Biol. Chem., Vol. 389, pp. 1513–1522, December 2008 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2008.171

# Role of tissue kallikrein-related peptidases in cervical mucus remodeling and host defense

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## Abstract

Human tissue kallikrein-related peptidases (KLKs) are 15 hormonally regulated genes on chromosome 19q13.4 encoding secreted serine proteases. Many KLKs are expressed throughout the female reproductive system and found in cervico-vaginal fluid (CVF). Immunohistochemistry was performed to determine KLK localization in the female reproductive system (fallopian tube, endometrium, cervix and vagina tissues). KLK levels were measured in CVF and saliva over the menstrual cycle to study whether KLKs are regulated by hormonal changes during the cycle. *In vitro* cleavage analysis was performed to establish whether KLKs may play a role in vaginal epithelial desquamation, mucus remodeling or processing of antimicrobial proteins. KLKs were localized in the glandular epithelium of the fallopian tubes and endometrium, the cervical mucus-secreting epithelium and vaginal stratified squamous epithelium. KLK levels peaked in CVF and saliva after ovulation. *In vitro* cleavage analysis confirmed KLKs 5 and 12 as capable of digesting desmoglein and desmocollin adhesion proteins and cervical mucin proteins 4 and 5B. KLK5 can digest defensin-1$\alpha$, suggesting it may aid in cervico-vaginal host defense. We provide evidence of potential physiological roles for KLKs in cervico-vaginal physiology: in desquamation of vaginal epithelial cells, remodeling of cervical mucus and processing of antimicrobial proteins.

**Keywords:** cervical mucus; cervico-vaginal fluid; desquamation; fertility; kallikrein; mucin.

## Introduction

Human tissue kallikrein-related peptidases (KLKs) are a family of 15 genes, located in tandem on chromosome 19q13.4 (Yousef et al., 2003). These genes encode hormonally regulated, secreted serine proteases with trypsin or chymotrypsin-like substrate specificity (Borgono et al., 2004). KLKs have either restricted or broad tissue expression patterns and are often co-expressed in tissues and biological fluids (Shaw and Diamandis, 2007). Immunohistochemical data has shown that KLKs are expressed primarily by glandular epithelia (Petraki et al., 2006). KLKs have also been shown to be co-regulated by steroid hormones in cancer cell lines (Paliouras and Diamandis, 2007) and a few are established or candidate biomarkers for cancer diagnosis and monitoring (Borgono and Diamandis, 2004). For example, KLK3 (prostate-specific antigen or PSA) is a well-known biomarker for the diagnosis and monitoring of prostate cancer (Barry, 2001).

The physiological functions of KLKs 1–3 have been rather well-characterized; however, the functions of the remaining KLKs remain largely unknown. KLK co-expression and co-regulation suggests their potential involvement in proteolytic cascades, similar to the coagulation and complement cascades. KLK cascades have been partially delineated in seminal plasma, involving KLKs 2, 3, 5 and 14 (Michael et al., 2006; Emami and Diamandis, 2007), and in skin, involving KLKs 5, 7 and 14 (Brattsand et al., 2005; Borgono et al., 2007). We recently found relatively large amounts of several KLKs in human cervico-vaginal fluid (CVF) (Shaw and Diamandis, 2007; Shaw et al., 2007), which is a pool of fluids originating from the fallopian tube, endometrium, cervix and vagina (Huggins and Preti, 1981). Here, we investigate the immunohistochemical localization and concentration of KLKs in the female reproductive system (fallopian tube, endometrium, cervix and vagina), in our attempts to determine potential KLK functions in CVF and in cervico-vaginal physiology.

Since all KLKs have been shown to be regulated by steroid hormones at the mRNA and protein level (Borgono et al., 2004; Paliouras and Diamandis, 2007) and given that the menstrual cycle is a hormonally regulated process, we hypothesized that KLKs may be regulated by hormonal changes during the menstrual cycle. Salivary levels of KLKs 1 and 3, as well as serum levels of KLK3 have previously been shown to be influenced by hormonal changes during the menstrual cycle (Zarghami et al., 1997). Here, we analyzed KLK levels throughout the menstrual cycle in CVF and saliva from a normally cycling pre-menopausal woman. KLK levels in CVF from pregnant women were also measured, since during pregnancy, steroid hormone levels are dramatically increased. For comparative purposes, we also analyzed expression and hormonal regulation of KLKs in cultured human vaginal epithelial cells. The post-ovulatory period is characterized by shedding of mature vaginal epithelial cells (Faro, 2004). Given that KLK levels increase during this period, we hypothesized that KLKs may play a role in vaginal epithelial cell shedding, similar to their role in the

desquamation of skin corneocytes (Brattsand and Egelrud, 1999; Borgono et al., 2007). We investigated whether KLKs found in CVF were capable of digesting desmoglein (DSG) and desmocollin (DSC) proteins and found that KLK12 was capable of processing these proteins *in vitro*, suggesting a role for this enzyme in vaginal epithelial cell desquamation.

We previously identified the primary cervical mucus proteins, mucin 4 (MUC 4) and mucin 5B (MUC 5B) in CVF (Shaw et al., 2007), which was not surprising given that cervical mucus contributes to the CVF milieu. The composition and pH of cervical mucus changes throughout the menstrual cycle in response to changing hormone levels. Outside the ovulatory window the pH of cervical mucus is approximately 6.2. Around the time of ovulation, the pH rises to approximately 7.8, the volume of mucus increases and it becomes less viscous (Bigelow et al., 2004). This highly hydrated mucus allows for migration of sperm through the cervix into the uterus (Bigelow et al., 2004). It has been suggested that proteolytic enzymes may affect the physical properties of mucin proteins, causing the changes in mucus observed over the menstrual cycle (Moghissi and Syner, 1970).

A primary function of CVF is host protection from microorganisms (Cole, 2006). KLKs 5 and 7 have recently been shown to play a role in host defense in skin and sweat through cleavage of the antimicrobial human cathelicidin protein, hCAP-18 (Yamasaki et al., 2006). We identified several members of the defensin family in CVF (Shaw et al., 2007) and investigated whether KLKs may also contribute to antimicrobial activity within CVF through processing of defensin proteins.

## Results

### Immunohistochemistry

We immunolocalized KLKs 5, 6, 11, 12 and 13 throughout the female reproductive system. More specifically, a diffuse, cytoplasmic staining of all KLKs was observed in the secretory and ciliated cells of the fallopian tube epithelium (Figure 1A–F). KLK12 immunoexpression (IE) was stronger than the IE of the other KLKs (Figure 1E).

