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Review

Human Tissue Kallikreins: A New Enzymatic Cascade Pathway?

George M. Yousef and Eleftherios P. Diamandis*

Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

* Corresponding author

Serine proteases are proteolytic enzymes with an active serine residue in their catalytic site. Kallikreins are a subgroup of the serine protease family which is known to have diverse physiological functions. The human kallikrein gene family has now been fully characterized and includes 15 members tandemly located on chromosome 19q13.4. Here we discuss the common structural features of kallikreins at the DNA, mRNA and protein levels and summarize their tissue expression and hormonal regulation patterns. Kallikreins are expressed in many tissues including the salivary gland, endocrine tissues such as testis, prostate, breast and endometrium, and in the central nervous system. Most genes appear to be under steroid hormone regulation. The occurrence of several splice variants is common among kallikreins, and some of the splice variants seem to be tissue-specific and might be related to certain pathological conditions. Kallikreins are secreted in an inactive 'zymogen' form which is activated by cleavage of an N-terminal peptide. Some kalikreins can undergo autoactivation while others may be activated by other kallikreins or other proteases. Most kallikreins are predicted to have trypsin-like enzymatic activity except three which are probably chymotrypsin-like. New, but mainly circumstantial evidence, suggests that at least some kallikreins may be part of a novel enzymatic cascade pathway which is turned-on in aggressive forms of ovarian and probably other cancers.

Key words: Alzheimer's disease / Cascade activation system / Enzymatic activity / Kallikrein / Serine proteases / Tumor markers.

Introduction: Serine Proteases

Proteolytic enzymes can be classified based on their catalytic mechanism. Classes of proteolytic enzymes include proteases which have an activated cysteine residue (cysteine proteases), aspartate (aspartate proteases), metal ion (metalloproteases) or serine (serine proteases). Serine proteases are a family of enzymes that utilizes a uniquely activated serine residue in the substrate-binding site to catalytically hydrolyze peptide bonds (Schultz and Liebman, 1997). This active site is characterized by the irreversible interaction with diisopropylfluorophosphate (DFP). Of all the serines in the protein, DFP can only react with the active serine to form a phosphate ester (Schultz and Liebman, 1997). Out of the estimated 400 – 500 proteases in the human genome, 32% are predicted to be serine proteases (Southan, 2001). This large family includes the digestive enzymes (*e.g.*, trypsin, chymotrtypsin), the kringle domain-containing growth factors (*e.g.*, tissue plasminogen activator), some of the blood clotting factors and the kallikreins. Serine proteases are involved in many vital functions such as digestion, coagulation and fibrinolysis, tissue remodelling, activation of hormones, growth factors and in extracellular matrix protein degradation. A number of serine proteases are secreted as inactive 'zymogens', which require limited proteolysis to release the active enzyme. Others are anchored to the cell membrane.

Kallikreins in Humans and Other Species

Kallikreins were named so because they were originally isolated from the pancreas (in Greek, the '*kallikreas*'). Traditionally, kallikreins were defined as enzymes which can release vasoactive peptides from high molecular weight precursors. Only one of the tissue kallikreins, salivary/renal/pancreatic kallikrein (KLK1), fulfills this criterion. More recently, a new structural concept has emerged to describe kallikreins. Richards and co-workers introduced the concept of a 'kallikrein multigene family' in mice to refer to those genes (van Leeuwen *et al.,* 1986). This definition was not based much on the enzymatic function, but rather on sequence homology and close chromosomal location.

In humans, there are two classes of kallikreins; the plasma kallikrein and the tissue kallikreins. The plasma kallikrein is encoded by a 15-exon single gene on chromosome 4q35. This enzyme (a serine protease) releases the vasoactive peptide bradykinin from a high molecular weight precursor synthesized in the liver (Asakai *et al.,* 1987). The human tissue kallikreins are a family of genes

localized on chromosome 19 and also encode for serine protease enzymes. This review will focus on the structural and biological aspects of the human tissue kallikrein gene family. There are a number of excellent reviews describing the rodent kallikrein family of genes (Evans *et al.,* 1987; Wines *et al.,* 1989; Murray *et al.,* 1990; Clements, 1997; Margolius, 1998). More detailed discussions about the clinical applications of kallikreins can be found in our recent reviews (Diamandis and Yousef, 2001; Yousef and Diamandis, 2001).

The Human Tissue Kallikrein Gene Family

Extensive recent work by many laboratories has led to the identification of all members of the human tissue kallikrein gene family, the establishment of their structural features (as described below) and of a uniform nomenclature (Anisowicz *et al.,* 1996; Yoshida *et al.,* 1998a,b; Brattsand and Egelrud, 1999; Nelson *et al.,* 1999; Stephenson *et al.,* 1999; Yousef *et al.,*1999a,b, 2000a,b,d,e,f, 2001b,c; Diamandis *et al.,* 2000a; Yousef and Diamandis, 2000). The human tissue kallikrein family currently includes 15 genes (KLK1 – 15). Table 1 summarizes the official names, GenBank accession numbers and synonyms of all genes and proteins (Diamandis *et al.,* 2000a).

Structure of the Human Kallikrein Genes and Proteins

Human kallikrein genes range from 4 – 10 kb with most of the size differences attributed to intron lengths. The common structural features of this family of genes are as fol-

Table 1 The Official and Additional Gene and Protein Names for Members of the Human Kallikrein Gene Family.

