Biol. Chem., Vol. 387, pp. 643–652, June 2006 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2006.083

Review

The kallikrein world: an update on the human tissue kallikreins

Miltiadis Paliouras[1,2] and Eleftherios P. Diamandis[1,2,3,*]

[1] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto M5G 1X5, ON, Canada
[2] Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto M5G 1L5, ON, Canada
[3] Department of Clinical Biochemistry, University Health Network and Toronto Medical Laboratories, Toronto M5G 1X5, ON, Canada

* Corresponding author
e-mail: ediamandis@mtsinai.on.ca

Abstract

Human tissue kallikreins (hKs) are attracting increased attention owing to their association with various forms of cancer and other diseases. Human tissue kallikrein genes represent the largest contiguous group of proteases within the human genome. There are many areas of kallikrein research that need to be further explored, including their tissue expression patterns, their regulation, identification of specific substrates, their participation in proteolytic cascades, and their clinical applicability as cancer biomarkers and therapeutic targets. In this review, we briefly describe the current status of kallikrein research and identify future avenues that will enhance our understanding of their function and involvement in human diseases.

Keywords: cancer; clinical applications; proteolytic cascade; regulation; serine proteases.

Introduction

The last decade has witnessed a wealth of new information on human tissue kallikreins, including the identification of all members of this serine protease family, the discovery of the kallikrein locus, understanding of the regulation of their expression and their biological functions, and their role in cancer biology and other disorders. These accomplishments have been thoroughly chronicled in three extensive reviews, as well as in many research articles (Yousef and Diamandis, 2001; Borgono and Diamandis, 2004; Borgono et al., 2004). The autumn of 2005 saw the 1st International Kallikrein Symposium in Lausanne, Switzerland, giving researchers an opportunity to discuss their findings. In this review we summarize the current status of kallikrein research and the ongoing efforts to understand their physiological functions and associations with human diseases.

Historical perspective

Of the 178 human serine proteases, accounting for the 32% of all proteases, the human tissue kallikreins represent the largest contiguous cluster of protease genes in the human genome. *Human kallikrein 1* (*hK1*), *human kallikrein 2* (*hK2*) and *prostate-specific antigen* (*PSA*, *hK3*), were the first members of this family to be studied, with hK1 showing abundant levels in the pancreas (derived from the Greek 'kallikreas'), from which these genes derived their name (Kraut et al., 1930). Between 1994 and 2001, the kallikrein family expanded to include 15 genes and a complete description of the human kallikrein locus was reported. The newly identified kallikreins share significant similarities to the hK1, hK2 and PSA kallikreins, and include *human stratum corneum chymotryptic enzyme* (*HSCCE*) */hK7*, *normal epithelial cell-specific gene 1* (*NES1*)*/hK10*, *protease M/zyme/neurosin/hK6*, *neuropsin/TADG-14/hK8*, *trypsin-like serine protease* (*TLSP*)*/hippostasin/hK11*, *prostase/KLK-L1/ARM1/PRS-S17/hK4*, *human stratum corneum tryptic enzyme* (*HSTCE*)*/KLK-L2/hK5*, *KLK-L3/hK9*, *KLK-L4/hK13*, *KLK-L5/hK12*, *KLK-L6/hK14*, and *prostinogen/hK15*, as well as the first kallikrein pseudogene, *ΨKLK1* (Yousef and Diamandis, 2001; Yousef et al., 2004). These newly discovered kallikreins all map to the same chromosomal region (19q13.4) as the three classical kallikreins.

Kallikrein locus and gene structure

The organization of the human kallikrein locus has been extensively described in several reviews; the locus spans approximately 300 kb on the long arm of chromosome 19 in the cytogenic region 13.4 (Yousef et al., 2000). The *KLK* genes are bound centromerically by the testicular acid phosphatase gene (*ACPT*) and telomerically by *Siglec-9* (a member of the sialic acid-binding immunoglobulin-like lectin family). These flanking genes have no structural or functional relationship to the human kallikreins. The *KLK* genes are tightly grouped and arranged tandemly without any intervention by any non-*KLK* genes. The three classical kallikreins and *KLK15* are clustered in a 60-kb region, followed by the pseudogene *ΨKLK1*, and the 11 other *KLK* genes, with the direction

In this article kallikrein genes are denoted as *KLK1* … *KLK15* and kallikrein proteins as hK1 … hK15, in accordance with the currently approved nomenclature (Diamandis et al., 2000b). Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., this issue pp. 637–641.

of transcription of all genes from telomere to centromere, with the exception of *KLK3* (*PSA*) and *KLK2*.

All the *KLK* genes share many common characteristics, including: five coding exons, similar or identical coding exon lengths, and conserved protease catalytic triad residues His, Asp and Ser in exons 1, 3 and 5, respectively. Most *KLK* genes also have a number of splice variants and/or alternative transcriptional start sites. With the exception of *KLK14*, all kallikreins have at least one alternative transcript, exclusive of their reference form, with *PSA* followed by *KLK13* having the highest number of alternative transcripts (Kurlender et al., 2005). Most of these alternative *KLK* transcripts are predicted to code for truncated proteins as a result of a frameshift in the coding sequence or an in-frame deletion. The biological and physiological significance, if any, of truncated hK proteins or the regulation of alternative *KLK* transcripts is unknown.

KLK transcripts code for a single-chain serine protease pre-proenzyme. The hK proteins, in addition to conservation of their catalytic residues, share an overall amino-acid sequence identity of 40–80%, with the highest degree of similarity between hK2 and PSA. Along with sharing common elements at the genomic level, all hK proteins possess a signal peptide, so that all hK pro-forms (zymogens) are expected to be secreted. Table 1 lists the amino acid residues around the activation site of all hKs. With the exception of hK4, all have a pro-peptide ending in Lys or Arg, suggesting that these zymogens are activated by enzymes with trypsin-like activity. The majority of hK proteins (hK1–2, hK4–6, hK8, hK10–14) have an Asp residue in their binding pocket (or Glu for hK15), indicating that they have trypsin-like substrate specificity. According to the most recent investigation (Malm et al., 2000), PSA cleaves at chymotryptic sites, but also after Gln, Asn and His – an activity that is unique or perhaps could be defined as extended chymotrypsin-like. Similarly, hK7, also known as stratum corneum chymotryptic enzyme, also contains an Asn in the catalytic pocket. It has recently been shown that pro-hK proteins can serve as substrates for activated hKs. The functional significance of this phenomenon is discussed later.

Table 1 Amino acid cleavage sites required for kallikrein activation.

Kallikrein	Predicted hK activity	hK amino acid position				
		P3	P2	P1	P1′	P2′
hK1 and hK2	Trypsin-like	Q	S	**R**	I	V
hK3	Chymotrypsin-like	L	S	**R**	I	V
hK4	Trypsin-like	C	S	**Q**	I	I
hK5	Trypsin-like	S	S	**R**	I	I
hK6	Trypsin-like	Q	N	**K**	L	V
hK7	Chymotrypsin-like	G	D	**K**	I	I
hK8	Trypsin-like	E	D	**K**	V	L
hK9	Chymotrypsin-like	D	T	**R**	A	I
hK10	Trypsin-like	D	T	**R**	L	D
hK11	Trypsin-like	E	T	**R**	I	I
hK12	Trypsin-like	T	P	**K**	I	F
hK13	Trypsin-like	S	S	**K**	V	L
hK14	Trypsin-like	E	N	**K**	I	I
hK15	Trypsin-like	G	D	**K**	L	L

Activation occurs by cleavage after the amino acid shown in bold (single letter code).

