Multiple tissue kallikrein mRNA and protein expression in normal skin and skin diseases

N. Komatsu,*†‡§ K. Saijoh,§ T. Toyama,§ R. Ohka,§ N. Otsuki,‡ G. Hussack,* K. Takehara‡ and E.P. Diamandis*†

*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada, M5G 1X5
†Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada, M5G 1L5
‡Departments of ‡Dermatology and §Hygiene, Graduate School of Medical Science, School of Medicine, Kanazawa University, Kanazawa, Japan
*Department of Dermatology, Maizuru Kyouai Hospital, Maizuru, Kyoto, Japan

Correspondence
Eleftherios P. Diamandis, MD PhD FRCPC.
E-mail: ediamandis@mtsinai.on.ca

Accepted for publication
9 December 2004

Key words:
atopic dermatitis, desquamation, human kallikreins, psoriasis, serine proteases, stratum corneum

Conflicts of interest:
None declared.

Summary
Background Human tissue kallikreins are a gene family (KLK1–KLK15) encoding for 15 secretory serine proteases (hK1–hK15). Two tissue kallikrein proteins, hK5 and hK7, were previously found in the stratum corneum (SC), stratum granulosum (SG) and appendages. hK8 was also shown to be secreted via lamellar granules and numerous KLK mRNAs were previously identified. KLKs are believed to be responsible for desquamation of corneocytes and sebum, sweat and hair maturation.

Objectives To demonstrate immunohistochemically the expression of hK6, hK8 and hK13 in normal skin tissue and to show an increased cell number expressing kallikrein mRNAs and proteins in psoriasis vulgaris (PV) and atopic dermatitis (AD).

Methods Samples of normal, PV and AD skin were obtained. hK6-, hK8- and hK13-specific antibodies were produced and used for immunohistochemical analysis. Multiple KLK mRNAs were synthesized and used for in situ hybridization study.

Results Three other hKs, namely hK6, hK8 and hK13, were immunohistochemically identified as new skin serine proteases in the whole SC, SG, sebaceous glands, eccrine sweat glands, hair follicles and nerves. We also demonstrated an increased number of cells expressing KLK mRNAs and hKs in PV and AD. In PV, KLK mRNAs/hKs were predominantly expressed in the upper epidermis. In AD, hK distribution was rather diffuse and expanded into the lower epidermis.

Conclusions The colocalization of various hKs seems to be essential for the regulation of serine protease activity in skin and for steady desquamation and skin barrier function. Moreover, the increased number of cells expressing multiple KLK mRNA and hK in PV and AD could be a clue to elucidate their pathogenesis.

The maintenance of a steady number of stratum corneum (SC) layers is an important factor for skin barrier function. The proliferation rate of keratinocytes to corneocytes is matched by the shedding of old corneocytes at the SC, and skin tissue maintains a steady number of SC layers regardless of age. The desquamation of corneocytes requires both SC trypsin-like and chymotrypsin-like serine protease activities for cornodesmosome degradation. It is likely that there is a regulatory system that maintains a constant serine protease activity, in order to maintain a steady number of SC layers.

The human tissue kallikrein (KLK) gene family localizes as a cluster to chromosome 19q13.4 and encodes for 15 secretory serine proteases (hK1–hK15). Despite the high homology among KLKs, their expression varies in organs, and their protease activities also differ, i.e. hK3, hK7 and hK9 have chymotrypsin-like activity, while the rest possess trypsin-like activity. It has been suggested that kallikreins represent a cascade enzymatic pathway operating in many tissues, including skin. To date, some hKs, i.e. hK5 (previously designated as SCTE15) and hK7 (SCCE15), have been identified immunohistochemically in the SC, stratum granulosum (SG) and appendages as skin serine proteases. hK8 is transported from the trans-Golgi network within lamellar granules, and the granules are released from the apical surface of most superficial
granular cells. In addition, previous in situ hybridization studies suggested that KLK 1, 4, 6, 9, 10, 11, 13 and 14 are also candidate skin serine proteases.

Human kallikrein involvement in skin diseases is not well understood, despite the fact that several studies have raised this possibility. In psoriasis vulgaris (PV), reverse transcriptase-polymerase chain reaction analysis has shown enrichment of KLK6 and KLK9 mRNAs in psoriatic lesions but not in the uninvolved, nonlesional skin samples. Enhanced conversion of inactive hK7 precursor to active hK7 also occurs in PV lesions. In atopic dermatitis (AD), the epidermal expression of hK7 was increased in chronic lesions.