Official gene name	Official protein name	Other names/symbols	GenBank accession no.	References
KLK1	hK1	Pancreatic/renal kallikrein, hPRK	M25629 M33105	(Evans *et al.,* 1988; Fukushima *et al.,* 1985)
KLK2	hK2	Human glandular kallikrein 1, hGK-1	M18157	(Schedlich *et al.,* 1987)
KLK3	hK3	Prostate-specific antigen, PSA, APS	X14810 M24543 M27274	(Lundwall 1989; Riegman *et al.,* 1988, 1989b; Sutherland *et al.,* 1988)
KLK4	hK4	Prostase, KLK-L1, EMSP1, PRSS17, ARM1	AF113141 AF135023 AF148532	(Hu *et al.,* 2000; Korkmaz *et al.,* 2001; Nelson *et al.,* 1999; Stephenson *et al.,* 1999; Yousef *et al.,* 1999b)
KLK5	hK5	KLK-L2, HSCTE	AF135028 AF168768	(Brattsand and Egelrud, 1999; Yousef and Diamandis, 1999)
KLK6	hK6	Zyme, Protease M, Neurosin, PRSS9	AF013988 AF149289 U62801 D78203	(Anisowicz *et al.,* 1996; Little *et al.,* 1997; Yamashiro *et al.,* 1997; Yousef *et al.,* 1999b)
KLK7	hK7	HSCCE, PRSS6	L33404 AF166330	(Hansson *et al.,* 1994; Yousef *et al.,* 2000f)
KLK8	hK8	Neuropsin; Ovasin; TADG-14, PRSS19, HNP	AB009849 AF095743 AB010780 AF055982	(Underwood *et al.,* 1999; Yoshida *et al.,* 1998a)
KLK9	hK9	KLK-L3 protein	AF135026	(Yousef and Diamandis, 2000)
KLK10	hK10	NES1, PSSSL1	AF055481 NM_002776	(Goyal *et al.,* 1998; Liu *et al.,* 1996; Luo *et al.,* 1998)
KLK11	hK11	TLSP/Hippostasin, PRSS20	AB012917	(Mitsui *et al.,* 2000b; Yoshida *et al.,* 1998b; Yousef *et al.,* 2000e)
KLK12	hK12	KLK-L5 protein	AF135025	(Yousef *et al.,* 2000d)
KLK13	hK13	KLK-L4 protein	AF135024	(Yousef *et al.,* 2000b)
KLK14	hK14	KLK-L6 protein	AF161221	(Yousef *et al.,* 2001b)
KLK15	hK15	prostinogen, HSRNASPH	AF303046	(Takayama *et al.,* 2001a; Yousef *et al.,* 2001c)

lows (Yousef *et al.,* 2000c; Yousef and Diamandis, 2000, 2001; Clements *et al.,* 2001):

(i) All genes are formed of 5 coding exons and most of them have one or more extra 5' untranslated exons. The first coding exon always contains a 5' untranslated region, followed by the methionine start codon, located ~50 bp away from the end of the exon. The stop codon is always located ~156 bp from the beginning of the last coding exon.
(ii) Exon sizes are very similar or identical.
(iii) The intron phases of the coding exons (*i.e*., the position where the intron starts in relation to the last codon of the previous exon) are conserved in all genes. The pattern of the intron phase is always I-II-I-0.
(iv) The positions of the residues of the catalytic triad of serine proteases are conserved, with the histidine always occurring near the end of the second coding exon, the aspartate in the middle of the third exon and the serine residue at the beginning of the last exon.
(v) All kallikrein proteins are synthesized as a pre/pro peptides with a signal peptide of about 17–20 amino acids at the amino terminus, followed by an activation peptide of about 4–9 amino acids (with the exception of hK5), followed by the mature (enzymatically active) protein.
(vi) The substrate specificity of the kallikrein enzymes is predicted to be mainly trypysin-like (in 12 out of the 15 kallikreins), as indicated by the presence of aspartate or glutamate in the substrate-binding pocket. The specificity is chymotrypsin-like in hK3, hK7 and probably hK9.
(vii) All genes seem to be under regulation by steroid sex hormones (see below).
(viii) All proteins contain 10–12 cysteine residues, that will form 5 (in hK1–3 and hK13) or 6 (in the rest) disulphide bonds. The positions of the cysteine residues are also fully conserved.

Classical or variant polyadenylation signals have been predicted 10–20 bases away from the poly A tail of all kallikreins (Yousef and Diamandis, 2001). In addition to the conservation of the catalytic amino acid triad, we recently identified seven protein motifs. Searching the SWISS-PROT and the EMBL protein databases revealed that these motifs are conserved in kallikreins of different species and in several other groups of serine proteases (Yousef and Diamandis, 2002). The biological function of these motifs has not been elucidated.

Three-Dimensional Structure

In contrast to rodent kallikreins, where the crystal structure has been revealed for some proteins, hK1 is the only human kallikrein whose crystal structure has been determined (Katz *et al.,* 1998), but the structural coordinates are not publicly available. Most of the discussion in this section is derived from comparative model building of the human kallikrein proteins.

Kallikreins can be roughly divided into two categories, the classical kallikreins (hK1–3) and the 'new' kallikreins. The new kallikreins appear to be unique in their three-dimensional structure, sharing some features with trypsins and others with the classical kallikreins. Comparative protein models show that the pattern of hydrophobic side chain packing in the protein core is nearly identical in all human kallikreins and the observed differences occur within the solvent-exposed loop segments.