All KLKs were immunoexpressed in the epithelium of the endometrium in both the proliferative and secretory phases. The staining was cytoplasmic with a characteristic infranuclear distribution (Figure 2A–F). KLK11, KLK12 and KLK13 showed a stronger IE (Figure 2E–F).

Cytoplasmic IE of the six KLKs was observed in the mucus-secreting epithelium of the endocervix and the tubular cervical glands. KLKs 11 and 12 were strongly expressed, while KLKs 5, 6 and 13 showed moderate IE (Figure 3A–F). The stratified squamous epithelium of the vagina showed a full-thickness IE, with varying intensities for the different KLKs analyzed. KLK12 IE was the strongest, followed by KLK13 (Figure 4E and F). KLKs 5, 6 and 11 were weakly immunoexpressed. The epithelium of the Batholin's glands, both the ductal and the mucus-secreting columnar cells of the acini, showed a moderate to strong IE for all KLKs (Figure 4B–D). KLK5 IE was stronger in the ductal epithelium than in the mucus-secreting columnar cells of the acini (Figure 4B).

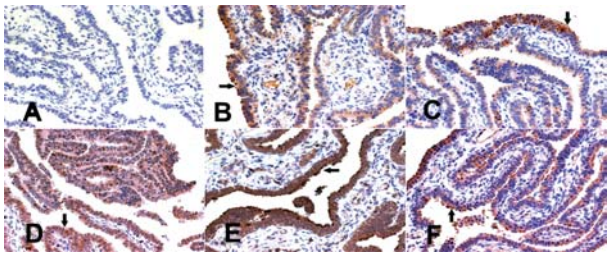


**Figure 1** Immunohistochemical expression analysis of KLKs in the epithelium of the fallopian tube.
The immunolocalization of the KLKs is indicated by arrows and magnification by ×. (A) Non-immune serum ×400 (no staining); (B) KLK5 ×400; (C) KLK6 ×400; (D) KLK11 ×400; (E) KLK12 ×400; (F) KLK13 ×400.

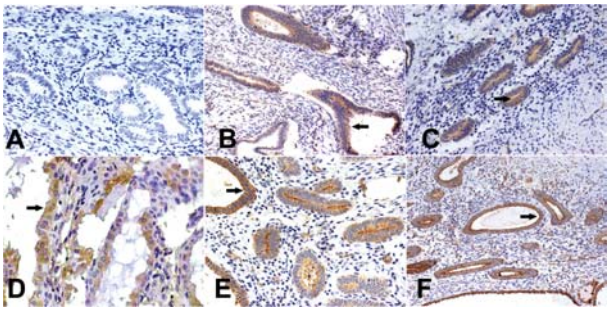


**Figure 2** Immunohistochemical expression analysis of KLKs in the epithelium of the endometrium.
Localization of the KLKs is indicated by arrows and magnification by ×. (A) Non-immune serum ×400 (no staining); (B) proliferative endometrium, KLK5 ×200; (C) proliferative endometrium, KLK6 ×400; (D) secretory endometrium, KLK11 ×400; (E) proliferative endometrium, KLK12 ×400; (F) proliferative endometrium, KLK13 ×200.

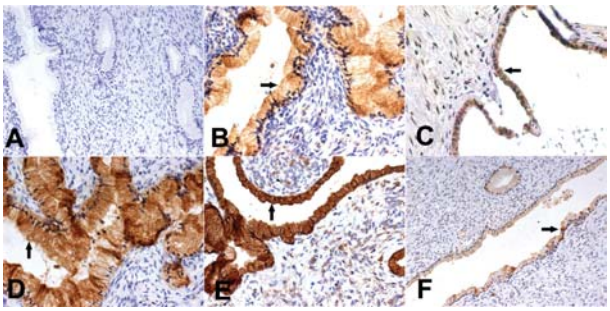


**Figure 3** Immunohistochemical expression analysis of KLKs in the epithelium of the cervix.
Localization of the KLKs is indicated by arrows at the indicated original magnification: (A) non-immune serum ×200 (no staining); (B) KLK5 ×400; (C) KLK6 ×400; (D) KLK11 ×400; (E) KLK12 ×400; (F) KLK13 ×200.

### Hormonal regulation of KLKs in CVF and saliva over the menstrual cycle

CVF and saliva samples were collected from a female subject and KLK levels were measured by ELISA. KLK levels were normalized for total protein and expressed as μg/g of total protein (Figure 5). In CVF, levels of KLKs 5, 6, 7, 11 and 12 were found to peak around day 20 (Figure

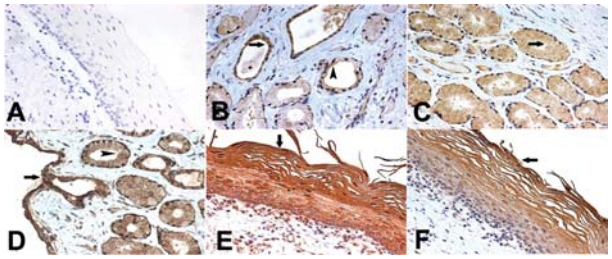


**Figure 4** Immunohistochemical localization of KLKs in the vagina.
The original magnification factor is indicated: (A) vaginal squamous epithelium, non-immune serum ×400 (no staining); (B) strong KLK5 immunohistochemical expression by the ductal epithelium of Bartholin's glands (arrow) and weaker expression by the mucus-secreting acinar cells (arrowhead) ×400; (C) KLK6 immunohistochemical expression by the mucus-secreting acinar columnar cells of Bartholin's glands (arrow); (D) KLK11 immunohistochemical expression by the ductal epithelium (arrow) and the mucus-secreting acinar cells (arrowhead) ×400; (E) KLK12 immunohistochemical expression in the squamous vaginal epithelium (arrow) ×400; (F) KLK13 immunohistochemical expression in the squamous vaginal epithelium (arrow) ×400.

5A), approximately 4 days post-ovulation. In saliva, KLK levels were found to peak slightly at mid-cycle and showed a major peak at day 25 (Figure 5B).

### Changes in trypsin-like activity in CVF and saliva over the menstrual cycle

Total trypsin-like activity in CVF and saliva was measured using the trypsin-like substrate, Val-Pro-Arg-amino-4-methylcoumarin (VPR-AMC). Activity levels are expressed as fluorescence units (FU) per min per microgram of total protein. Trypsin-like activity was found to peak around the time of ovulation (mid-cycle) in CVF (Figure 5C) and on day 25 in female saliva (Figure 5D).