Analytical measurement technologies

To facilitate quantitative analysis of kallikrein expression, technologies that can accurately measure kallikreins from different biological sources have been developed. Currently, two well-established technologies are in use to identify and measure kallikrein expression, RT-PCR and ELISAs. Initial kallikrein expression was assessed using RT-PCR to detect the presence of any individual kallikrein transcript directly from a tissue source. Much of the tissue expression profiles, along with steroid hormone studies of kallikrein gene regulation, took advantage of this simple and highly sensitive technique. However, because hK proteins are secreted into the extracellular matrix and fluids, ELISAs were developed to measure protein concentration from a wide variety of biological samples, such as serum and seminal plasma. To date, ELISAs have been developed for all hK proteins except for hK9, hK12, and hK15 (Table 2).

hK ELISAs have contributed to our understanding of the potential importance of hKs in cancer biology. Thus, many hKs have been identified as new biomarkers for several different forms of cancer. The same technologies are beginning to implicate hKs in several non-cancer diseases, such as skin disorders, diabetes and neurodegenerative diseases. The role of hK proteins in cancer biology is further elaborated in other sections.

Tissue expression

Using Northern blot, RT-PCR, and ELISA methodologies, it has been shown that tissue kallikreins are expressed in multiple organs. Interestingly, groups of *KLK* genes are often expressed within a specific tissue. For example, *KLK2*, *KLK3*, *KLK4*, *KLK5*, *KLK11*, and *KLK15* mRNA and/or proteins are found in the prostate. In addition, almost every kallikrein is expressed in the salivary gland, while other groups are found in the skin (*KLK5*, *KLK7*, *KLK8*, *KLK9*, *KLK11*, *KLK13*, and *KLK14*), breast (*KLK3*, *KLK4*, *KLK5*, *KLK6*, *KLK8*, *KLK10*, *KLK13*, *KLK14*), pancreas (*KLK1*, *KLK10*, *KLK12*), and the central nervous system (*KLK5*-*KLK9*, *KLK11*, *KLK14*). hK proteins have also been found in biological fluids such as serum, seminal plasma, and milk of lactating women, confirming that these are secreted proteins. Tissue-specific expression patterns have also been identified for a few alternative *KLK* transcripts. Some splice variants of both *KLK2* and *KLK3* seem to be exclusively expressed in the prostatic epithelium. In addition, splice variants of *KLK4*, *KLK8*, and *KLK13* are frequently found in the skin. The expression of multiple kallikreins within several tissues indicates the existence of a complex coordinating regulatory mechanism that links their expression to their downstream physiological function (reviewed by Borgono et al., 2004).

Table 2 ELISAs used in the present study.

Kallikrein	Coating/detection antibody	Dynamic range to (ng/l)	Detection limit (ng/l)	Reference
hK2	Mono/mono	2 000	6	Black et al., 1999
hK3	Mono/mono	2 000	1	Ferguson et al., 1996
hK4	Mono/poly	20 000	100	Obiezu et al., 2002
hK5	Mono/mono	25 000	100	Yousef et al., 2003a
hK6	Mono/mono	50 000	100	Diamandis et al., 2003
hK7	Mono/mono	20 000	200	Kishi et al., 2004
hK8	Mono/mono	20 000	200	Kishi et al., 2003
hK10	Mono/mono	20 000	50	Luo et al., 2001b
hK11	Mono/mono	50 000	100	Diamandis et al., 2002
hK13	Mono/mono	20 000	50	Kapadia et al., 2003
hK14	Mono/poly	20 000	100	Borgono et al., 2003

Mono, monoclonal mouse antibody; poly, polyclonal rabbit antibody.

Regulation of *KLK* gene expression

Hormonal regulation

The regulation of gene expression by steroid hormones plays an important role in the normal development and function of many organs, as well as in the pathogenesis of endocrine-related cancers. A number of experiments in endocrine-related tissues, in both cell culture and *in vivo*, have shown that most, if not all, *KLKs* are under steroid hormone regulation.

By far, the *KLK* for which regulation by steroid hormones has been most thoroughly studied is *KLK3*. Initially, two androgen response elements (ARE-I and ARE-II) were identified in the upstream promoter region (-170 and -400 bp), functionally tested and found to be active in LNCaP, a prostate cancer cell line (Riegman et al., 1991; Cleutjens et al., 1996). An additional ARE was found at -4316 bp, which induced a dramatic increase in *KLK3* transcription in comparison to ARE-I and ARE-II (Schuur et al., 1996). AREs have also been identified in *KLK2*, including one at position -170 bp and another in an enhancer region approximately 3000 bp upstream from the transcriptional start site, a similar organization of regulatory elements to *KLK3* (Murtha et al., 1993; Yu et al., 1999). Along with androgen sensitivity in prostate cancer cell lines, *KLK2* and *KLK3* expression is also up-regulated by androgens and progestins in the breast cancer cell lines BT-474, T-47D and MFM 223 (Magklara et al., 2000). *KLK4* was also found to be up-regulated by androgens in the prostate cancer cell line LNCaP and the breast cancer cell line BT-474. Putative AREs have been identified in the immediate upstream promoter region of *KLK4*, although they have not been functionally tested. Such similarities could account for the shared expression patterns observed between these three genes, especially in androgen-sensitive organs such as the prostate (Obiezu et al., 2002, 2005).

Of the remaining kallikrein genes, many show sensitivity to steroid hormones in various cancer cell lines. Most notable are *KLK6* and *KLK10*, which are highly responsive to estrogens in breast cancer cell lines (Yousef et al., 1999; Luo et al., 2000, 2003a). However, promoter deletion analysis of these two genes could not identify any functional response elements, either in immediate upstream promoter sequences or in potential enhancer regions, to mediate transcriptional activation by steroid hormones. Difficulties that have arisen from traditional analysis of promoter deletion constructs include the possibility that transcriptional gene activation may require the coordinated binding of a number of coactivating factors along with the hormone receptor or be mediated indirectly via other hormone-dependent activated *trans*-acting factors (Luo et al., 2003a).

More recently, several studies suggest the possibility that signal transduction pathways may influence the hormonal regulation of kallikrein gene expression. The traditional understanding of androgen receptor (AR)-induced gene expression simply relied on binding of the hormone to the receptor and binding of the complex to the ARE upstream of the gene. The AR has been shown to be activated by several pathways, including MEK through the RAS pathway, AKT kinases and PKC, which sensitizes the receptor to low circulating levels of androgen (Blok et al., 1998; Lin et al., 2001; Rochette-Egly, 2003). Using RAS effector-loop gain-of-function RAS mutant stable cell lines, it has been shown that constitutive MEK activation can hyper-induce PSA protein expression in LNCaP cells under normal levels of androgen (Bakin et al., 2003). It is currently being investigated whether other kallikreins are also influenced by the RAS-MEK-ERK signal transduction pathway.

It is interesting to note that many kallikreins show both coordinated tissue and hormone-regulated gene expression. It is not clear whether these ‘cassettes’ of kallikrein expression are regulated by the same or different molecular mechanisms within the cell or tissue. The possibility exists for a single or multiple transcriptional control loci that would coordinate kallikrein expression in groups.