An 11-aminoacid residue insertion relative to the trypsin sequences in loop E (residues 91–103 in the bovine chymotrypsinogen consensus numbering*),* known also as 'the kallikrein loop', is a unique feature for the three classical kallikreins. Loop E is located between the fifth (residues 81–90) and the sixth β-strand (residues 104–108) and loop G between the seventh and the eighth β-strand (residues 156–163). None of the new human kallikreins contains this loop in its entirety. Loop E in hK10 is longest, with an 8-residue long insertion relative to the trypsin sequences. Loop E overhangs the substrate-binding groove on the surface of the protease molecule and its length and sequence can directly influence substrate recognition.

The KLK15 gene is particularly interesting, as it lies between two classical glandular kallikrein genes, KLK1 and KLK3 (Yousef *et al.,* 2001c), yet the sequence and structure of hK15, with six disulphide bonds and no insertion in the so-called kallikrein loop (E), clearly place it among the new kallikreins. Moreover, in loop G, hK15 has an 8-residue insertion (Yousef *et al.,* 2001c) that is not found in any other kallikrein. In the three-dimensional structure, the extended loop G lies on the opposite side of the active site relative to loop E and, although it is more distant to the substrate-binding groove, loop G may also participate in substrate and inhibitor recognition.

Isoforms and Splice Variants of the Human Kallikreins

The presence of more than one mRNA form for the same gene is common among kallikreins. These forms may result from alternative splicing, retained intronic segment (Riegman *et al.,* 1989a), or the utilization of an alternative transcription initiation site (Chen *et al.,* 1994). A list of all reported splice variants of kallikreins can be found in our recent review (Yousef and Diamandis, 2002).

Characterization of all splice variants for each gene is important. In addition to any physiological significance, these variants have the potential of being used for diagnostic applications. Slawin *et al.* (2000) reported the prognostic significance of a splice variant-specific RT-PCR for KLK2, in detecting prostate cancer metastasis. Nakamura *et al.* (2001) reported differential expression of the brain and prostate-types of KLK11 between benign, hyperplastic and malignant prostate cancer cell lines. In

Table 2 Functional and Structural Features of Serine Proteases That Are Conserved in Kallikreins.

– The presence of most of the 29 invariable amino acids (Dayhoff, 1978).
– Only one serine residue of the protein is catalytically active.
– Two residues, a histidine and an aspartate are always associated with the activated serine in the catalytic site.
– The conserved amino acid motifs surrounding the catalytic triad.
– The catalytically essential histidine and serine are almost immediately adjacent to their exon boundary.
– Initially produced in a 'zymogen' form.
– High degree of sequence similarity.
– The active serine is situated in an internal pocket and the aspartate and histidine residues are c losely located in the three-dimensional structure.

addition, misleading results may be obtained, due to the presence of the splice variants, during measuring the active forms of the genes or proteins.

Some of these alternatively spliced forms were also found to be tissue-specific. A 1.5 kb transcript of KLK14 was only found in the prostate, and another 1.9 kb transcript only in skeletal muscle (Hooper *et al.,* 2001). Several splice variants of KLK13 were found to be testis-specific (Chang *et al.,* 2001). Type 2 neuropsin (KLK 8) is preferentially expressed in human adult brain and hippocampus (Mitsui *et al.,* 1999), and a new splice variant of KLK4 was isolated from prostatic tissue (Obiezu and Diamandis, 2000). It is also important to mention that some of these splice variants were found to be translated (Heuze *et al.,* 1999; Tanaka *et al.,* 2000). Despite the many splice variants reported for the human kallikrein genes, there is only one report indicating the presence of splice variants in the mouse hippostasin gene (Mitsui *et al.,* 2000a).

Tissue Expression and Cellular Localization of the Kallikrein Genes

Many kallikreins are transcribed predominantly in few tissues, as indicated by Northern blotting. By using the more sensitive RT-PCR technique, kallikreins were found to be expressed at lower amounts in several other tissues. The tissue expression of all kallikreins, assessed by RT-PCR and/or Northern blot, is summarized in our recent reviews (Yousef and Diamandis, 2001, 2002). Many kallikreins are expressed in the salivary gland, the tissue where most of the rodent kallikreins are expressed. It will be interesting to investigate the function of human kallikreins in this tissue. Also, several kallikreins were found in the central nervous system and preliminary reports suggest that they are involved in brain physiology and/or pathobiology.

In contrast to hK3 (PSA) and hK2, which are mainly expressed in the prostate, immunohistochemical analysis showed that other kallikreins are localized in many tissues. For example, hK6 protein was found in glandular epithelia, including those of the breast, prostate, endometrium, colon, and pancreas (Petraki *et al.,* 2001). Its presence in fluids, including milk and cerebrospinal fluid confirms that hK6 is a secreted protein (Diamandis *et al.,* 2000d). Similar comments apply to hK10 (Luo *et al.,* 2001b) and hK11 (our unpublished results). hK6 was shown to be immmunolocalized in the microglial cells of Alzheimer's disease brain (Little *et al.,* 1997). We have recently observed that the expression of hK6 in the pancreas, co-localizes with insulin in the pancreatic islets, raising the possibility that it might have a role in the processing of islet hormones. hK7 was localized in the cytoplasm and cell membrane of epithelial ovarian cancer cells (Tanimoto *et al.,* 1999). hK9 was recently found to localize in the nuclei, but not the cytoplasm, of epithelial cells of ovarian cancer tissues. In general, then, all immunohistochemically detected kallikreins are present in the cytoplasm of epithelial cells and are secreted into the lumen of glandular tissues. An interesting exception is the observation that Green Fluorescent protein (GFP)-tagged hK4 has a distinct perinuclear localization (Korkmaz *et al.,* 2001).