### KLK levels in CVF from pregnant and non-pregnant women

CVF samples from 7 non-pregnant and 47 pregnant women were analyzed. Mean KLK levels between the two groups were compared using the Mann-Whitney test. KLK5, 6, 7, 8, 10, 11, 12 and 13 levels were higher in CVF from pregnant versus non-pregnant women (Table 1); however, only differences of KLKs 10, 11 and 12 reached statistical significance.

### Constitutive expression and hormonal regulation of KLKs in vaginal epithelial cells

KLKs are constitutively expressed and secreted into the culture supernatant of the human vaginal epithelial cell

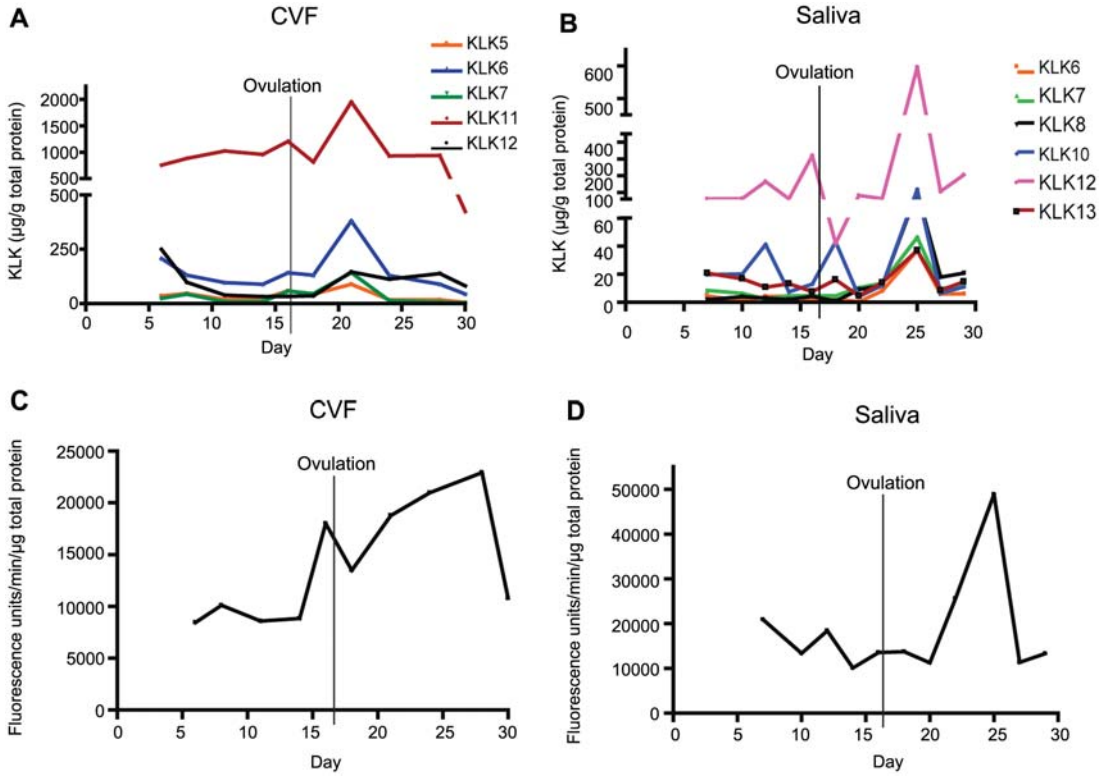


**Figure 5** KLK levels in CVF and saliva over the menstrual cycle and trypsin-like activity in CVF and saliva over the menstrual cycle. KLK levels were measured using ELISA immunoassays specific for each KLK of interest. KLK levels were normalized for total protein and expressed µg/g of total protein (TP). In CVF, expression of KLKs 5, 6, 7, 11 and 12 peaked around day 20 of the menstrual cycle (A). In saliva, expression of KLKs 6, 7, 8, 10, 12 and 13 peaked slightly at mid-cycle and showed a major peak in expression on day 25 (B). The same panel of KLKs was measured in CVF and saliva. Those KLKs not showing a peak in expression were removed to reduce congestion in the Figure. Total trypsin-like activity in CVF and saliva was measured using the fluorogenic substrate, valine-proline-arginine (VPR)-AMC. CVF or saliva was diluted 20-fold in 100 mM Tris, 100 mM NaCl (pH 8.0) and 0.2 mM VPR-AMC in a total volume of 100 µl. Fluorescence was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Enzymatic activity is expressed as fluorescence units per min per µg of total protein in each saliva sample, (C) CVF menstrual cycle 1, (D) saliva.

**Table 1** KLK levels in CVF from pregnant and non-pregnant women as measured by ELISA and normalized for total protein levels.

| KLK | Pregnant (μg/g TP) median | range | Non-pregnant (μg/g TP) median | range |
|---|---|---|---|---|
| 5 | 33 | 1.3–575 | 17 | 0.4–130 |
| 6 | 249 | 8.4–4739 | 144 | 31–1113 |
| 7 | 58 | 1.9–1050 | 35 | 7.6–252 |
| 8 | 21 | 3.9–199 | 19 | 9.4–39 |
| **10** | **209** | **30.7–1102** | **21** | **2.5–520** |
| **11** | **1263** | **412–5167** | **503** | **10.6–1348** |
| **12** | **1601** | **75–12 899** | **275** | **41–700** |
| 13 | 434 | 95–2205 | 327 | 20.6–736 |

Data in bold font denote significant differences using the Mann-Whitney test ($p<0.05$).

line VK2, as shown in Table 2. KLKs 5, 6, 7, 10 and 11 are downregulated by dexamethasone and/or estradiol in VK2 cells.

### KLK cleavage of cell-cell adhesion molecules

We investigated the *in vitro* processing of DSG1, DSC2 and DSC3 by active KLKs 5, 6, 11, 12 and 13, which are highly expressed in CVF. Recombinant DSG1/Fc, a chimeric protein comprising the five extracellular domains of DSG1, followed by a peptide linker and the Fc region of human IgG1 was incubated with active KLK5, 6, 11, 12 and 13 in Tris-buffered saline (TBS), pH 7.8 at 37°C for various time points. Cleavage of DSG1 was examined by silver staining, as shown in Figure 6, KLKs 5, 6 and 12 were able to cleave DSG1. KLK12 was found to process DSG1 quite extensively, compared with the other two KLKs. There was no cleavage by KLKs 11 or 13 (data not shown).