Here, we demonstrate immunohistochemically the expression of hK6, hK8 and hK13 in normal skin tissue and show an increased cell number expressing kallikrein mRNAs and proteins in PV and AD.

Materials and methods

Western blotting

The kallikreins used in this study were produced as follows: hK3 (prostate-specific antigen) was expressed in an Escherichia coli expression system (unpublished), hK4 in yeast, hK5 in yeast, hK6 in a stable mammalian cell line, hK7 in a stable mammalian cell line, hK8 in a baculovirus expression system, hK9 in E. coli (unpublished), hK10 in yeast, hK11 in yeast, hK12 in E. coli (unpublished), hK13 in yeast, hK14 in yeast and hK15 in yeast (unpublished). hK1 and hK2 proteins were not currently available. Proteins were purified by using ion-exchange and reverse-phase chromatography.

Anti-hK6 rabbit polyclonal antibody (hK6-pAb), anti-hK8 rabbit polyclonal antibody (hK8-pAb), and anti-hK13 mouse monoclonal antibody (clone 13C1) (hK13-mAb) were developed in our laboratory as described elsewhere. Ten ng well$^{-1}$ for Western blotting and 1 µg well$^{-1}$ for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of each hK protein were mixed with NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, CA, U.S.A.) and dithiothreitol (Sigma, St Louis, MO, U.S.A.), heated and resolved by SDS–PAGE (4–12% Bis-Tris gel; Invitrogen). Proteins were stained by coomassie blue (SimplyBlue™ SafeStain; Invitrogen) or electrotransferred to reinforced nitrocellulose membranes (Protran; Schleicher and Schuell, Germany); the membranes were incubated with primary antibodies after blocking in 5% skim milk in TBS–TWEEN® 20. Secondary antibodies and chemiluminescent substrate were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, U.S.A.) and Pierce (Rockford, IL, U.S.A.) respectively. Nonimmune rabbit or mouse serum was substituted as the primary antibody for negative control experiments. For quantitative comparison, blotting intensity of each band over background staining was assessed using the public domain NIH Image program written by Wayne Rasband at the U.S. National Institutes of Health (http://www.zippy.nimh.nih.gov).

Skin samples

Normal human skin samples were obtained from six individuals (three females and three males, 47 ± 11 years old; mean ± SD). Chronic plaque lesions of PV from six patients (all males, 49 ± 7 years old) and chronic lichenified plaques of AD from seven patients (four females and three males, 29 ± 12 years old) were obtained during histological diagnostic biopsy while the patients were free of any medication. All patients gave informed consent and our procedures have been approved by the IRB of Kanazawa University Hospital. The formalin-fixed paraffin-embedded specimens were cut into 4-µm sections and mounted on silane-coated glass slides for immunohistochemistry and in situ hybridization studies.

In situ hybridization for KLK mRNAs

The bidirectional cRNA probes for KLK 1, 4, 5, 6, 9, 10, 11, 12, 13 and 14 mRNAs were synthesized from I.M.A.G.E. consortium clones (cDNA-IDs; 2470227, 2321042, 342591, 740000, 1088637, 740780, 4227367, 1644236 and 2465019, respectively; Research Genetics, Inc., Livermore, CA, U.S.A.). The expression of KLK2 and KLK3 mRNAs has not been confirmed in skin, and these genes were not included in this study. Also, KLK15 clones were unavailable. The experimental conditions and procedures have been described elsewhere, that is, although the intensity appeared to be almost identical within each area, measurements performed at five different points were used to evaluate each sample as described elsewhere. The intensities from six individuals were compared using Friedman’s analysis. The intensities were categorized into six groups: –, no significant difference over background staining; ±, less than 2 times; +, 2–4 times; ++, 4–6 times; +++; 6–8 times; and ++++, > 8 times over the background staining. Sense cRNAs were used as negative controls in all cases.