Transcriptional control by DNA methylation

Epigenetic control of kallikrein gene expression through DNA methylation is another means by which *KLKs* have been shown to be regulated. The most widely characterized kallikrein to be regulated by this mechanism is *KLK10*. This kallikrein gene has been shown to be down-regulated in breast cancer and lymphoblastic leukemia as a result of hypermethylation of CpG islands within exon 3 of the gene. *KLK10* DNA methylation control has also been shown *in vitro* in a wide variety of cancer cell lines (Li et al., 2001; Sidiropoulis et al., 2005). There is

emerging evidence that *KLK6* may also be regulated by a similar mechanism in breast cancer cells. DNA methylation control of other kallikreins is quite preliminary and varies between different cancer cell lines (G. Sortiropoulou, personal communication, and our unpublished data).

Kallikrein dysregulation in cancer

Much has already been alluded to in this review about the up-regulation of several kallikreins in different cancers. Overall, carcinogenesis is a complex process that is a result of alterations in gene expression. One of the goals of cancer research is to identify these alterations and to determine their effects on tumor phenotype. All 15 kallikrein genes show differential expression patterns in many cancers (primarily endocrine or hormone-related cancers) at the mRNA and protein levels. Identifying gross genetic aberrations within the kallikrein locus of diseased tissues that show kallikrein dysregulation is also currently being examined. We briefly analyze the role of kallikreins in ovarian, breast and prostate cancers.

Ovarian cancer Ovarian cancer is a common malignancy among women in North America. It has been shown that *KLK4*, *5*, *6*, *7*, *8*, *10*, *11*, *13*, *14*, and *15* are overexpressed in ovarian carcinoma tissues, serum, and cell lines at either the mRNA or protein level or both (Obiezu and Diamandis, 2005). In particular, *KLK4* and *KLK5* mRNAs have been shown to be overexpressed and are indicators of poor prognostic outcome in grade 1 and grade 2 tumors, suggesting that these genes are associated with more aggressive forms of ovarian cancer (Kim et al., 2001; Obiezu et al., 2001). *KLK6*/hK6 appears to be one of the most promising ovarian cancer biomarkers among the kallikreins. Initially discovered by differential display to identify serine proteases with a strong expression pattern in ovarian cancer cell lines and ovarian carcinomas, the work was followed by examining hK6 protein expression in 44 ovarian tumors. Upon comparison with 10 normal ovarian tissues, it was shown that hK6 was overexpressed more often in tumors. Ovarian cancer patients who show high levels of hK6 protein in serum are not responsive to chemotherapies and have lower disease-free and overall survival (Diamandis et al., 2000a, 2003; Tanimoto et al., 2001; Hoffman et al., 2002). At the genetic level, Southern blot analysis of ovarian tumor samples suggests that amplification of the *KLK6* gene may be a possible explanation for the dysregulated expression of hK6 (Ni et al., 2004).

KLK10/hK10 is another kallikrein that is an unfavorable ovarian cancer prognostic/predictive biomarker. Overexpression of hK10 protein was observed in primary ovarian tissue lysates and mRNA by *in situ* hybridization and was also overexpressed in tumor tissue versus normal epithelial or stromal cells. A study of ovarian cancer tissue extracts indicated that high concentrations of hK10 were significantly associated with serous histotype, advanced stage, and large residual tumor size. hK10 protein levels are also found in high concentrations in the serum of the majority of ovarian cancer patients in comparison to healthy controls (Luo et al., 2001a; Shvartsman et al., 2003; Yousef et al., 2003b). Other data indicate that for stage III and IV patients, hK10 was an independent indicator of reduced overall and progression-free survival. Taken together, all these studies suggest that hK10 is a new serological marker for diagnosis and monitoring of ovarian cancer. Combination of hK6 with CA125 (a well-characterized and widely used ovarian cancer marker) yielded a 21% increase in sensitivity (at 90% specificity) over sensitivity of CA125 alone (Luo et al., 2003b).

Whereas *KLK6*/hK6 and *KLK10*/hK10 are unfavorable markers of ovarian cancer, studies of *KLK11*/hK11 and *KLK13*/hK13 expression have found them to be independent indicators of favorable outcome for overall survival. In these studies, hK11- and hK13-positive tumors were associated with early stage (I and II) of the cancer and complete or partial response to chemotherapy (Scorilas et al., 2004; Shigemasa et al., 2004).

Breast cancer Early diagnosis of breast cancer is very important, as 5-year survival rates drop dramatically from 97% for localized tumors to 79% for regionally spread tumors and to 23% for metastatic tumors (Jemal et al., 2004). Many kallikreins have been assessed as prognostic indicators in breast cancer.

Using quantitative RT-PCR analysis, *KLK5* expression was shown to be an indicator of poor prognosis in all patients, as well as in a subgroup of early stage (I and II) tumors (Yousef et al., 2002b, 2003a). hK6 protein is another kallikrein found to be expressed in primary mammary carcinoma cell lines, but it is absent in corresponding cell lines of metastatic origin (Yousef et al., 1999; Pampalakis et al., 2004). It is unclear at the molecular level as to why these kallikreins are up-regulated in breast cancer. *KLK6* and *KLK5* expression in breast cancer cell lines does not seem to be influenced by DNA methylation. As mentioned earlier, we are now realizing that many signal transduction pathways play a role in regulating kallikrein gene expression. It has been found that approximately 30% of all breast cancers either have a deletion or mutation in the gene encoding the tumor suppressor protein phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*). PTEN is a negative regulator of AKT function, resulting in increases in cell growth and proliferation. Therefore, it is also currently being investigated as to whether kallikrein gene expression can be regulated through AKT function (De-Graffenried et al., 2004). It is worth noting that *PTEN*-deficient cells are no longer sensitive to current therapeutics agents such as CCI-779 and tamoxifen (Peralba et al., 2003; Noh et al., 2004).

Originally cloned as a putative tumor suppressor, with loss of expression in breast cancer cell lines, *KLK10* has been extensively studied in breast tumors. Study of *KLK10* mRNA by *in situ* hybridization on tissue sections from normal breast, typical and atypical hyperplasia, as well as infiltrating ductal carcinoma, has shown that while all normal and a large majority of hyperplasia samples showed *KLK10* expression, more than half of the ductal carcinoma and 29 of 30 infiltrating ductal carcinomas completely lacked *KLK10* expression (Liu et al., 1996; Luo et al., 2001b). This suggests that loss of *KLK10* expression is required for tumor progression. *KLK14* expression also relates to stage and breast cancer pro-

gression. Analysis of 178 breast carcinoma samples suggested that higher *KLK14* expression was more frequently present in patients with advanced stage disease, indicating that *KLK14* expression is associated with poor prognosis for the disease (Yousef et al., 2002a; Borgono et al., 2003).

Prostate cancer As discussed above, several kallikreins are normally expressed in the prostate. PSA is the best prostate tumor-screening marker available to date. This screening tool has shortcomings, one of which is its inability to distinguish between benign prostatic hyperplasia (BPH) and prostate cancer (Rittenhouse et al., 1998; de Koning et al., 2002). Therefore, many kallikreins have been examined at the RNA and protein level as candidate prostate cancer biomarkers.

A study of 90 pairs of non-cancerous and cancerous prostate tissue samples using quantitative RT-PCR showed clear up-regulation of *KLK15* mRNA. Levels of *KLK15* were significantly higher in patients with pT3/pT4 than in pT2 patients (Stephan et al., 2003). This indicates that *KLK15* levels may have utility in assessing the aggressiveness of prostate cancer.

hK11 protein is proving to be another promising novel biomarker for prostate cancer, as ELISA studies showed that this kallikrein is found at highest levels in prostate tissue, as well as in seminal plasma. The levels of hK11 in seminal plasma are comparable to those of hK2, although 300-fold lower than PSA (Nakamura et al., 2003). Analysis of plasma samples from 65 cancer patients has shown that hK11 is elevated in comparison to healthy controls. To investigate whether hK11 could distinguish between BPH and prostate cancer, the ratios of hK11 to total PSA (tPSA) were examined. This ratio was significantly lower in serum from prostate cancer patients than in serum of BPH patients (Stavropoulou et al., 2005). These data indicate that the combination of serum hK11 and total PSA could be used to reduce the number of prostatic biopsies.