Partial recombinant proteins, DSC2 and DSC3 comprising the extracellular cadherin 5 domain of each protein linked to a GST tag, were incubated with active KLKs 5, 6, 11, 12 and 13 in TBS, pH 7.8 at 37°C for various time points. Cleavage was monitored by silver staining and Western blotting using a GST antibody, as shown in Figure 7. Both KLK5 and KLK12 were found to process DSC2 and DSC3 extensively after 2 h of incubation. KLKs 6, 11 and 13 did not cleave DSC2 or DSC3, even at higher ratios and after longer incubation times (data not shown).

### KLK cleavage of mucin proteins

To investigate whether KLKs are able to process the primary mucin proteins found in cervical mucus, mucin 4 and mucin 5B, partial recombinant MUC4 (comprising an extracellular portion of the protein) and MUC5B (comprising a C-terminal portion of the protein), both linked to a GST tag, were incubated with active KLKs 5, 6, 11, 12 and 13 in TBS, pH 7.8 at 37°C for various time points. Cleavage of the mucin proteins by the KLKs was monitored by silver staining and Western blotting, as shown in Figure 8. KLKs 5 and 12 were both found to cleave MUC4 and MUC5B extensively by the end of 2 h incubation, whereas KLKs 6, 11 and 13 did not process either of these proteins even after longer incubation times (data now shown).

### KLK cleavage of defensin proteins

Recombinant GST-linked full-length defensin-1α (DEFα) and defensin-1β (DEFβ) were incubated with active KLKs 5, 6, 11, 12 and 13 for various time points in TBS, pH 7.8. The silver stained gels and Western blots in Figure 9 show cleavage of DEFα by only KLK5. KLKs 6, 11, 12 and 13 did not cleave DEFα (data not shown) and no KLKs were found to cleave DEFβ (Figure 9).

### *Ex vivo* cleavage of substrates by proteases in CVF

Recombinant GST-linked DSC2, DSC3, MUC4, MUC5B, DEFα and DEFβ were incubated for 2 h with CVF collected at day 22 of the menstrual cycle (DSC2, DSC3, MUC4 and MUC5B) or 14 h (DEFα and DEFβ). The recombinant proteins were also incubated alone as a control. The Western blots in Figure 10 show that all of these proteins, with the exception of DEFβ, were cleaved by proteases within CVF.

## Discussion

The presence of large amounts of many KLKs in CVF is explained by their IE in the epithelium of all studied tissues (fallopian tube, endometrium, cervix and vagina) (Figures 1–4). Each of these tissues is known to contribute to the CVF milieu through secretions or exfoliation of cells. Furthermore, the higher levels of some KLKs (mainly KLK11) in the CVF, as well as in tissue extracts (Shaw

**Table 2** Constitutive expression and hormonal regulation of KLKs in the vaginal epithelial cell line VK2.

| Hormone | KLK | No stimulation (mean±SD) | After stimulation (mean±SD) | Fold change |
|---|---|---|---|---|
| Dexamethasone | 5 | 4324±444 | ↓(2959±327)‡ | -0.7 |
| | 6 | 30±0.8 | ↓19±1.3‡ | -0.6 |
| | 7 | 417±19 | ↓277±14‡ | -0.7 |
| | 10 | 308±61 | ↓(245±32)‡ | -0.8 |
| Estradiol | 6 | 30±0.8 | ↓(24±2.0)‡ | -1.3 |
| | 10 | 308±61 | ↓(197±61)‡ | -1.6 |
| | 11 | 51±0.4 | ↓(48±1.8)† | -1.1 |

†$p<0.05$; ‡$p<0.01$.

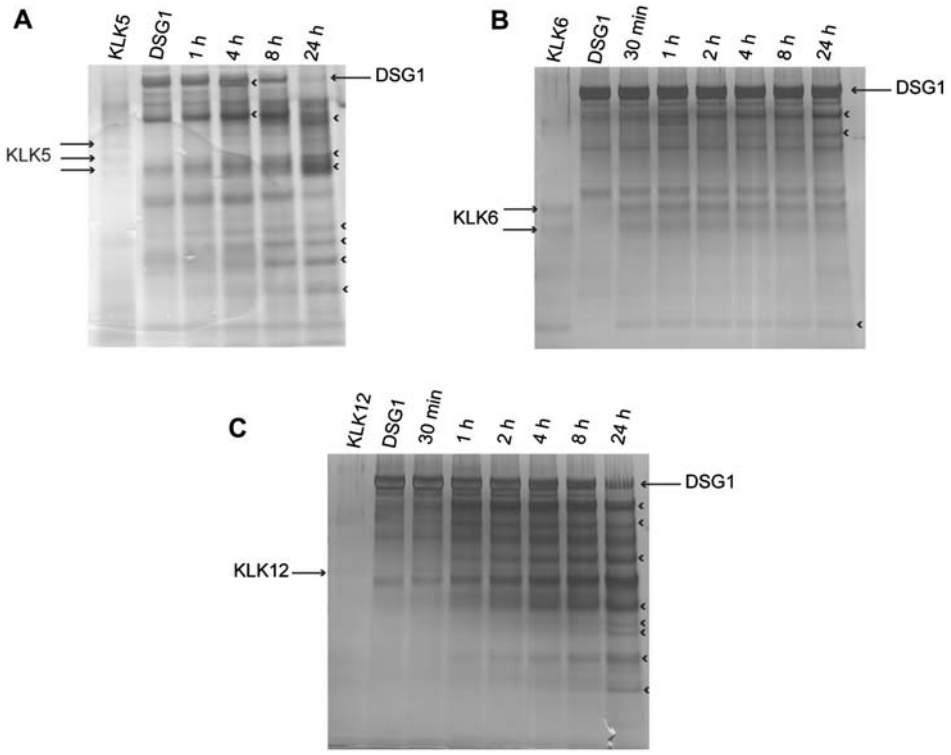


**Figure 6** Silver stains showing cleavage of desmoglein-1 (DSG1) by KLKs 5, 6 and 12.
A total of 100 ng of recombinant Fc-DSG1 was incubated with 10 ng of active KLK5 or KLK12 in TBS, pH 7.8 for various time points. Samples were resolved using 4–12% gradient gels and proteins were stained with silver. KLK 5 (A), 6 (B) and 12 (C) were found to cleave DSG1. Cleaved fragments are marked with arrowheads.

and Diamandis, 2007) matches with our findings of stronger IE of these KLKs in the corresponding tissues.