Immunohistochemistry

Staining procedures included deparaffinization in xylene and rehydration through graded ethanol. Endogenous peroxidase activity was blocked with 0.3% H$_2$O$_2$ in methanol for 20 min, and the sections were pretreated with 10 mmol L$^{-1}$ citrate buffer (pH 6.0) at 95 °C for 10 min. The primary antibodies were diluted in 0.1% bovine serum albumin/phosphate-buffered saline. The normal serum blocking reagent, secondary antibodies and peroxidase-labelled streptavidin complex were purchased from DAB Laboratories (London, Ontario, Canada). Samples were stained with 3,3′-diaminobenzidine substrate (DAB substrate kit for peroxidase; Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 5 min. The sections were counterstained with haematoxylin for 1 min. Nonimmune rabbit or mouse serum was substituted as the primary antibody for negative control staining. The method of evaluation is the same as with the in situ hybridization study.
Signal intensity evaluation

For in situ hybridization and immunohistochemistry, signal intensity measurements performed at five different points were used to evaluate each sample using the public domain NIH Image program. The intensities were categorized into six groups, as noted above for in situ hybridization.12

Statistical analysis

The intensities of signals from Western blotting, immunohistochemistry and in situ hybridization experiments were compared, using Friedman’s analysis and Statview v.5 (SAS Institute, Cary, NC, U.S.A.).

Results

Immunohistochemical expression of hKs in normal skin tissue

A weak cross-reactivity of the hK6-pAb against hK4 was observed, but the intensity of the band was less than 2%, in comparison with hK6. The other two antibodies (hK8-pAb and hK13-mAb) were highly specific for hK8 and hK13, respectively. To indicate the hK protein positions in the gel, coomassie blue staining was also provided. These are all shown in Figure 1.

Although our previous in situ hybridization study demonstrated negative KLK8 mRNA and a very weak KLK13 mRNA expression in normal skin tissue,12 both hK8 and hK13 were immunohistochemically detected in the SG and SC of normal epidermis, where prominent cytoplasmic expression of hK6 was also observed (Fig. 2, Table 1). The appendages also showed specific distribution of hKs (Fig. 2).

Enhanced expression of KLK mRNAs in psoriasis vulgaris and atopic dermatitis

In PV, compared with normal epidermis and described elsewhere,12 the number of cell layers expressing KLK mRNAs

Fig 1. Specificity of antibodies by Western blotting. Testing was done for hK3–hK15. hK8-pAb and hK13-mAb specifically recognize hK8 and hK13, respectively, without any detectable cross-reactivity from other hKs. hK6-pAb displayed a weak cross-reactivity against hK4, but the intensity of the band was less than 2%, in comparison with hK6. hK5 appears on sodium dodecyl sulphate–polyacrylamide gel electrophoresis as four bands around 38–49 kDa due to glycosylation.

Fig 2. Immunohistochemical localization of hK6, hK8 and hK13 in normal human skin: (a) normal epidermis (scale bar, 50 μm); (b) hair follicular epithelium (scale bar, 100 μm); (c) sebaceous glands (100 μm); (d) eccrine sweat glands (50 μm); (e) intradermal sensory nerves (50 μm). (a) hK6, hK8 and hK13 were predominantly stained in the stratum corneum (SC) and stratum granulosum. Mild expression of hK6 and hK13 was also observed in the stratum spinosum. hKs were detected in whole layers of SC; however, it was not clear where hKs were distributed, i.e. in the cytoplasm of cornocytes or their intercellular space. (b) In the lower portion of the hair follicle, hK6 and hK8 were predominantly positive in the inner root sheath but faint in the outer root sheath. Ascending to the middle portion (isthmus), expression was gradually shifted to the outer root sheath. For hK13, the inner root sheath and outer root sheath showed a similar intensity even in the lower portion of hair follicle. In addition, hK13 was also expressed in the hair shaft. (c) In the sebaceous glands, the intensity of kallikrein expression was higher in the cytoplasm of basal layer cells than in the thin cytoplasm of matured cells compressed with droplets. (d) In eccrine sweat glands, hK6, hK8 and hK13 showed diffuse cytoplasmic staining in the secretory segment, whereas in the ductal segment, their expression prevailed in the inner lumen side of the cytoplasm. (e) In the intradermal sensory nerve, a diffuse cytoplasmic labelling was observed for hK6, hK8 and hK13.
was drastically increased in the upper epidermis for all KLKs (Fig. 3a). An extremely intense hybridization for KLK13 mRNA was seen along the dermal papilla of basal cells in two of six cases (Fig. 3a). The leukocytes in the Munro’s microabscesses were negative (Fig. 3b). In AD, the KLK mRNA distribution was elongated to the lower epidermis for KLKs 1, 4, 5, 6, 10 and 14 (Fig. 3c), in contrast with the normal skin in which a predominant expression was seen in the SC.12 Three cases of AD also demonstrated strong expression of KLK13 mRNA along the dermal papilla in the stratum basale (Fig. 3c, column 4, row 2). Appendages in both PV and AD showed no significant findings compared with those in normal skin (data not shown). KLK12 mRNAs were not detected in any section (data not shown). Semiquantitative analysis of these data is shown in Tables 2 and 3.