Enzymatic Activity and Substrate Specificity of Kallikreins

From the functional point of view, kallikreins are serine proteases. Proteolytic activity of some kallikrein proteins has been already experimentally proven (*e.g*. hK2 – 5, and hK7) (Hansson *et al.,* 1994; Rittenhouse *et al.,* 1998; Brattsand and Egelrud, 1999). Although experimental evidence is lacking for the rest of them, structural analyses indicate that kallikreins have all the essential criteria to be classified as serine proteases. Table 2 summarizes the essential features of serine proteases that are preserved in the kallikreins. Phylogenetic analysis also supports the grouping of the kallikrein proteins among the serine protease family of enzymes. Activation reactions catalyzed by serine proteases (including kallikreins) are an example of 'limited proteolysis' where the hydrolysis is limited to one or two particular peptide bonds. Hydrolysis of peptide bonds starts by the oxygen atom of the hydroxyl group of the serine residue which attacks the carbonyl carbon atom of the susceptible peptide bond. At the same time, the serine transfers a proton to the histidine residue of the catalytic triad, then to the nitrogen atom of the susceptible peptide bond which is then cleaved and released. The other part of the substrate is now covalent-

ly bound to the serine by an ester bond. The charge that develops at this stage is partially neutralized by the third (asparate) residue of the catalytic triad. This process is followed by 'deacylation' where the histidine draws a proton away from a water molecule and the hydroxyl ion attacks the carbonyl carbon atom of the acyl group that was attached to the serine. The histidine then donates a proton to the oxygen atom of the serine, which will then release the acid component of the substrate.

In addition to the release of vasoactive peptides (as is the case with hK1), kallikreins can also cleave other important molecules, such as growth factors and hormones. Examples of other kallikrein biological substrates are discussed below. Another recently investigated mechanism of action of serine proteases is the activation of the proteinase-activated receptors (PAR). PAR is a novel family of G protein-coupled receptors which is stimulated by cleavage of their N-termini by a serine protease rather than by ligand-receptor occupancy (Macfarlane *et al.,* 2001). Activation of these receptors elicits different responses in several tissues. In addition, they switch-on cell signaling pathways, *e.g*. the MAP-kinase pathway, leading to cell growth and division (Macfarlane *et al.,* 2001).

Substrate Specificity

Serine proteases exhibit preference for hydrolysis of peptide bonds adjacent to a particular class of amino acids. In the trypsin-like group (to which most of the kallikreins belong), the protease cleaves peptide bonds following basic amino acids such as arginine or lysine, since it has an aspartate (or glutamate) in the substrate-binding pocket which can form a strong electrostatic bond with these residues. The chymotrpsin-like proteases (*e.g*., hK3) have non-polar substrate-binding pockets, and thus require an aromatic or bulky non-polar amino acid such as tryptophan, phenylalanine, tyrosine and leucine. The elastase-like enzymes, on the other hand, have bulky amino acids (valine and threonine) in their binding pockets, thus requiring small hydrophobic residues, such as alanine. Substrate specificity has been experimentally confirmed for some kallikreins. hK3 (PSA) has been shown to have restricted chymotrryptic-like activity (Akiyama *et al.,* 1987). PSA cleaves lysozyme, insulin and seminogelin I on the carboxy terminal side of certain leucines, tyrosines and phenylalanines (Robert *et al.,* 1997). hK2 cleaves substrates following single or double arginine residues, confirming its trypsin-like activity (Lovgren *et al.,* 1999). hK7 has a chymotrypsin-like primary substrate specificity (Halprin, 1972). More recently, hK4 was found to have trypsin-like activity (Takayama *et al.,* 2001b). hK15 has a glutamic acid in the substrate binding pocket and showed significant activity against substrates which have an Arg-$_p$NA cleavage site, suggesting a trypsin-like activity (Takayama *et al.,* 2001a). Again, experimental results showed that hK11 has a preference to cleave substrates after an arginine residue (Mitsui *et al.,* 2000b). Multiple alignment of the deduced protein sequences of all 15 kallikreins predicted that 12 out of the 15 kallikreins will have a trypsin-like substrate specificity (as indicated by the presence of an aspartate or glutamate residue in the substrate binding pocket) (Yousef *et al.,* 2000c; Yousef and Diamandis, 2001). Two important points are worth mentioning here: (1) the activity of a specific protease for a certain type of amino acid only indicates its preference, still it might be able to cleave other substrates, although at a much slower rate. (2) The secondary interaction, outside of S_1-S'_1 region plays an important role in determining the substrate specificity. The differences in substrate specificity of kallikreins have been shown to be dependent on the amino acids located at positions P3-P8 on the C-terminal site of the cleavage site (Brillard-Bourdet *et al.,* 1995). This also explains the inability of hK4 to cleave certain substrates although they have the essential arginine site (Takayama *et al.,* 2001b).