During the menstrual cycle, we found that KLK levels peaked following ovulation in the CVF and saliva of one woman (Figure 5). We chose to measure KLK levels in saliva, in addition to CVF, because hormonal levels in saliva are often measured when monitoring fertility status in women (Ellison, 1993; Hofman, 2001). These results are comparable to those of KLK1 and KLK3 levels in saliva and serum, respectively, over the menstrual cycle (Bhoola et al., 1978; Zarghami et al., 1997). We also found that total trypsin-like activity in CVF and saliva closely paralleled changes in KLK expression levels (Figure 5), suggesting that KLKs may be active in CVF.

Following ovulation, estrogen levels begin to fall and progesterone levels rise (Faro, 2004), suggesting that KLKs may be regulated by progesterone during the menstrual cycle. In saliva, progesterone levels are found to peak around day 25 (Gandara et al., 2007), further suggesting that KLKs may be regulated by progesterone during the menstrual cycle. During pregnancy, progesterone levels are highly elevated (Parker, 1998). We found that KLK levels increase in CVF from pregnant women compared to non-pregnant women (Table 1), further suggesting that KLKs may be regulated by progesterone. The menstrual cycle stage was not determined at the time of CVF collection from non-pregnant women. This may explain the wide variation in KLK levels between individuals. The stage of pregnancy was known for the CVF samples collected from pregnant women; however, we did not find any correlation between pregnancy stage and KLK levels (data not shown).

During the ovulatory and post-ovulatory period, rising progesterone levels result in the shedding of the vaginal epithelium. KLKs are known to play a role in the desquamation of skin corneocytes through cleavage of desmoglein (DSG1) and desmocollin (DSC1) cell-cell adhesion molecules (Caubet et al., 2004; Borgono et al., 2007). Processing of DSC2 and DSC3 (found in CVF; Shaw et al., 2007) by KLKs has not previously been investigated. We demonstrated cleavage of these two proteins by KLKs 5 and 12 (Figure 7). KLK12 processing of cell-cell adhesion molecules has not previously been investigated. Here, we show that KLK12 can digest DSG1, DSC2 and DSC3 *in vitro* (Figures 6 and 7) and suggest that KLK12 may also play a role in desquamation, at least in the context of the vaginal epithelium. Examination of DSC2 and DSC3 cleavage by enzymes within CVF *ex vivo* (Figure 10) revealed a cleavage pattern for DSC2 similar to DSC2 cleavage by KLK12 and a cleavage pattern for DSC3 similar to that produced by incubation of this protein with KLK5 (Figure 7). In addition, KLK12 was found to be highly expressed in the stratified squamous epithelium of the vagina (Figure 4). These results further implicate KLK12 in desquamation of the vaginal epithelium during the menstrual cycle.

It has been suggested that proteolytic enzymes may affect the physical properties of mucin proteins causing the changes in mucus observed over the menstrual cycle (Moghissi and Syner, 1970). We found that KLKs 5 and

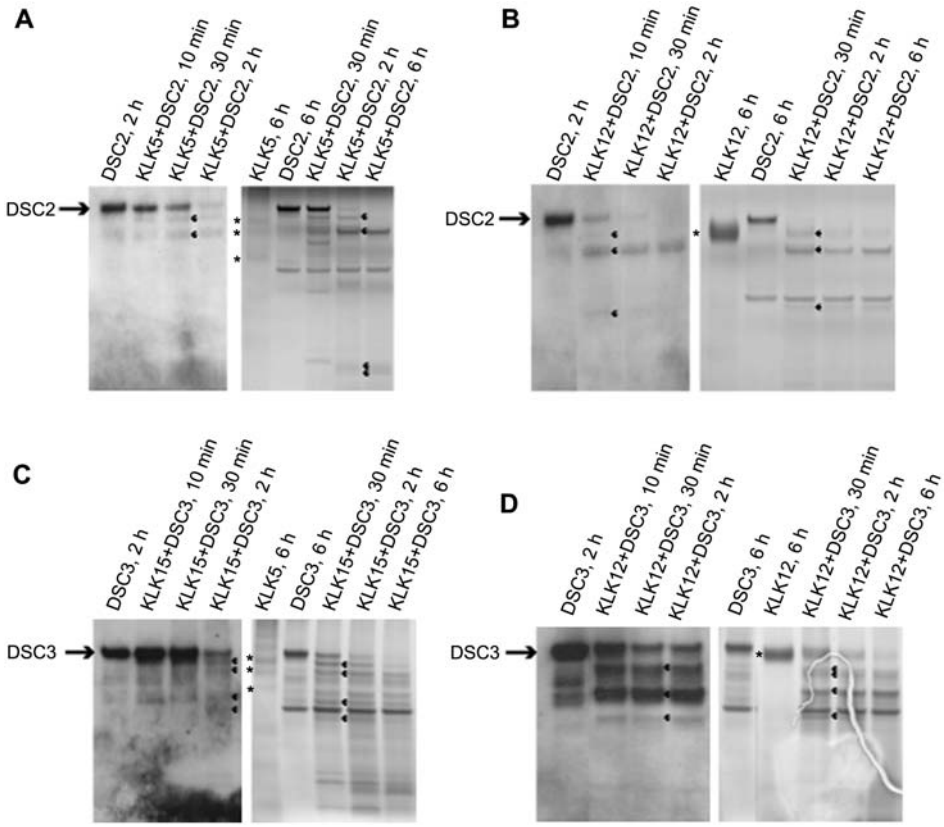


**Figure 7** Silver stains and Western blots showing cleavage of desmocollin-2 (DSC2) and desmocollin-3 (DSC3) by KLKs 5 and 12. A total of 100 ng of recombinant GST-linked DSC2 and DSC3 were incubated with 10 ng of active KLK5 or KLK12 in TBS, pH 7.7 at 37°C for various time points. The proteins were resolved and visualized by silver staining (right panels) as described above. Proteins were also transferred to nitrocellulose for Western blotting using a GST antibody (left panels). Cleavage products are noted with arrowheads, KLK proteins are indicated with stars. (A) DSC2 cleavage by KLK5, (B) DSC2 cleavage by KLK12, (C) DSC3 cleavage by KLK5, (D) DSC3 cleavage by KLK12.

