leu9 and Arg34/Ser35 (Fig. 3b lane 3; data confirmed by N-terminal sequencing). These cleavages additionally yielded 6 and 3 kD hGH fragments. Although KLK14 has been predicted to be a trypsin-like serine protease[12], in this assay, KLK14 (0.2 μg) acted as both chymotrypsin-like (Tyr143/Ser144) and trypsin-like enzyme (Arg64/Glu65) (Fig. 3f lane 3). The trypsin and chymotrypsin-like activity of KLK14 was recently confirmed by using a substrate phage display library [49]. No visible bands remained when 1.0 μg of KLK14 was added (Fig. 3f lane 3'). The results of Fig. 3 show that many KLK proteins are capable of proteolytically modifying hGH to the 2-chain form and that an excess of KLKs can further digest hGH to smaller fragments.

The inhibition of KLK activity by SPINK5 protein fragment derivatives was examined by using the recombinant SPINK5 protein fragments, sp6 (SPINK5 protein domains 6–9) and sp9 (SPINK5 protein domains 9–12) [27,38,44], which are frequently absent in Netherton syndrome patients (Fig. 4) [29]. KLK4 efficiently digested sp6/sp9, even when hGH was also present, but hGH cleavage was significantly suppressed (Fig. 4b). KLK5 did not digest sp6 and sp9 nor hGH in the presence of sp6 and sp9 (Fig. 4c). Despite the fact that sp6 and sp9 were substrates for KLK4 but not for KLK5, they had a specific inhibitory effect on hGH digestion by both KLK4 and KLK5. Both sp6 and sp9 were entirely digested by KLK14, while sp6 alone prevented autolysis of KLK14 (Fig. 4d). In addition, sp6 alone partially inhibited hGH proteolysis by KLK14.

4. Discussion

Previously, it was reported that rat KLK2 proteolytically cleaved rat prolactin [50], suggesting that rat KLK2 is a putative
prolactin-processing protease [51]. Rat KLK1 catalyzes the release of kinins, which are capable of stimulating prolactin and GH secretion [52]. In human, KLK1 was co-localized with prolactin in prolactin-secreting adenomas of the human anterior pituitary [23]. KLK1 was associated with prolactin-secreting cells within human GH-secreting adenomas [24]. To complement these preceding studies, our study demonstrates for the first time that many human tissue kallikrein enzymes are expressed in the normal human pituitary gland, and that they are able to generate various proteolytically modified hGH isoforms, such as the 22 kDa 2-chain form and 15, 14, 6, 5 and 3 kDa fragments. Based on these data, we developed a model describing the proteolytic processing of hGH by KLKs (Fig. 5). The preferred cleavage site for most of the KLKs was Arg134/Thr135.

Fig. 5. A model for regulation of growth hormone proteolysis in normal individuals and Netherton syndrome patients (modified from [29]). (a) Upper panel: In normal pituitary SPINK5 proprotein is proteolytically processed to at least 14 individual bioactive domains. Cleavage occurs at Lys/Arg (K/R)–Xn–Lys/Arg (K/R); (n=0, 2, 4, and 6) motifs, which are repeatedly found in-between an inhibitory domain and other domains of SPINK5 proprotein, by subtilisin-like proprotein convertases (SPCs), such as furin [29,30,58,59]. Since it has not been conclusively determined whether KLKs are involved in SPINK5 proprotein processing KLKs are indicated with a question mark. Cleaved domains may be further processed by carboxypeptidases (CPs) [29] to bioactive SPINK5 domains. Middle panel: SPINK5 domains are capable of inhibiting KLK serine protease activities, (blue circles with arrows), leaving some in active form (red circles). Thus, SPINK5 domains are believed to be negative regulators of KLKs [29,41]. Bottom panel: KLKs may contribute to hGH proteolytic processing, yielding a 2-chain form in pituitary and/or periphery. (b) In Netherton syndrome patients, all mutations in SPINK5 reported so far [29,54,55], lead to premature translation termination in and truncated protein. Consequently, Netherton syndrome patients possess fewer SPINK5 inhibitory domains. This leads to higher KLK enzymatic activity and higher proteolytic processing of GH which may become biologically inactive. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
or Tyr\textsuperscript{143}/Ser\textsuperscript{144} for KLK14. Both cleavages convert hGH to the 2-chain form (Fig. 5b). Several other Arg residues are also targeted, probably as a pathway that ultimately leads to hGH degradation (Fig. 5c). Furthermore, SPINK5 protein fragments can suppress KLK protease activity, leading to decreased hGH proteolytic processing (Fig. 5d).

Our preliminary immunohistochemical data suggest that human kallikreins are produced by cells that also produce hGH, as well as by endothelial cells of pituitary small veins. It is thus possible that hGH, and possibly other pituitary hormones, are proteolytically modified by KLKs before entering the systemic circulation. This proteolysis may be under the control of inhibitors such as SPINK5 protein, which was also localized in hGH-producing cells and endothelial cells (Fig. 2). These preliminary data need confirmation with double-staining techniques. We further demonstrated that excess KLKs can digest hGH into smaller, and likely inactive fragments (Fig. 3), and that SPINK5 protein fragments can inhibit this process (Fig. 4).

Groups of human kallikrein enzymes, their inhibitors, and probably other proteases, may participate in cascade enzymatic pathways, e.g., in the skin and cancer tissues [13,39,53] (Fig. 6a). SPINK5 pro-protein may be proteolytically processed by furin (and possibly some kallikreins) to generate at least 14 inhibitory domains [29,30]. SPINK5 inhibitory domains may then control multiple kallikrein activities [37,38] (Figs. 4 and 6a). It is likely that similar pathways are operating in the pituitary and/or the periphery, leading to hormone processing and degradation (Fig. 6a).
In Netherton syndrome, all SPINK5 gene mutations identified so far lead to premature stop codons [29,54,55] (Fig. 6b). These mutations result in the production of truncated SPINK5 proteins, lacking inhibitory domains downstream of the mutations. The reduction of SPINK5-derived inhibitory domains would explain the elevated corneocyte trypsin-like activity in Netherton syndrome and the clinical picture, which is associated with increased desquamation [29,41]. We hypothesize that in the same patients, proteolytic over-processing of hGH in the pituitary may be responsible for the growth retardation [40], which is a consistent finding in these patients (Fig. 6b). Further examination of this hypothesis may lead to design of rational therapies of Netherton syndrome, including development of selective inhibitors for certain human kallikreins. Strategies for designing such inhibitors have recently been published [56].

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