Biological function of kallikreins

The first part of the past decade dealt with the identification, cloning, and characterization of tissue expression of kallikreins. More recently, efforts focused on understanding the physiological and biological functions of kallikreins in different tissues and diseases. Specific degradomic tools have been developed, such as phage display and combinatorial peptide-based specific profiling, to identify specific kallikrein substrates. Phage-display technology allows for the expression of all possible combinations of pentapeptide substrates and screening with selective kallikreins (Wu et al., 2000; Cloutier et al., 2002). Using similar principles as phage display, specific fluorogenic substrates have also been developed to determine the enzyme kinetics of kallikrein activity (Rehault et al., 2002). These methods have allowed for the characterization of highly selective substrates and potential kallikrein biological targets.

Phage-display technology has been applied to examine substrates for hK2, hK14, hK5 and hK8 proteins (Cloutier et al., 2002; Felber et al., 2005; and our unpublished data). Results from these experiments have identified several substrates that may have physiological relevance to kallikrein-associated disease manifestations, most importantly tumorigenesis. Phage display analysis of hK14 identified specificity towards both trypsin-like and chymotrypsin-like substrates. These substrates also displayed high selectivity for hK14 in comparison to other kallikreins, such as hK1, hK2, and PSA. Many of the substrates identified suggest that hK14 is able to cleave extracellular matrix (ECM) proteins, including laminin α-5 chain precursor, matrilin-4, and collagen IV (Felber et al., 2005). *In vitro* analysis has shown that hK5, hK6 and hK13 are also able to hydrolyze a variety of ECM proteins, including laminin, fibronectin and collagen I, II, and II (Ghosh et al., 2004; Kapadia et al., 2004a; Michael et al., 2005). Taken together, the discovery that these kallikreins are able to hydrolyze ECM proteins and the dysregulated expression of these proteins in breast and ovarian cancer cells indicate the possibility that kallikreins could contribute to the invasiveness and/or angiogenesis of these cancers. Other kallikreins have already been suspected of favoring migration of cancer cells, such as PSA and hK4. Prostate cancer cells overexpressing these proteins showed both increases in cell migration, linked to loss of E-cadherin, and an increase in vimentin, providing compelling evidence that these kallikreins have a role in prostate cancer progression through their promotion of tumor cell migration (Matsumura et al., 2005).

There are also several non-ECM-related proteins that are hydrolyzed by kallikreins. hK2, PSA, and hK4 may be regulators of the insulin-like growth factors (IGFs) in prostate carcinogenesis. IGF1 and IGF2 are important mitogens involved in regulating cellular proliferation, differentiation, apoptosis, and transformation. The action of IGFs requires their release from IGF-binding proteins (IGFBPs), a family of six proteins that block the binding of IGFs to the IGF1 receptor. It has been shown that hK2 and PSA are IGFBP proteases that can collectively degrade IGFBP2, IGFBP3, IGFBP4 and IGFBP5, resulting in release of IGF1, which in turn can interact with IGF1 receptor, stimulating the growth of normal, stromal, and malignant prostate cells (Cohen et al., 1992; Rehault et al., 2001).

hK5, 6, and 14 are able to cleave and activate protease-activated receptor (PAR) signaling (Oikonomopoulou et al., 2006). PARs are a family of four G protein-coupled cell-surface receptors that are activated by serine proteases by cleavage of their N-terminal extracellular segment, thus revealing a cryptic ligand, which in turn binds to the extracellular receptor domains initiating cell signaling. PAR signaling has been implicated in a variety of physiological processes, including regulation of muscle contraction, inflammation, cell adhesion, metastasis, and proliferation, along with apoptosis (Hollenberg and Compton, 2002). It was shown that these three kallikreins can selectively activate a set of PARs, resulting in PAR-related physiological changes in cellular biology. The evidence that hK proteins are functionally associated and interact with specific cell-surface receptors raises the possibility that they may be able to regulate their own expression through these signal transduction pathways.

Another important substrate of kallikrein activity is uPA. Both hK2 and hK4 can activate uPA, along with hK2 inactivation of plasminogen activator inhibitor 1 leading to activation of uPA (Frenette et al., 1997; Takayama et al., 2001). uPA, when bound to its cell surface receptor, uPAR, converts plasminogen to plasmin, leading to pericellular ECM degradation and the release and/or activation of tumor growth factors.

Finally, another kallikrein substrate subgroup that has been studied are the kallikreins themselves. The possibility that kallikreins can serve as substrates for other kallikreins was alluded to earlier when discussing the cleavage sites for activation (Table 1). It has been shown that hK5 can activate pro-PSA and pro-hK2 (our unpublished data). hK5 can also activate pro-hK7 under *in vitro* conditions (Caubet et al., 2004). Along with activating these enzymes, kallikreins can also deactivate each other by further degrading the protein. hK2 and hK6 have been shown to autoactivate themselves (Mikolajczyk et al., 1997; Magklara et al., 2003; Bayes et al., 2004). Taken together with the above-mentioned substrates, the coordinated 'cassette' expression of kallikreins in specific tissues and their dysregulation in several cancers creates a dynamic environment where the spatial expression of these proteins can have a profound impact on overall cell physiology.

Kallikrein proteolytic cascades

The idea that kallikrein enzymes participate in cascade pathways originated from the discovery of putative substrates combined with their expression patterns in different tissues. Currently, the functional characterization of a kallikrein proteolytic cascade pathway is being explored in two settings: skin desquamation and semen liquefaction. These pathways are discussed in some detail below.

Skin desquamation

In the skin, the degradation of corneodesmosomes leads to desquamation. Moreover, the proteolytic cleavage of extracellular cell adhesion molecules was shown to be important for this process in plantar and non-palmoplantar stratum corneum regeneration. Three extracellular proteins have been described as components of the corneodesmosomes, desmoglein 1 (DGS1), desmocollin 1 (DCS1) and corneodesmosin (CDSN). CDSN is progressively proteolyzed in the stratum corneum, strongly suggesting that its degradation is necessary for cell desquamation. The cleavage of DSG1 is also linked to scale shedding and its persistence is a characteristic of hyperkeratosis. hK5 and hK7 are both highly expressed in granular keratinocytes and are present in the intracellular spaces of the stratum corneum (Komatsu et al., 2003, 2005).

It was found that active hK5 and hK7 could proteolytically cleave CDSN, DGS1 and DCS1, with hK5 also activating pro-hK7. This is the first time that non-classical kallikrein enzyme function has been linked to a specific biological process. In turn, the proteolytic activities of hK5 and hK7 are regulated by their biochemical microenvironment. Present in the stratum corneum are the serine and/or cysteine protease inhibitors elafin, secretory leukocyte protease inhibitor (SLPI), and lumphoepithelial Kazal-type 5 serine protease inhibitor (LEKTI/*spink5*) (Caubet et al., 2004).