12 were both able to cleave MUC4 and MUC5B *in vitro* (Figure 8). Given that KLKs are expressed by the mucus-secreting columnar epithelial cells of the cervix, these results suggest that KLKs may be involved in the remodeling of cervical mucus. The *ex vivo* cleavage pattern of MUC4 by CVF (Figure 10) was similar to the pattern produced by incubating KLK5 with MUC4 (Figure 8), whereas the pattern of MUC5B cleavage by CVF (Figure 8) did not resemble cleavage by either KLK5 or KLK12 (Figure 8). These results further suggest a role for KLKs 5 and 12 in the remodeling of cervical mucus during the menstrual cycle.

Approximately 200–500 million spermatozoa are deposited into the cervix during a normal ejaculation episode. For fertilization to take place, sperm must migrate through the cervical mucus into the uterus and subsequently into the fallopian tube, where fertilization most often takes place (Moghissi, 1973). Around the time of ovulation cervical mucus changes and the levels of MUC4 and, particularly, MUC5B increase, causing the mucus to lose viscosity (Gipson, 2001; Brunelli et al., 2007). Here, we suggest that KLKs may play a role in the remodeling of cervical mucus through their cleavage of mucins 4 and 5B (Figure 8), the primary mucins found in cervical mucus (Gipson, 2001). We suggest that KLKs may be important for the return and maintenance of cervical mucus as scant and viscous, in the post-ovulatory period. These changes in cervical mucus are an important component of the actions of many contraceptives, particularly those which contain progesterone (Fisher and Black, 2007). It is also possible that KLKs contribute to the action of progesterone-based contraceptives through the processing of mucin proteins.

The vagina is open to the outside world and thus exposed to many microorganisms, particularly during sexual intercourse. As such, host defense is a prominent aspect of vaginal physiology and CVF plays an important role in this defense (Cole, 2006). In particular, cationic peptides, such as defensins and the human cathelicidin, have been shown to be fundamental in defending the vagina from infectious agents (Cole, 2006).

Defensin proteins are found abundantly expressed in tissues and biological fluids involved in host defense (Ganz, 2003). Human CVF is known to play an important role in host defense (Cole, 2006) and we found defensin-1$\alpha$ and 1-$\beta$ in human CVF by proteomic analysis (Shaw et al., 2007). The $\alpha$-defensins are proteins of approximately 100 amino acids with a signal peptide of approximately 20 amino acids. Proteolytic processing of these proteins results in active antimicrobial peptides of approximately 30 amino acids in length (Ganz, 2003). Interestingly, defensin levels in CVF have been found to

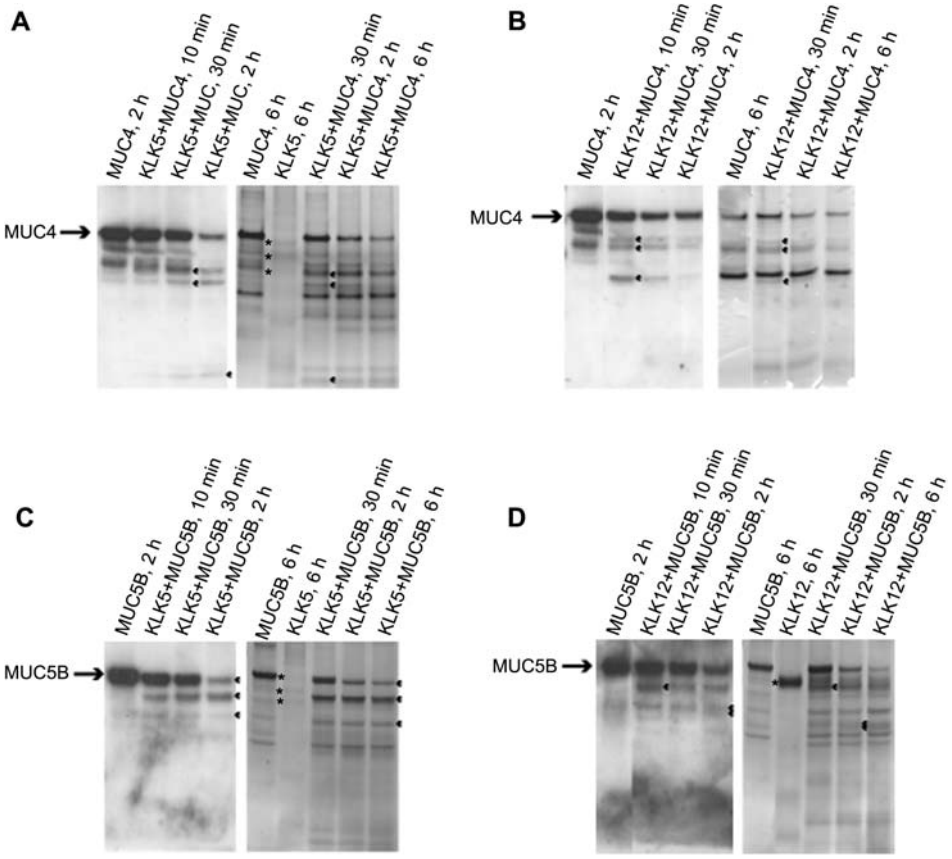


**Figure 8** Silver stains and Western blots showing cleavage of mucin-4 (MUC4) and mucin-5B (MUC5B) by KLKs 5 and 12. Recombinant GST-linked MUC4 and MUC5B were incubated with active KLK5 or KLK12 as described in the materials and methods section. The proteins were resolved by SDS-PAGE and cleavage was monitored by silver staining and Western blotting as described in the materials and methods section. (A) MUC4 cleavage by KLK5, (B) MUC4 cleavage by KLK12, (C) MUC5B cleavage by KLK5, (D) MUC5B cleavage by KLK12.

be highest during the secretory, post-ovulatory stage of the menstrual cycle, suggesting that they may be regulated by progesterone (Quayle et al., 1998).

We examined whether members of the KLK family are involved in the processing of $\alpha$ and defensin-1$\beta$ proteins *in vitro* and found that KLK5 can process defensin-1$\alpha$ (Figure 9). KLK5 has been shown previously to fulfill a similar role in skin, where it processes the human cathelicidin antimicrobial protein (Yamasaki et al., 2006). These results, together with those presented here, suggest that KLK5 may be an important regulator of antimicrobial activity. Incubation of defensin-1$\alpha$ with CVF containing high levels of KLKs revealed a cleavage pattern similar to that of KLK5 (Figure 10), further suggesting that KLK5 in CVF may be responsible for the processing of defensin-1$\alpha$. Given that KLKs also appear to be regulated by progesterone during the menstrual cycle, we suggest that KLKs may play an antimicrobial role through their processing and activation of active defensin peptides in the vagina during this time.