Physiological and Pathological Roles of Kallikreins

Little is known about the exact physiological functions of kallikreins in normal tissues. However, accumulating evidence show that kallikreins might have diverse functions, depending on the tissue and circumstances of expression. hK1 exerts its biological activity mainly through the release of lysyl-bradykinin (kallidin) from low molecular weight kininogen, leading to regulation of blood pressure. However, the diverse expression pattern of hK1 has led to the suggestion that the functional role of this enzyme may be specific to different cell types (Schachter, 1979; Bhoola *et al.,* 1992). Apart from its kininogenase activity, tissue kallikrein has been implicated in the processing of growth factors and peptide hormones (Mason *et al.,* 1983) in light of its presence in pituitary, pancreas and other tissues. As summarized by Bhoola *et al.* (1992), hK1 has been shown to cleave pro-insulin, low density lipoprotein, the precursor of atrial natriuretic factor, prorenin, vasoactive intestinal peptide, procollagenase and angiotensinogen.

hK2 has been reported to be able to activate the proform of PSA (Kumar *et al.,* 1997; Lovgren *et al.,* 1997; Takayama *et al.,* 1997). Seminal plasma hK2 was found to be able to cleave seminogelin I and seminogelin II but at different cleavage sites and with lower efficiency than PSA (Deperthes *et al.,* 1996). Since the amount of hK2 in seminal plasma is much lower than PSA (1–5%), the contribution of hK2 in the process of seminal clot liquefaction is expected to be relatively small (Rittenhouse *et al.,* 1998).

Since PSA is present at very high levels in seminal plasma, most studies focused on its biological activity within this fluid. Lilja (1985) has shown that PSA rapidly hydrolyzes both semenogelin I and semenogelin II, as well as fibronectin, resulting in liquefaction of the seminal plasma clot after ejaculation. Several other potential sub-

strates for PSA have been identified, including IGFBP-3 (Sutkowski *et al.,* 1999), TGF-β (Killian *et al.,* 1993), parathyroid hormone-related peptide (Iwamura *et al.,* 1996) and plasminogen (Heidtmann *et al.,* 1999). The physiological relevance of these findings is still not clear.

The mouse and porcine orthologs of hK4 were originally designated 'enamel matrix serine protease' because of their predicted role in the normal teeth development (Hu *et al.,* 2000). The human KLK4, however, was shown to be highly expressed in the prostate, pointing out to the possibility of having a different function in humans. hK7, and more recently hK5, were found to be highly expressed in the skin, and it is believed that they are involved in the mechanism of skin keratinization (Egelrud and Lundstrom, 1990). hK6, hK8 and hK11 are highly expressed in the central nervous system where they are thought to be involved in neural plasticity.

The most well-documented relationships of kallikreins to disease are those with endocrine-related malignancies. Several kallikreins have been shown to be differentially expressed in prostate (Diamandis, 1998; Yousef *et al.,* 2001c, 2002b; Diamandis *et al.,* 2002), breast (Liu *et al.,* 1996; Yousef *et al.,* 2000b, 2001b), testicular (Luo *et al.,* 2001d), and ovarian (Kim *et al.,* 2001; Magklara *et al.,* 2001; Obiezu *et al.,* 2001; Yousef *et al.,* 2001a) cancers. In addition to hK3 (PSA), other kallikreins are emerging as new cancer biomarkers (Diamandis *et al.,* 2000c,d; Luo *et al.,* 2001a). A more detailed description of kallikreins as cancer biomarkers has been published previously (Diamandis *et al.,* 2000b; Diamandis and Yousef, 2001). Another growing field of investigation involves kallikreins and diseases of the central nervous system. For further details, see Yousef and Diamandis (2001).

Regulation of Kallikrein Activity

At the mRNA Level

Promoter analysis and hormonal stimulation experiments allowed us to obtain some insights into the mechanisms that regulate expression of the human kallikrein genes. Besides KLK3 and KLK2, no other kallikrein gene promoter has been functionally tested.

Foot printing and mutation analysis have confirmed the presence of a TATA-box and a GC-box at the early promoter region of the prostate-specific antigen (KLK3) (Riegman *et al.,* 1989b, 1991b). Transfection experiments with deletion constructs revealed the presence of two androgen response elements (ARE-I and ARE-II) at positions -170 and -400 of the KLK3 promoter (Cleutjens *et al.,* 1996). Another ARE (ARE-III) was mapped in the far upstream enhancer region of the gene and shown to be functional and tissue-specific (Schuur *et al.,* 1996; Cleutjens *et al.,* 1997; Pang *et al.,* 1997; Brookes *et al.,* 1998). More recently, five additional low affinity AREs have been identified close to ARE-III (Huang *et al.,* 1999), and three distinct regions surrounding ARE-III were found to bind ubiquitous and cell-specific proteins. These regions were shown by mutation analysis to be required for maximal activity in the LNCaP prostate cancer cell line (Farmer *et al.,* 2001). Cell line transfection with a series of 5' deletion constructs of the KLK2 promoter revealed the presence of a functional ARE, although less homologous to the consensus glucocorticoid response element (GRE) and less palindromic than that of KLK3, at position -170 (Murtha *et al.,* 1993), which is the exact position where ARE-I of KLK3 was found. In both KLK2 and KLK3, these response elements were experimentally proven to be activated by androgen. Interestingly, a negative regulatory element was also found in position -468 to -323 of KLK2 (Murtha *et al.,* 1993). Henderson *et al.* identified another ARE at position -3819 to -3805 of the KLK2 promoter which is identical to ARE-II of KLK3 (Yu *et al.,* 1999). Computer analysis revealed the presence of highly conserved CREB, AP-1 binding site and c-Fos serum response elements at comparable positions in the enhancer regions of both genes (Yu *et al.,* 1999). There is also about 75% homology between both promoters in the region around ~– 3.5 and 5.2 kb. Apart from KLK2 and KLK3, no obvious TATA boxes were found in the promoter of other kallikreins. Although no typical TATA box or CCAAT sequence was detected in the KLK 8 promoter, a weak TATA box-like sequence (TTAAAA) and other transcription factor binding sites were predicted (Yoshida *et al.,* 1998a). A putative TATA box was also predicted for the KLK4 gene (Hu *et al.,* 2000). Two major obstacles exist in predicting the promoter response elements; the inaccurate localization of the transcription start site, and the presence of more than one splice variant with more than one start codon.