The skin disorder Netherton syndrome is associated with epidermal hyperplasia, in which the stratum corneum is often detached from the underlying epidermis or is entirely missing. The granular layer is frequently absent, revealing a phenotype that appears to result from an increase in cell desquamation. Mutations in *LEKTI* have been found in patients with Netherton syndrome (Chavanas et al., 2000; Komatsu et al., 2002). *spink 5* knock-out mice also mimic Netherton syndrome-like phenotypes (Descargues et al., 2005). LEKTI consists of 15 potential Kazal-type serine proteinase inhibitory domains (D1–D15). Different domains of the proteins are able to inhibit different proteases. Full-length recombinant LEKTI protein inhibits trypsin, plasmin and elastase. Domains D5 and D6 inhibit trypsin only, and domains D6–D9 inhibit trypsin and subtilisin A, but not plasmin or elastase (Jayakumar et al., 2004; Kreutzmann et al., 2004). It is currently being investigated which domains of LEKTI are able to inhibit kallikreins found in the stratum corneum.

Semen liquefaction

Much of the data on the role of kallikreins in semen liquefaction are still preliminary; however, what is known to occur is a biological process similar to that observed during skin desquamation. Kallikrein activity in seminal plasma (hK2, PSA, hK5, and hK11) is sensitive to the presence of Zn^{2+}. These ions stall kallikrein activity until ejaculation (Lovgren et al., 1999; Malm et al., 2000). Upon ejaculation, Zn^{2+} ions are then redistributed to semenogelin I/II (SEMG1/2) and fibronectin, major extracellular components of semen that carry the spermatozoa. Active kallikreins then hydrolyze SEMG1/2 and fibronectin, leading to semen liquefaction (Lilja, 1985; Jonsson et al., 2005; Michael et al., 2005).

Future directions of clinical applications

Figure 1 illustrates the continuing diversity of work related to this unique family of serine proteases. With the use of kallikreins as new biomarkers for the diagnosis and prognosis of cancer, together with the understanding of their regulation and the discovery of substrates, models are being developed to understand their physiological function. Pathways are starting to emerge in our efforts to understand tumor biology as it relates to cell invasion and angiogenesis (Borgono and Diamandis, 2004). Kallikreins are also beginning to represent a promising new source of potential targets for the development of novel cancer therapeutics. Kallikrein inactivation in the processes of skin desquamation and semen liquefaction is also currently being studied. There is also increasing evidence that a group of serine protease inhibitors (collectively known as serpins) may play a role in blocking hK activity.

Figure 1 Summary of research fronts for kallikrein enzymes.
For a brief discussion, see the text and previous reviews. SNP, single nucleotide polymorphism; ELISA, enzyme-linked immunosorbent assay; ECM, extracellular matrix.

The design of specific kallikrein serpins exploits the flexible reactive-site loop (RSL) of the inhibitors, which is implicated in the interaction with the putative protease. Upon binding of the enzyme and cleavage of the serpin, a covalent bond is formed between the two proteins, irreversibly trapping the protease. The specificity of serpin inhibition depends on both the amino acid sequence and length of the RSL. Several serpins have been identified as inhibitors of kallikrein activity; however, serpin to kallikrein specificity is one of the therapeutic aims of future work (Markland et al., 1996; Kapadia et al., 2004b; Michael et al., 2005). Using phage-display technology in a similar manner as for identifying hK protein substrates, amino acid substitutions were made in the RSL of α-antichymotrypsin (ACT) to construct novel hK2 specific inhibitors. Several potential serpin inhibitors were identified and tested against other serine proteases, including chymotrypsin, PSA and uPA, with one showing hK2 specific inhibition (Cloutier et al., 2004). The same technology is being used to discover additional kallikrein-specific serpin inhibitors.

Other therapeutic strategies have taken advantage of kallikrein activity or tissue specificity (e.g., PSA in prostate) to deliver tissue-specific toxic genes and induce active immunotherapy using hK-based vaccines. Preclinical investigations have shown that an adenoviral or non-viral/liposomal vector delivery system containing a cell suicide gene under the regulation of prostate-specific *KLK3* promoter and enhancer elements is able to selectively stimulate gene expression within PSA-producing prostate cancer cells, resulting in prostate cancer cell death *in vitro* and inhibition of tumor growth in xenografted mice (Latham et al., 2000; Suzuki et al., 2001; Yoshimura et al., 2001).

Although there is some 'catch-up' to be made with the other kallikreins in the field of therapeutics, the development of clinical assays and biochemical techniques in combination with the increased understanding of their genetic regulation and biological activities will facilitate a faster transition. These applications will be useful not only in cancer therapies, but also in non-cancer disorders. With much focus placed on serious maladies, the possibility exists of taking advantage of kallikrein function to develop products for more common ailments.

Acknowledgments

This work was supported by a University-Industry grant to E.P. Diamandis from the Natural Sciences and Engineering Research Council of Canada and IBEX Technologies Inc.

References

Bakin, R.E., Gioeli, D., Sikes, R.A., Bissonette, E.A., and Weber, M.J. (2003). Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res. *63*, 1981–1989.

Bayes, A., Tsetsenis, T., Ventura, S., Vendrell, J., Aviles, F.X., and Sotiropoulou, G. (2004). Human kallikrein 6 activity is regulated via an autoproteolytic mechanism of activation/inactivation. Biol. Chem. *385*, 517–524.

Black, M.H., Magklara, A., Obiezu, C.V., Melegos, D.N., and Diamandis, E.P. (1999) Development of ultrasensitive immunoassay for human glandular kallikrein with no cross-reactivity from prostate-specific antigen. Clin. Chem. *45*, 790–799.

Blok, L.J., de Ruiter, P.E., and Brinkmann, A.O. (1998). Forskolin-induced dephosphorylation of the androgen receptor impairs ligand binding. Biochemistry *37*, 3850–3857.

Borgono, C.A. and Diamandis, E.P. (2004). The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer *4*, 876–890.

Borgono, C.A., Grass, L., Soosaipillai, A., Yousef, G.M., Petraki, C.D., Howarth, D.H., Fracchioli, S., Katsaros, D., and Diamandis, E.P. (2003). Human kallikrein 14: a new potential

biomarker for ovarian and breast cancer. Cancer Res. *63*, 9032–9041.

Borgono, C.A., Michael, I.P., and Diamandis, E.P. (2004). Human tissue kallikreins: physiologic roles and applications in cancer. Mol. Cancer Res. *2*, 257–280.

Caubet, C., Jonca, N., Brattsand, M., Guerrin, M., Bernard, D., Schmidt, R., Egelrud, T., Simon, M., and Serre, G. (2004). Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J. Invest. Dermatol. *122*, 1235–1244.

Chavanas, C., Bodemer, C., Rochat, A., Hamel-Teillac, D., Ali, M., Irvine, A.D., Bonafe, J.L., Wilkinson, J., Taieb, A., Barrandon, Y., et al. (2000). Mutations in *SPINK5*, encoding a serine protease inhibitor, cause Netherton syndrome. Nat. Genet. *25*, 141–142.

Cleutjens, K.B., van Eekelen, C.C., van der Korput, H.A., Brinkmann, A.O., and Trapman, J. (1996). Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J. Biol. Chem. *271*, 6379–6388.

Cloutier, S.M., Chagas, J.R., Mach, J.P., Gygi, C.M., Leisinger, H.J., and Deperthes, D. (2002). Substrate specificity of human kallikrein 2 (hK2) as determined by phage display technology. Eur. J. Biochem. *269*, 2747–2754.

Cloutier, S.M., Kundig, C., Felber, L.M., Fattah, O.M., Chagas, J.R., Gygi, C.M., Jichlinski, P., Leisinger, H.J., and Deperthes, D. (2004). Development of recombinant inhibitors specific to human kallikrein 2 using phage-display selected substrates. Eur. J. Biochem. *271*, 607–613.