### Discussion

In order to clarify the mechanism responsible for maintaining a steady number of SC layers, it is important to understand the regulation of serine protease activity in the epidermis. In the present study, we confirmed immunohistochemically the expression of hK6, hK8 and hK13 in skin, using highly specific antibodies. In addition to the previously reported expression of hK5 and hK7,12 we now know that at least five hKs are present in skin tissue. Moreover, our findings with in situ hybridization suggest that KLK 1, 4, 9, 10, 11 and 14 are additional candidate players, balancing serine protease activity in the epidermis.12

In normal keratinocytes, KLK mRNA/hK expression may begin in the stratum spinosum but the most abundant pool of kallikreins would be in the SG. hK7 and hK8 were found to be transported as aggregates forming lamellar bodies (lamellar granules) directly from the trans-Golgi network, and the granules are released to the intercellular space from the apical surface of the uppermost layer of the SG.11 hK7 was shown to directly cleave intracellular adhesion molecules of SC corneodesmosomes such as corneodesmosin and desmocollin1.6 The desquamation of corneocytes requires both SC trypsin-like and chymotrypsin-like serine protease activities for corneodesmosome degradation.1,4 The present study demonstrated that hK6, hK8 and hK13 are secreted into the intracellular space of corneocytes and are responsible for desquamation.

In hair follicles, hKs are always dominantly expressed at sites adjacent to hair, that is, at the inner root sheath in the lower portion, and at the outer root sheath in the upper portion. hKs could be important for hair growth and differentiation and trichilemmal keratinization. In the case of hK13, not only the inner root sheath but also the outer root sheath and even the hair shaft expressed hK13. hK13 could have a specific role for the hair follicle. In sebaceous glands, both KLK mRNAs12 and hKs are intensely expressed in the basal layer and hKs may be involved in sebum maturation. The present study demonstrated that hK6, hK8 and hK13 are transferred to the inner luminal side in the ductal segment of the sweat glands; so, these hKs are expected to be found in sweat and across the skin surface. Various hKs, including hK6, hK8 and hK13, have been detected in sweat by enzyme-linked immunosorbent assay (ELISA) (our unpublished data). In the central nervous system, especially in the hippocampus, hK8 may be involved in synapse formation, myelin degradation27 and neurite outgrowth/fasciculation.28 As well, the presence of hK6, hK8 and hK13 in intradermal sensory nerves has been determined; however, little is known about the role of hKs in the peripheral nervous system.
Our in situ hybridization and immunohistochemical studies confirmed an increased number of cells expressing both KLK mRNAs and hKs in the upper epidermis of PV. In PV, the increased transcriptional KLK mRNA may be followed by increased hK protein expression. The leucocytes in Munro’s microabscesses were negative for KLK mRNAs, and KLK6 mRNA was also positive in the deeper parts of the acanthotic rete ridges of psoriatic lesions. In two of six cases, KLK13 mRNA was found along the dermal papilla of basal cells (arrows). In AD, KLK1 and KLK14 mRNAs were positive in whole suprabasal layers, and the intensity was almost homogeneous among layers. KLK4 mRNA was positive in the whole layers, and the upper stratum spinosum (SS) and the stratum granulosum (SG) showed predominant expression. KLK6 mRNA was distributed diffusely in the whole layers. Some cases showed positive expression in the dermal papilla. Leucocytes that are infiltrating into epidermis or the stratum corneum.

Table 2. KLK mRNA expression in psoriasis vulgaris lesions by in situ hybridization

<table>
<thead>
<tr>
<th>Stratum</th>
<th>KLK1</th>
<th>KLK4</th>
<th>KLK5</th>
<th>KLK6</th>
<th>KLK9</th>
<th>KLK10</th>
<th>KLK11</th>
<th>KLK13</th>
<th>KLK14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinosum (upper)</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>(middle)</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Spinosum (lower)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basale</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>± or +++b</td>
</tr>
</tbody>
</table>

*The hybridization intensities were evaluated in the same way as in Table 1. Based on conventional terminology, the psoriatic lesion lacks the stratum granulosum. Thus, for ease of explanation, the stratum malpighii was divided into four parts. Some cases showed positive expression in the dermal papilla. Leucocytes that are infiltrating into epidermis or the stratum corneum.
mRNA transcription seemed to be limited to the epidermis. hK staining was not always apparent in the upper SC. As previously, an increased amount of active hK7 in the upper SC of psoriatic skin was reported,14 and our preliminary data by ELISA also showed a significantly elevated amount of hK6, hK8 and hK13 from psoriatic lesions (data not shown). This result could be explained by a possible abnormal autodegradation of the proteases or epitope masking in the compact layer. Further study is necessary to explain it.