Steroid hormones, acting through their receptors, play important roles in normal development and function of many organs. In addition, they seem to be involved in the pathogenesis of many types of cancer (Trapman and Cleutjens, 1997). Several reports confirmed that many kallikreins are under steroid hormone regulation in endocrine-related tissues and cell lines (Riegman *et al.,* 1991a,b; Murtha *et al.,* 1993; Nelson *et al.,* 1999; Yousef and Diamandis, 1999; Yousef *et al.*, 1999b, 2000a,b,e,f, 2001a,b,c; Myers and Clements, 2001). An interesting observation is the tissue-specific pattern of regulation of some genes (*e.g.* the prostate-specific expression of PSA) and the different pattern of hormonal regulation in different tissues; *e.g*. KLK4 is up-regulated by androgen in prostate and breast cancer cell lines (Nelson *et al.,* 1999) and by estrogen in endometrial cancer cell lines (Myers and Clements, 2001). Also, KLK12 was found to be up-regulated by androgens and progestins in prostate cancer cell lines and by estrogens and progestins in breast cancer cell lines (Yousef *et al.,* 2000d).

At the Protein Level

There are different mechanisms for controlling serine protease activity in order to avoid any unwanted function

and to allow special and temporal regulation of the proteolytic activity. One important mechanism is by producing kallikreins in an inactive 'preproenzyme' form which will be activated when necessary. The N-terminal extension of the mature enzyme, or the 'prosegment' sterically blocks the active site and thus prevents binding of substrates. It is also possibly implicated in folding, stability and intracellular sorting of the zymogen. For more detailed discussion see the recent review by Khan and James (Khan and James 1998). All kallikreins are predicted to be synthesized as preproenzymes with the N-terminal end formed of the signal peptide followed by the activation peptide. The activation of the zymogen (also called the pro-enzyme) can occur intracellularly, *i.e*., in the trans-Golgi or the secretory granules, or extracellularly after secretion, and it can be autolytic or dependent on the activity of another enzyme (see below). Interestingly, all of the 'proforms' of the kallikrein enzymes, with the exception of hK4, are activated by cleavage at the carboxy terminal end of either arginine or lysine (the preferred trypsin cleavage site), indicating that they will need an enzyme with trypsin-like activity for their activation. This observation has been experimentally proven for some kallikreins. hK5 and hK7 can be converted to the active enzyme by trypsin treatment (Hansson *et al.,* 1994; Brattsand and Egelrud 1999) and hK11 can be activated by entrokinase (Mitsui *et al.,* 2000b). Autoactivation is reported among kallikreins. hK2, but not hK3, is capable of autoactivation (Denmeade *et al.,* 2001). The reason for this may be that hK3 has chymotryptic activity while it needs a trypsin-like activating enzyme. hK4 is also autoactivated during the refolding process (Takayama *et al.,* 2001a), and experimental evidence showed that hK6 is capable of autoactivation (Little *et al.,* 1997). hK13 is also autoactivated upon secretion (G. Sotiropoulou, personal communication). This can be explained by the finding that many kallikreins have trypsin-like substrate activity and the same type of activity is needed for their activation. Proteolytic activation is irreversible. Hence, other means of switching off the activity of these enzymes are needed. One way to achieve that is by binding of kallikreins to serine protease inhibitors, known as 'serpins' (for serine protease inhibitors). These are usually poor substrates with strong inhibition by the inhibitor requiring hydrolysis of a peptide bond in the inhibitor by the protease. hK3 has been shown to form complexes with many extracellular protease inhibitors such as α_1-antichymotrypsin, α_2-macrogolbulin and α1-antitrypsin (Christensson *et al.,* 1993; Christensson and Lilja, 1994) and hK2 was found to be bound to α_2-antiplasmin, antithrombin III, plasminogen activator inhibitor-1, and α_2-macroglobulin (Stephan *et al.,* 2000). Another mechanism for controlling the activity is by internal cleavage and subsequent degradation. Self-digestion is reported for hK7 (Hansson *et al.,* 1994). Around 30% of hK3 in seminal plasma is inactivated by internal cleavage between lysine 145 and lysine 146 (Christensson *et al.,* 1990), and about 25% of hK2 was found to be internally cleaved between amino acids 145–146 (Arg-Ser) (Lovgren *et al.,* 1997).

Do Tissue Kallikreins Represent a Novel Enzymatic Cascade Pathway?