Cohen, P., Graves, H.C., Peehl, D.M., Kamarei, M., Giudice, L.C., and Rosenfeld, R.G. (1992). Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J. Clin. Endocrinol. Metab. *75*, 1046–1053.

de Koning, H.J., Auvinen, A., Berenguer Sanchez, A., Calais da Silva, F., Ciatto, S., Denis, L., Gohagan, J.K., Hakama, M., Hugosson, J., Kranse, R., et al. (2002). Large-scale randomized prostate cancer screening trials: program performances in the European Randomized Screening for Prostate Cancer trial and the Prostate, Lung, Colorectal and Ovary cancer trial. Int. J. Cancer *97*, 237–244.

DeGraffenried, L.A., Fulcher, L., Friedrichs, W.E., Grunwald, V., Ray, R.B., and Hidalgo, M. (2004). Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway. Ann. Oncol. *15*, 1510–1516.

Descargues, P., Deraison, C., Bonnart, C., Kreft, M., Kishibe, M., Ishida-Yamamoto, A., Elias, P., Barrandon, Y., Zambruno, G., Sonnenberg, A., and Hovnanian, A. (2005). *Spink5*-deficient mice mimic Netherton syndrome through degradation of desmoglein 1 by epidermal protease hyperactivity. Nat. Genet. *37*, 56–65.

Diamandis, E.P., Yousef, G.M., Soosaipillai, A.R., and Bunting, P. (2000a). Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin. Biochem. *33*, 579–583.

Diamandis, E.P., Yousef, G.M., Clements, J., Ashworth, L.K., Yoshida, S., Egelrud, T., Nelson, P.S., Shiosaka, S., Little, S., Lilja, H., et al. (2000b). New nomenclature for the human tissue kallikrein gene family. Clin. Chem. *46*, 1855–1858.

Diamandis, E.P., Okui, A., Mitsui, S., Luo, L.Y., Soosaipillai, A., Grass, L., Nakamura, T., Howarth, D.J., and Yamaguchi, N. (2002). Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. Cancer Res. *62*, 295–300.

Diamandis, E.P., Scorilas, A., Fracchioli, S., Van Gramberen, M., De Bruijn, H., Henrik, A., Soosaipillai, A., Grass, L., Yousef, G.M., Stenman, U.H., et al. (2003). Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis of ovarian carcinoma. J. Clin. Oncol. *21*, 1035–1043.

Felber, L.M., Borgono, C.A., Cloutier, S.M., Kundig, C., Kishi, T., Ribeiro Chagas, J., Jichlinski, P., Gygi, C.M., Leisinger, H.J., Diamandis, E.P., and Deperthes, D. (2005). Enzymatic profiling of human kallikrein 14 using phage-display substrate technology. Biol. Chem. *386*, 291–298.

Ferguson, R.A., Yu, H., Kalyvas, M., Zammit, S., and Diamandis, E.P. (1996). Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the Immulite immunochemiluminescent third-generation assay: potential applications in prostate and breast cancers. Clin. Chem. *42*, 675–684.

Frenette, G., Tremblay, R.R., Lazure, C., and Dube, J.Y. (1997). Prostatic kallikrein hK2, but not prostate-specific antigen (hK3), activates single-chain urokinase-type plasminogen activator. Int. J. Cancer *71*, 897–899.

Ghosh, M.C., Grass, L., Soosaipillai, A., Sotiropoulou, G., and Diamandis, E.P. (2004). Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol. *25*, 193–199.

Hoffman, B.R., Katsaros, D., Scorilas, A., Diamandis, P., Fracchioli, S., Rigault de la Longrais, I.A., Colgan, T., Puopolo, M., Giardina, G., Massobrio, M., and Diamandis, E.P. (2002). Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new independent unfavourable prognostic biomarker. Br. J. Cancer *87*, 763–771.

Hollenberg, M.D. and Compton, S.J. (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol. Rev. *54*, 203–217.

Jayakumar, A., Kang, Y., Mitsudo, K., Henderson, Y., Frederick, M.J., Wang, M., El-Naggar, A.K., Marx, U.C., Briggs, K., and Clayman, G.L. (2004). Expression of LEKTI domains 6–9′ in the baculovirus expression system: recombinant LEKTI domains 6–9′ inhibit trypsin and subtilisin A. Protein Expr. Purif. *35*, 93–101.

Jemal, A., Tiwari, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J., and Thun, M.J. (2004). Cancer statistics, 2004. CA Cancer J. Clin. *54*, 8–29.

Jonsson, M., Linse, S., Frohm, B., Lundwall, A., and Malm, J. (2005). Semenogelins I and I bind zinc and regulate the activity of prostate-specific antigen. Biochem. J. *387*, 447–453.

Kapadia, C., Chang, A., Sotiropoulou, G., Yousef, G.M., Grass, L., Soosaipillai, A., Zing, X., Howarth, D.H., and Diamandis, E.P. (2003). Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin. Chem. *49*, 77–86.

Kapadia, C., Ghosh, M.C., Grass, L., and Diamandis, E.P. (2004a). Human kallikrein 13 involvement in extracellular matrix degradation. Biochem. Biophys. Res. Commun. *323*, 1084–1090.

Kapadia, C., Yousef, G.M., Mellati, A.A., Magklara, A., Wasney, G.A., and Diamandis, E.P. (2004b). Complex formation between human kallikrein 13 and serum protease inhibitors. Clin. Chim. Acta *339*, 157–167.

Kim, H., Scorilas, A., Katsaros, D., Yousef, G.M., Massobrio, M., Fracchioli, S., Piccinno, R., Gordini, G., and Diamandis, E.P. (2001). Human kallikrein gene 5 (*KLK5*) expression is an indicator of poor prognosis in ovarian cancer. Br. J. Cancer *84*, 643–650.

Kishi, T., Grass, L., Soosaipillai, A., Shimizu-Okabe, C., and Diamandis, E.P. (2003) Human kallikrein 8: immunoassay development and identification in tissue extracts and biological fluids. Clin. Chem. *49*, 87–96.

Kishi, T., Soosaipillai, A., Grass, L., Little, S.P., Johnstone, E.M., and Diamandis, E.P. (2004). Development of an immunofluorometric assay and quantification of human kallikrein 7 in tissue extracts and biological fluids. Clin. Chem. *50*, 709–716.

Komatsu, N., Takata, M., Otsuki, N., Ohka, R., Amano, O., Takehara, K., and Saijoh, K. (2002). Elevated stratum corneum hydrolytic activity in Netherton syndrome suggests an inhibitory regulation of desquamation by SPINK5-derived peptides. J. Invest. Dermatol. *118*, 436–443.

Komatsu, N., Takata, M., Otsuki, N., Toyama, T., Ohka, R., Takehara, K., and Saijoh, K. (2003). Expression and localization of tissue kallikrein mRNAs in human epidermis and appendages. J. Invest. Dermatol. *121*, 542–549.

Komatsu, N., Saijoh, K., Toyama, T., Ohka, R., Otsuki, N., Hussack, G., Takehara, K., and Diamandis, E.P. (2005). Multiple tissue kallikrein mRNA and protein expression in normal skin and skin diseases. Br. J. Dermatol. *153*, 274–281.

Kraut, H., Frey, E.K., and Werle, E. (1930) Der Nachweis eines Kreislaufhormons in der Pankreasdrüse. Hoppe-Seyler's Z. Physiol. Chem. *192*, 1–21.