Table 3 KLK mRNA expression in atopic dermatitis by in situ hybridization

<table>
<thead>
<tr>
<th>Stratum</th>
<th>KLK1</th>
<th>KLK4</th>
<th>KLK5</th>
<th>KLK6</th>
<th>KLK9</th>
<th>KLK10</th>
<th>KLK11</th>
<th>KLK13</th>
<th>KLK14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulosome</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Spinousum (upper half)</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Spinousum (lower half)</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Basale</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

*The intensities were evaluated in the same way as in Table 1. Some cases showed positive expression in the dermal papilla.

Fig 4. Immunohistochemistry of hKs in psoriasis vulgaris and atopic dermatitis (AD). (a) Psoriatic lesions (scale bars indicate 100 μm); (b) and (c) Munro’s microabscesses (50 μm for b, and 25 μm for c); (d) parakeratosis (25 μm); (e) AD (50 μm). (a) Localization of hK6, hK8 and hK13 was comparable to their mRNA distribution in psoriatic lesions; namely, they were strongly positive in the upper epidermis and elongated into rete ridges. (b,c) Despite the intense hK staining of the upper epidermis (keratinocytes), corneocytes, including parakeratosis rarely showed hK positivity. (d) hKs were also occasionally detected in parakeratotic lesions. The leucocytes that form Munro’s microabscesses in the stratum corneum (SC) expressed hKs (b and c). (e) In AD, hK6 was diffusely expressed in the whole layers including SC, being comparable to KLK6 mRNA distribution in AD as seen in Figure 3c. hK8 and hK13 were detected mainly in the SC, stratum granulosum and upper stratum spinosum.

A proteasome inhibitor, selectively affecting the chymotryptic and trypsinic activities, significantly reduced both superantigen-mediated T-cell activation and T-cell (HLA-DR) expression.10 This inhibitor was proven to be therapeutically effective in a xenogeneic psoriasis transplantation model. These results imply a possible correlation between an increased number of cells expressing KLK mRNA and hK and the onset of psoriasis.

In AD, an increased number of cells expressing KLK mRNAs and hKs was also observed. However, compared with PV, where the increase was restricted to the upper epidermis, the increase in AD was rather homogeneous with expansion to all
epidermal layers. It seems that the increasing of cell numbers expressing kallikreins in PV and AD is mediated by different mechanisms.

Proteases and kinins can cause itching or potentiate histamine release. Certain proteases work as signalling molecules through cleavage of members of the protease-activated receptor (PAR) family. Trypsin is an activator for both PAR-1 and PAR-2 receptors and their activation can induce cell proliferation, differentiation, pain transmission and inflammatory responses. Immunohistochemically, PAR-2 protein was localized in the SG, hair follicles and myoepithelial cells of sweat glands. In AD, enhanced PAR-2 expression expanded to the lower epidermis, indicating that PAR-2 has almost the same distribution with hKs in both normal skin and AD. Simultaneous altered distribution of hKs and PAR-2 receptor to the lower epidermis in AD and the increase seems to be controlled by distinct mechanisms. More studies are necessary to expand our understanding of the roles of this group of enzymes in skin tissue and their involvement in skin diseases.

### Acknowledgments

We thank the patients for their generous cooperation, M. Takata for critical reading of this manuscript and K. Hama, Y. Obata, R. Hase and Y. Yamada for technical help.

### References


---

**Table 4** hK expression in psoriasis vulgaris by immunohistochemistry

<table>
<thead>
<tr>
<th>Stratum</th>
<th>hK6</th>
<th>hK8</th>
<th>hK13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneum</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spinosum (upper)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Spinosum (middle)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basale</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

*The intensities were evaluated in the same way as in Table 1.

**Table 5** hK expression in atopic dermatitis by immunohistochemistry

<table>
<thead>
<tr>
<th>Stratum</th>
<th>hK6</th>
<th>hK8</th>
<th>hK13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneum</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Spinosum (upper)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Spinosum (lower)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basale</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

*The intensities were evaluated in the same way as in Table 1.*