Interactions between serine proteases are common, and substrates of serine proteases are usually other serine proteases that are activated from an inactive precursor (Schultz and Liebman, 1997). The involvement of serine proteases in cascade pathways is well documented. One important example is the blood coagulation cascade. Blood clots are formed by a series of zymogen activations. In this enzymatic cascade, the activated form of one factor catalyzes the activation of the next factor. Very small amounts of the initial factors are sufficient to trigger the cascade because of the catalytic nature of the process. These numerous steps yield a large amplification, thus ensuring a rapid and amplified response to trauma (Silverthorn 1995). Signal amplification is an important characteristic of cascades. A single molecule of enzyme A will be able to activate two or more substrate enzymes (B) and each in turn will activate two or more of enzyme (C) and so on. By the end of the cascade, a single molecule will be able to activate many molecules of the end product. Several of the activated factors of this cascade are serine proteases. A similar mechanism is involved in the dissolution of blood clots where activation of plasminogen activators lead to conversion of plasminogen to plasmin which is responsible for lysis of the fibrin clot. A third important example of the coordinated action of serine proteases is the intestinal digestive enzymes. The digestion of proteins in the duodenum requires the concurrent action of several proteolytic enzymes. Coordinated control is achieved by the action of trypsin as the common activator of all pancreatic zymogens; trypsinogen, chymotrypsinogen, proelastatse, and procarboxypeptidase. Trypsin is first activated by the enteropeptidase enzyme, and a small amount of the active trypsin activates more trtypsin in addition to all other proteases. The apoptosis pathway is another important example of coordinated action of proteases.

The cross talk between kallikreins and the hypothesis that they are involved in a cascade enzymatic pathway are supported by strong, but mostly circumstantial evidence, as follows:

- The co-expression of many kallikreins in the same tissue.
- The ability of some kallikreins to activate each other (see below).
- The ability of other serine proteases to activate kallikreins.
- The common patterns of hormonal regulation (by steroids).
- The parallel patterns of expression in normal tissues (*e.g*., the adjacently localized kallikrei genes KLK2-5 all highly expressed in prostate).

– The parallel pattern of differential expression of many kallikreins in different malignancies (*e.g*., at least 7 kallikrein genes are up-regulated in ovarian cancer, KLK4, 5, 6, 7, 8, 10 and 11; and at least 7 kallikreins are down-regulated in breast cancer, KLK2, 3,6,10, 12, 13, and 14). Table 3 summarizes the data available so far about the differential expression of kallikreins in endocrine-related malignancies.
– Serine proteases commonly utilize other serine proteases as substrates.

Recent experimental evidence has shown that hK3 (PSA) can be activated by hK15 (Takayama *et al.,* 2001a). hK4 have also recently been shown to activate hK3 much more efficiently compared to hK2 (Takayama *et al.,* 2001b). hK5 is predicted to be able to activate hK7 in the skin (Brattsand and Egelrud, 1999). The activation of kK3 by hK2 is possible. While Takayama *et al.*, reported the ability of hK2 to activate hK3 (Takayama *et al.,* 1997), Denmeseade *et al.* (2001) reported the opposite and hypothesized that additional proteases may be required, further supporting the proposed hypothesis. It will be interesting to study all possible combinations of interactions among kallikreins, especially those with expression in the same tissues. Bhoola *et al.* (2001) have recently provided strong evidence of the involvement of a 'kallikrein cascade' in initiating and maintaining systemic inflammatory responses and immune-modulated disorders. Another way of kallikreins initiating a cascade type of reaction is by activating membrane receptors (*e.g*., protease-activated receptors, PAR).

Kallikreins might also be involved in cascade reactions involving other non-kallikrein serine proteases. This is evident from the reported but questionable ability of hK3 to activate insulin-like growth factor-binding protein (IGF-BP-3) (Cohen *et al.,* 1992), and also to inactivate the amino terminal fragment of the parathyroid hormone-related protein (PTHrP) by cleavage (Iwamura *et al.,* 1996).

Table 3 Differential Expression of Human Kallikreins in Various Cancers.

Cancer	Differentially expressed kallikreins[a]	Prognostic value of higher expression	References
Breast	hK2		
	hK3,		
	KLK5	Poor prognosis	(Yousef *et al.,* 2002c)
	hK6	Association with hormone receptors	Our unpublished data
	KLK10	Down-regulated in breast cancer	(Dhar *et al.,* 2001)
		Favorable prognosis.	Our unpublished data
	KLK12	Down-regulated in breast cancer	(Yousef *et al.,* 2000d)
	KLK13	Down-regulated in breast cancer,	(Yousef *et al.,* 2000b)
		Favorable prognosis	(Chang *et al.,* 2002)
	KLK14	Down-regulated in breast cancer	(Yousef *et al.,* 2001b)
	KLK15	Favorable prognosis	Our unpublished data
Prostate	hK2	Diagnostic marker	(Partin *et al.,* 1999)
	hK3	Diagnosis, prognosis and follow-up	(Diamandis, 1998)
	KLK5	Favorable prognosis	(Yousef *et al.,* 2002b)
	hK11	Up-regulated in prostate cancer	(Diamandis *et al.,* 2002)
	KLK15	Poor prognosis	(Yousef *et al.,* 2001c)
Ovarian	KLK4	Poor prognosis	(Obiezu *et al.,* 2001)
		Poor prognosis	(Dong *et al.,* 2001)
	KLK5	Poor prognosis	(Kim *et al.,* 2001)
	hK6	Poor prognosis	(Diamandis *et al.,* 2000c)
	KLK7	Overexpression in ovarian cancer	(Tanimoto *et al.,* 1999)
	KLK8	Favorable prognosis	(Magklara *et al.,* 2001)
		Overexpression in ovarian cancer	(Underwood *et al.,* 1999)
	KLK9	Favorable prognosis	(Yousef *et al.,* 2001a)
	hK10	Diagnosis of ovarian cancer	(Luo *et al.,* 2001a)
		Poor prognosis	(Luo *et al.,* 2001c)
	hK11	Up-regulated in ovarian & prostate cancer	(Diamandis *et al.,* 2002)
	KLK14	Down-regulated in ovarian cancer	(Yousef *et al.,* 2001b)
	KLK15	Poor prognosis	Our unpublished data
Testicular	KLK5	Favorable prognosis	(Yousef *et al.,* 2002a)
	hK10	Down-regulated in testicular cancer	(Luo *et al.,* 2001d)
	KLK14	Down-regulated in testicular cancer	(Yousef *et al.,* 2001b)