In summary, here, we propose plausible functions of many KLKs in CVF. We also show evidence for the regulation of KLKs by hormonal changes during the menstrual cycle. KLK levels predominate during the progesterone-rich post-ovulatory phase of the menstrual cycle. Through *in vitro* cleavage analysis we show that KLKs, in particular KLK12, may play a role in the shedding of vaginal epithelial cells following ovulation. We also suggest that KLKs may process cervical mucin proteins and contribute to cervical mucus during the post-ovulatory period. Finally, we show that KLK5 is capable of processing defensin-1$\alpha$ and may be important in vaginal host defense.

## Materials and methods

### Materials

Recombinant human desmoglein-1/Fc chimera was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human desmocollin-2 (DSC2), desmocollin-3 (DSC3), mucin 4 (MUC4), and mucin 5B (MUC5B), defensin-1$\alpha$ (DEF-$\alpha$) and defensin-1$\beta$ (DEF-$\beta$)-GST fusion proteins were purchased from Abnova Corporation (Taipei, Taiwan). Recombinant KLKs 5, 6, 11 and 13 were produced in our laboratory. These proteins were expressed, purified and activated as described elsewhere (Kapadia et al., 2003; Michael et al., 2005; Oikonomopoulou et al., 2006). The proform of KLK12 was purchased from R&D Systems and was auto-activated according to the manufacturer's recommendations. All KLK proteins were confirmed to be active against their preferred synthetic substrates.

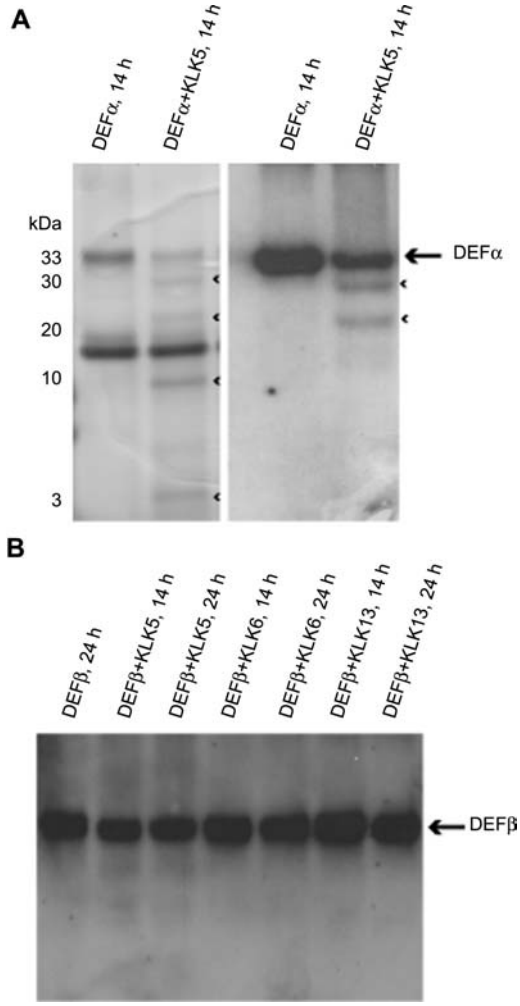


**Figure 9** Silver stains and Western blots showing cleavage of defensin-1α (DEFα) and defensin-1β (DEFβ) by KLK5.
Recombinant GST-linked DEFα and DEFβ were incubated with active KLK5 and cleavage was monitored by silver staining and Western blotting as described in the materials and methods section. (A) DEFα cleavage by KLK5, (B) DEF β cleavage by KLKs (no cleavage by any KLK; for more details, see text).

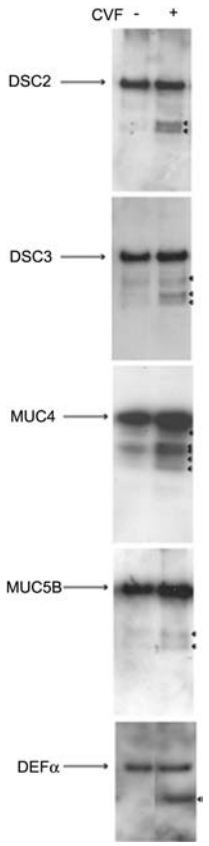


**Figure 10** *Ex vivo* cleavage of desmocollins (DSCs), mucins (MUCs) and defensins (DEFα) by CVF proteases.
A total of 100 ng of recombinant DSC, MUC or DEFα was incubated in PBS, pH 7.2 or in CVF (diluted in PBS, pH 7.2) for 2 h (DSC and MUC) or 14 h (DEFα) at 37°C. Samples were resolved and cleavage was monitored as described in the materials and methods section. For more details, see text.

Synthetic substrates, Val-Pro-Arg-amino-4-methylcoumarin (VPR-AMC) and Val-Leu-Lys-thiobenzyl-ester (VLK-SBzl) were purchased from Bachem Bioscience (King of Prussia, PA, USA).

All steroid hormones were purchased from Sigma-Aldrich (St. Louis, MO, USA). All steroid hormone stock solutions ($10^{-5}$ M) and dilutions were prepared in 100% ethanol.

## Cell line

VK2 vaginal epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). This epithelial cell line was established from the normal vaginal mucosa of a pre-menopausal woman. The cells were immortalized with the retroviral vector LXSN-16E6E7 and are characteristic of stratified squamous, non-keratinizing epithelia.

## Cell culture

VK2 cells were maintained in keratinocyte serum-free medium supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen Canada Inc., Burlington, ON, Canada). Cells were grown to 60–90% confluence and then seeded at a density of 500 000 cells/well in a 6-well plate. Cells were left for 24 h, after which the medium was removed and replaced with RPMI containing 10% charcoal-dextran stripped fetal bovine serum. At this point, cells were stimulated once with either alcohol (<1% ethanol final concentration as a control), dexamethasone, norgestrel or estradiol (all at $10^{-8}$ M final concentration). Cells were incubated for 7 days and the supernatant was collected and frozen at -20°C until use. All hormonal stimulations were performed in triplicate.