Kreutzmann, P., Schulz, A., Standker, L., Forssmann, W.G., and Magert, H.J. (2004). Recombinant production, purification and biochemical characterization of domain 6 of LEKTI: a temporary Kazal-type-related serine proteinase inhibitor. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *803*, 75–81.

Kurlender, L., Borgono, C., Michael, I. P., Obiezu, C., Elliott, M.B., Yousef, G.M., and Diamandis, E.P. (2005). A survey of alternative transcripts of human tissue kallikrein genes. Biochim. Biophys. Acta *1755*, 1–14.

Latham, J.P., Searle, P.F., Mautner, V., and James, N.D. (2000). Prostate-specific antigen promoter/enhancer driven gene therapy for prostate cancer: construction and testing of a tissue-specific adenovirus vector. Cancer Res. *60*, 334–341.

Li, B., Goyal, J., Dhar, S., Dimri, G., Evron, E., Sukumar, S., Wazer, D.E., and Band, V. (2001). CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. Cancer Res. *61*, 8014–8021.

Lilja, H. (1985). A kallikrein-like serine protease in prostate fluid cleaves the predominant seminal vesicle protein. J. Clin. Invest. *75*, 1899–1903.

Lin, H.K., Yeh, S., Kang, H.Y., and Chang, C. (2001). Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc. Natl. Acad. Sci. USA *98*, 7200–7205.

Liu, X.L., Wazer, D.E., Watanabe, K., and Band, V. (1996). Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res. *56*, 3371–3379.

Lovgren, J., Airas, K., and Lilja, H. (1999). Enzymatic action of human glandular kallikrein 2 (hK2). Substrate specificity and regulation by Zn^{2+} and extracellular protease inhibitors. Eur. J. Biochem. *262*, 781–789.

Luo, L.Y., Grass, L., and Diamandis, E.P. (2000). The normal epithelial cell-specific 1 (*NES1*) gene is up-regulated by steroid hormones in the breast carcinoma cell line BT-474. Anticancer Res. *20*, 981–986.

Luo, L.Y., Bunting, P., Scorilas, A., and Diamandis, E.P. (2001a). Human kallikrein 10: a novel tumor marker for ovarian carcinoma? Clin. Chim. Acta *306*, 111–118.

Luo, L.Y., Grass, L., Howarth, D.J., Thibault, P., Ong, H., and Diamandis, E.P. (2001b). Immunofluorometric assay of human kallikrein 10 and its identification in biological fluids and tissues. Clin. Chem. *47*, 237–246.

Luo, L.Y., Grass, L., and Diamandis, E.P. (2003a). Steroid hormone regulation of the human kallikrein 10 (*KLK10*) gene in cancer cell lines and functional characterization of the *KLK10* gene promoter. Clin. Chim. Acta *337*, 115–126.

Luo, L.Y., Katsaros, D., Scorilas, A., Fracchioli, S., Bellino, R., van Gramberen, M., de Bruijn, H., Henrik, A., Stenman, U.H., Massobrio, M., et al. (2003b). The serum concentration of human kallikrein 10 represents a novel biomarker for ovarian cancer diagnosis and prognosis. Cancer Res. *63*, 807–811.

Magklara, A., Grass, L., and Diamandis, E.P. (2000). Differential steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in breast cancer cell lines. Breast Cancer Res. Treat. *59*, 263–270.

Magklara, A., Mellati, A.A., Wasney, G.A., Little, S.P., Sotiropoulou, G., Becker, G.W., and Diamandis, E.P. (2003). Characterization of the enzymatic activity of human kallikrein 6: autoactivation, substrate specificity, and regulation by inhibitors. Biochem. Biophys. Res. Commun. *307*, 948–955.

Malm, J., Hellman, J., Hogg, P., and Lilja, H. (2000). Enzymatic action of prostate-specific antigen (PSA or hK3): substrate specificity and regulation by Zn^{2+}, a tight-binding inhibitor. Prostate *45*, 132–139.

Markland, W., Roberts, B.L., and Ladner, R.C. (1996). Selection for protease inhibitors using bacteriophage display. Methods Enzymol. *267*, 28–51.

Matsumura, M., Bhatt, A.S., Andress, D., Clegg, N., Takayama, T.K., Craik, C.S., and Nelson, P.S. (2005). Substrates of the prostate-specific serine protease prostase/KLK4 defined by positional-scanning peptide libraries. Prostate *62*, 1–13.

Michael, I.P., Sotiropoulou, G., Pampalakis, G., Magklara, A., Ghosh, M., Wasney, G., and Diamandis, E.P. (2005). Biochemical and enzymatic characterization of human kallikrein 5 (hK5), a novel serine protease potentially involved in cancer progression. J. Biol. Chem. *280*, 14628–14635.

Mikolajczyk, S.D., Millar, L.S., Marker, K.M., Grauer, L.S., Goel, A., Cass, M.M., Kumar, A., and Saedi, M.S. (1997). Ala217 is important for the catalytic function and autoactivation of prostate-specific human kallikrein 2. Eur. J. Biochem. *246*, 440–446.

Murtha, P., Tindall, D.J., and Young, C.Y. (1993). Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. Biochemistry *32*, 6459–6464.

Nakamura, T., Stephan, C., Scorilas, A., Yousef, G.M., Jung, K., and Diamandis, E.P. (2003). Quantitative analysis of hippostasin/*KLK11* gene expression in cancerous and noncancerous prostatic tissues. Urology *61*, 1042–1046.

Ni, X., Zhang, W., Huang, K.C., Wang, Y., Ng, S.K., Mok, S.C., Berkowitz, R.S., and Ng, S.W. (2004). Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br. J. Cancer *91*, 725–731.

Noh, W.C., Mondesire, W.H., Peng, J., Jian, W., Zhang, H., Dong, J., Mills, G.B., Hung, M.C., and Meric-Bernstam, F. (2004). Determinants of rapamycin sensitivity in breast cancer cells. Clin. Cancer Res. *10*, 1013–1023.

Obiezu, C.V. and Diamandis, E.P. (2005). Human tissue kallikrein gene family: applications in cancer. Cancer Lett. *224*, 1–22.

Obiezu, C.V., Scorilas, A., Katsaros, D., Massobrio, M., Yousef, G.M., Fracchioli, S., Rigault de la Longrais, I.A., Arisio, R., and Diamandis, E.P. (2001). Higher human kallikrein gene 4 (*KLK4*) expression indicates poor prognosis of ovarian cancer patients. Clin. Cancer Res. *7*, 2380–2386.

Obiezu, C.V., Soosaipillai, A., Jung, K., Stephan, C., Scorilas, A., Howarth, D.H., and Diamandis, E.P. (2002). Detection of human kallikrein 4 in healthy and cancerous prostatic tissues by immunofluorometry and immunohistochemistry. Clin. Chem. *48*, 1232–1240.

Obiezu, C.V., Shan, S.J., Soosaipillai, A., Luo, L.Y., Grass, L., Sotiropoulou, G., Petraki, C.D., Papanastasiou, P.A., Levesque, M.A., and Diamandis, E.P. (2005). Human kallikrein 4: quantitative study in tissues and evidence for its secretion into biological fluids. Clin. Chem. *51*, 1432–1442.

Oikonomopoulou, K., Hansen, K.K., Saifeddine, M., Vergnolle, N., Tea, I., Blaber, M., Blaber, S.I., Scarisbrick, I., Diamandis, E.P., and Hollenberg, M.D. (2006). Kallikrein-mediated cell signalling: targeting proteinase-activated receptors (PARs). Biol. Chem. *387*, 817–824.