[a]When differential expression was studied by RT-PCR we refer to the genes (KLK); when it was studied by immunoassay or immunohistochemistry, we refer to the protein (hK).

Similar properties were reported for rodent kallikreins (Matsui and Takahashi 2001). Experimental evidence has also shown that hK2 and hK4 can activate the pro-type of another serine protease, the urokinase plaminogen activator (uPA) (Lovgren *et al.,* 1997; Takayama *et al.,* 2001b). Urokinase activates plasmin (another serine protease) from its inactive form (plasminogen) which is ubiquitously located in the extracellular space, leading to degradation of extracellular matrix proteins. This might explain how kallikreins are involved in cancer progression (Kim *et al.,* 2001; Luo *et al.,* 2001d; Obiezu *et al.,* 2001; Yousef *et al.,* 2000b, 2001a,c). As mentioned above, other serine proteases, such as enterokinase and trypsin are able to activate many kallikreins. Furthermore, hK4 can degrade prostatic acid phosphatase in seminal plasma (Takayama *et al.,* 2001b). hK7 degrades the alpha chain of the native human fibrinogen and it is also hypothesized that it is involved in an apoptotic-like mechanism that leads to desquamation of the skin (Smyth, 1998). Figure 1 represents a proposed model for the possible role of a kallikrein cascade in the progression of ovarian cancer.

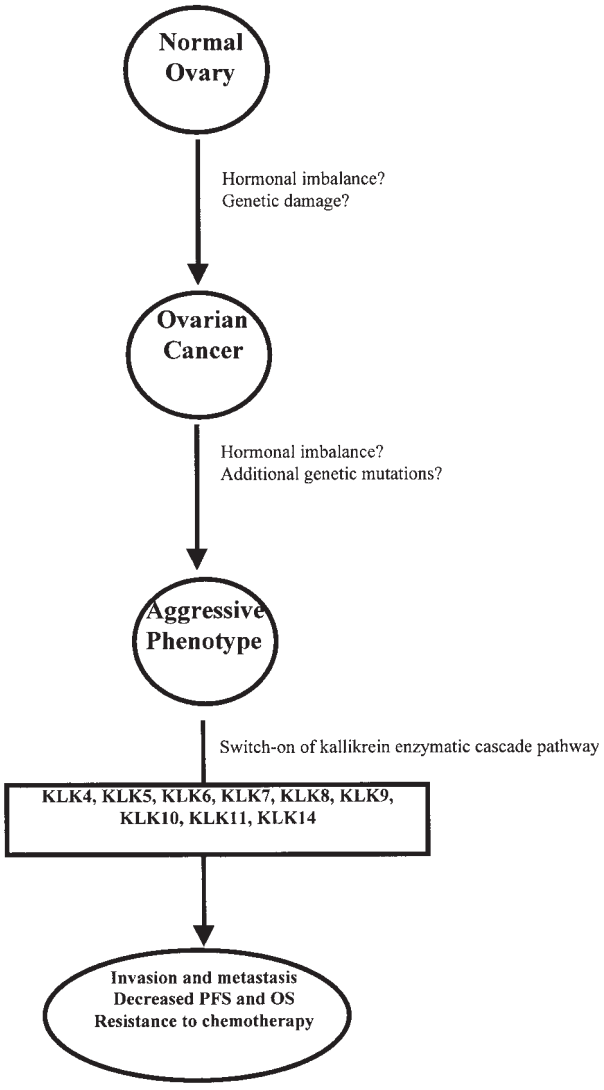

Fig. 1 Schematic Presentation of the Possible Role of a Potential Kallikrein Cascade in the Progression of Ovarian Cancer. PFS, progresssion-free survival; OS, overall survival.

Conclusions

Knowledge on human kallikrein genes is evolving rapidly. Now that the structure of these genes is well-characterized, it will be interesting to study the physiological function of the proteins and their possible connection to pathological processes. Also, interesting is the recent association of kallikrein gene expression with cancer and other diseases and the finding that many circulating kallikreins are biomarkers for cancer (Anisowicz *et al.,* 1996; Liu *et al.,* 1996; Underwood *et al.,* 1999; Diamandis *et al.,* 2000b; Diamandis and Yousef, 2001; Luo *et al.,* 2001a; Magklara *et al.,* 2001; Yousef *et al.,* 2001a,b,d). The examination of tissue kallikreins as therapeutic targets (through activation or inhibition) may also be important in selected cases. Clearly, over the next three to five years, the physiology and pathobiology of this large family of serine proteases will be more precisely defined.

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