## Immunohistochemistry

The immunohistochemical staining was performed on 3 μm thick paraffin sections of tissues fixed in buffered formalin. Staining procedures included deparaffinization in warm xylene for 5 min with two changes of xylene at room temperature, followed by re-hydration by transfer through graded alcohols and then rinsing with distilled water. The Trilogy antigen retrieval system (Cell Marque, Rocklin, CA, USA) was used for 1 h in order to expose antigen epitopes. After 20 min at room temperature and rinsing with distilled water, the sections were put in 3% $H_2O_2$ for 10 min in the dark. After washing with tap water, the sections were dipped twice for 5 min in TBS and incubated with the KLK polyclonal antibodies at the following dilutions: KLK5, 1:300; KLK6, 1:600; KLK11, 1:600; KLK12, 1:1000; KLK13, 1:400 for 30 min, rinsed with TBS for 10 min and then incubated with the Envision detection system peroxidase/DAB+, Rabbit/Mouse (DAKO Cytomation; Dako, Glostrup, Denmark) for 30 min. This was followed by rinsing with TBS for 10 min, incubation in diaminobenzidine (DAB) solution for 10 min at room temperature

and rinsing with tap water. The sections were then counterstained with hematoxylin, dehydrated, cleared in xylene and mounted. Negative controls were performed for all studied tissues by omitting the primary antibody or by replacing it with non-immune serum (dilution 1:500).

### CVF and saliva sample collection

Tampons were provided to a healthy, 30-year-old female volunteer, who was not pregnant. The subject was asked to insert the tampon into her vagina for 1 h, every other day, for an entire menstrual cycle. The tampons were then removed and stored in 50 ml plastic conical tubes (BD Biosciences, Mississauga, ON, Canada), at -20°C until use.

CVF was collected from pregnant women using a polyester vaginal swab. The swab was rolled across the posterior vaginal fornix to absorb fluid. The swab was then inserted into 1 ml of sterile phosphate-buffered saline (PBS) and stored at -80°C until use.

Saliva samples were also collected from one female over one menstrual cycle. Saliva was mixed 1:1 with PBS (pH 7.2) and stored at -20°C until use.

Our protocols have been approved by the Institutional Review Boards of Mount Sinai Hospital and the University of Toronto. Written informed consent was obtained from all subjects.

### CVF extraction

Tampons used to collect CVF were thawed, 20 ml of sterile PBS was added and the tube was mixed by rotation for 14 h. The extract was removed from the tampon using a 20 ml syringe, which was used to squeeze the fluid out of the tampon. The CVF samples were stored at -20°C until use.

### KLK ELISA immunoassays

The ELISA immunoassays used to measure KLK levels in hormonally stimulated vaginal epithelial cells, CVF and saliva have been described in detail elsewhere (Shaw and Diamandis, 2007).

### Analysis of trypsin-like activity in CVF and saliva

Total trypsin-like activity in CVF and saliva was measured using the fluorogenic substrate, VPR-AMC (Bachem Bioscience). CVF or saliva was diluted 20-fold in 100 mM Tris, 100 mM NaCl (pH 8.0) and 0.2 mM VPR-AMC in a total volume of 100 μl. Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Wallac fluorometer (Perkin Elmer, Waltham, MA, USA). Enzymatic activity was expressed as fluorescence units per min per microgram of total protein.

### KLK *in vitro* cleavage experiments

For analysis of substrate cleavage, 10 ng of KLK was incubated with 100 ng of recombinant protein in 1× TBS (pH 7.6 or pH 6.2) in a final volume of 20 μl at 37°C for 10 min, 30 min and 2 h for Western blotting (for desmocollins and mucins) and for 30 min, 2 h and 6 h for silver staining (for desmocollins and mucins). Control reactions containing recombinant protein alone were also performed for 2 h for Western blot analysis (for desmocollins and mucins). KLK alone and recombinant protein alone reactions were carried out for 6 h for silver stain analysis (for desmocollins and mucins). Cleavage and control reactions were carried out for 1, 2, 4, 8 and 24 h for desmoglein-1 and for 14 h for the defensins. After the specified amount of time, samples were flash-frozen in liquid nitrogen and stored at -80°C until use. For silver stain analysis, samples were thawed, resolved by SDS-PAGE using the NuPAGE bis-Tris gel electrophoresis system and 4–12% gradient gels (Invitrogen) under reducing conditions. Protein mixtures were visualized by silver staining using the Silver Xpress® silver stain kit (Invitrogen) according to the manufacturer's directions.

For Western blot analysis, samples were thawed and resolved by SDS-PAGE as described above, then transferred to Hybond-C blotting membrane (GE Healthcare, Baie d'Urfe, QC, Canada). Membranes were blocked in 5% milk in Tris-buffered saline-Tween (TBST) for 1 h at room temperature. Membranes were probed overnight at 4°C with an anti-GST antibody (Cell Signaling, Boston, MA, USA), diluted 1000-fold in 1% milk in TBST. Membranes were then washed 3 times for 15 min in TBST and incubated with alkaline phosphatase-conjugated goat-anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA), diluted 5000-fold in 1% milk in TBST for 1 h at room temperature. Membranes were washed again, as above, and fluorescence was detected using a chemiluminescent substrate (Diagnostic Products Corp., Los Angeles, CA, USA).

### CVF *ex vivo* cleavage experiments

A total of 100 ng of recombinant substrate protein (DSC2, DSC3, MUC4, MUC5B, DEFα or DEFβ) was incubated with CVF (day 22, the sample containing the highest levels of KLKs) in a total reaction volume of 20 μl. For DSCs and MUCs, incubations were for 2 h. For DEFα and DEFβ, samples were incubated for 14 h. These incubations were performed at 37°C with constant shaking. Following incubation, samples were flash-frozen in liquid nitrogen and stored at -80°C until use.

Cleavage analysis was performed by Western blotting with a GST antibody, as described above.

### Statistical analysis

The detection limit of each immunoassay was $\leq$0.2 μg/l. KLK levels undetectable by immunoassay upon alcohol stimulation were assigned a value of 0.2 μg/l for statistical analysis. Statistical analysis was performed using Prism software (version 4.02, Graphpad Software, La Jolla, CA, USA). The differences in mean expression levels between alcohol and each of the hormonal stimulations were calculated using one-way analysis of variance followed by Dunnett's post-hoc analysis. Differences in means with p-values $<$0.05 were considered to be statistically significant.

Differences in mean KLK levels in CVF from pregnant women versus non-pregnant women were compared using the Mann-Whitney test. Differences in means with p-values $<$0.05 were considered statistically significant.

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Received July 16, 2008; accepted September 8, 2008