Pampalakis, G., Kurlender, L., Diamandis, E.P., and Sotiropoulou, G. (2004). Cloning and characterization of novel isoforms of the human kallikrein 6 gene. Biochem. Biophys. Res. Commun. *320*, 54–61.

Peralba, J.M., DeGraffenried, L., Friedrichs, W., Fulcher, L., Grunwald, V., Weiss, G., and Hidalgo, M. (2003). Pharmacodynamic evaluation of CCI-779, an inhibitor of mTOR, in cancer patients. Clin. Cancer Res. *9*, 2887–2892.

Rehault, S., Brillard-Bourdet, M., Bourgeois, L., Frenette, G., Juliano, L., Gauthier, F., and Moreau, T. (2002). Design of new and sensitive fluorogenic substrates for human kallikrein hK3 (prostate-specific antigen) derived from semenogelin sequences. Biochim. Biophys. Acta *1596*, 55–62.

Rehault, S., Monget, P., Mazerbourg, S., Tremblay, R., Gutman, N., Gauthier, F., and Moreau, T. (2001). Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hK2 and hK3. Eur. J. Biochem. *268*, 2960–2968.

Riegman, P.H., Vlietstra, R.J., van der Korput, J.A., Brinkmann, A.O., and Trapman, J. (1991). The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol. Endocrinol. *5*, 1921–1930.

Rittenhouse, H.G., Finlay, J.A., Mikolajczyk, S.D., and Partin, A.W. (1998). Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. Crit. Rev. Clin. Lab. Sci. *35*, 275–368.

Rochette-Egly, C. (2003). Nuclear receptors: integration of multiple signalling pathways through phosphorylation. Cell. Signal. *15*, 355–366.

Schuur, E.R., Henderson, G.A., Kmetec, L.A., Miller, J.D., Lamparski, H.G., and Henderson, D.R. (1996). Prostate-specific antigen expression is regulated by an upstream enhancer. J. Biol. Chem. *271*, 7043–7051.

Scorilas, A., Borgono, C.A., Harbeck, N., Dorn, J., Schmalfeldt, B., Schmitt, M., and Diamandis, E.P. (2004). Human kallikrein 13 protein in ovarian cancer cytosols: a new favorable prognostic marker. J. Clin. Oncol. *22*, 678–685.

Shigemasa, K., Gu, L., Tanimoto, H., O'Brien, T.J., and Ohama, K. (2004). Human kallikrein gene 11 (*KLK11*) mRNA overexpression is associated with poor prognosis in patients with epithelial ovarian cancer. Clin. Cancer Res. *10*, 2766–2770.

Shvartsman, H.S., Lu, K.H., Lee, J., Lillie, J., Deavers, M.T., Clifford, S., Wolf, J.K., Mills, G.B., Bast, R.C. Jr., Gershenson, D.M., and Schmandt, R. (2003). Overexpression of kallikrein 10 in epithelial ovarian carcinomas. Gynecol. Oncol. *90*, 44–50.

Sidiropoulis, M., Pampalakis, G., Sotiropoulou, G., Katsaros, D., and Diamandis E.P. (2005). Downregulation of human kallikrein 10 (*KLK10*/*NES1*) by CpG island hypermethylation in breast, ovarian and prostate cancers. Tumour Biol. *26*, 324–336.

Stavropoulou, P., Gregorakis, A.K., Plebani, M., and Scorilas, A. (2005). Expression analysis and prognostic significance of human kallikrein 11 in prostate cancer. Clin. Chim. Acta *357*, 190–195.

Stephan, C., Yousef, G.M., Scorilas, A., Jung, K., Jung, M., Kristiansen, G., Hauptmann, S., Bharaj, B.S., Nakamura, T., Loening, S.A., and Diamandis, E.P. (2003). Quantitative analysis of kallikrein 15 gene expression in prostate tissue. J. Urol. *169*, 361–364.

Suzuki, S., Tadakuma, T., Kunitomi, M., Takayama, E., Sato, M., Asano, T., Nakamura, H., and Hayakawa, M. (2001). Liposome-mediated gene therapy using HSV-TK/ganciclovir under the control of human PSA promoter in prostate cancer cells. Urol. Int. *67*, 216–223.

Takayama, T.K., McMullen, B.A., Nelson, P.S., Matsumura, M., and Fujikawa, K. (2001). Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. Biochemistry *40*, 15341–15348.

Tanimoto, H., Underwood, L.J., Shigemasa, K., Parmley, T.H., and O'Brien, T.J. (2001). Increased expression of protease M in ovarian tumors. Tumour Biol. *22*, 11–18.

Wu, P., Leinonen, J., Koivunen, E., Lankinen, H., and Stenman, U.H. (2000). Identification of novel prostate-specific antigen-binding peptides modulating its enzyme activity. Eur. J. Biochem. *267*, 6212–6220.

Yoshimura, I., Suzuki, S., Tadakuma, T., and Hayakawa, M. (2001). Suicide gene therapy on LNCaP human prostate cancer cells. Int. J. Urol. *8*, S5–8.

Yousef, G.M. and Diamandis, E.P. (2001). The new human tissue kallikrein gene family: structure, function, and association to disease. Endocr. Rev. *22*, 184–204.

Yousef, G.M., Luo, L.Y., Scherer, S.W., Sotiropoulou, G., and Diamandis, E.P. (1999). Molecular characterization of zyme/protease M/neurosin (PRSS9), a hormonally regulated kallikrein-like serine protease. Genomics *62*, 251–259.

Yousef, G.M., Chang, A., Scorilas, A., and Diamandis, E.P. (2000). Genomic organization of the human kallikrein gene family on chromosome 19q13.3-q13.4. Biochem. Biophys. Res. Commun. *276*, 125–133.

Yousef, G.M., Borgono, C.A., Scorilas, A., Ponzone, R., Biglia, N., Iskander, L., Polymeris, M.E., Roagna, R., Sismondi, P., and Diamandis, E.P. (2002a). Quantitative analysis of human kallikrein gene 14 expression in breast tumours indicates association with poor prognosis. Br. J. Cancer *87*, 1287–1293.

Yousef, G.M., Scorilas, A., Kyriakopoulou, L.G., Rendl, L., Diamandis, M., Ponzone, R., Biglia, N., Giai, M., Roagna, R., Sismondi, P., and Diamandis, E.P. (2002b). Human kallikrein gene 5 (*KLK5*) expression by quantitative PCR: an independent indicator of poor prognosis in breast cancer. Clin. Chem. *48*, 1241–1250.

Yousef, G.M., Polymeris, M.E., Grass, L., Soosaipillai, A., Chan, P.C., Scorilas, A., Borgono, C., Harbeck, N., Schmalfeldt, B., Dorn, J., et al. (2003a). Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res. *63*, 3958–3965.

Yousef, G.M., Polymeris, M.E., Yacoub, G.M., Scorilas, A., Soosaipillai, A., Popalis, C., Fracchioli, S., Katsaros, D., and Diamandis, E.P. (2003b). Parallel overexpression of seven kallikrein genes in ovarian cancer. Cancer Res. *63*, 2223–2227.

Yousef, G.M., Borgono, C.A., Michael, I.P., and Diamandis, E.P. (2004). Cloning of a kallikrein pseudogene. Clin. Biochem. *37*, 961–967.

Yu, D., Sakamoto, G.T., and Henderson, D.R. (1999). Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res. *59*, 1498–1504.