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Functional proteomics of kallikrein-related peptidases in ovarian cancer ascites fluid*

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Abstract

Kallikrein-related peptidases (KLKs) are secreted serine proteinases with trypsin or chymotrypsin-like activity. Several family members, such as KLKs 6 and 10, are potential ovarian cancer biomarkers. Recently, using a newly developed assay for active KLK6, we found that only a very small proportion of immunoreactive KLK6 in tumor-derived clinical samples (malignant ascites fluid), in cerebrospinal fluid, and in cancer cell line supernatants is enzymatically active. We therefore hypothesized that a proportion of other immunoreactive KLKs in such samples could be present, but might be partly complexed to endogenous serine proteinase inhibitors. Using a combination of immunological isolation of the enzymes, activity-based probe analysis and proteomics, we identified active KLK10 in ovarian cancer ascites and we provide preliminary data that the activity of other KLKs present in these samples can be decreased by known proteinase inhibitors (e.g., α2-macroglobulin, α1-antitrypsin). Our data suggest that the enzymatic activity of ovarian cancer-released KLKs that are detected by regular immunoassays is low *in vivo* and very likely regulated by proteinase inhibitors.

Keywords: activity-based probe (ABP); ascites fluid; chymotrypsin-like activity; human kallikrein-related peptidase (KLK); serine proteinase; serine proteinase inhibitor; trypsin-like activity.

Introduction

Kallikrein-related peptidases (KLKs) belong to a family of serine proteinases with trypsin or chymotrypsin-like activity (Borgoño et al., 2004; Borgoño and Diamandis 2004; Yousef et al., 2005). The human family consists of 15 enzymes, which are secreted as inactive zymogens and are proteolytically activated by removal of a short N-terminal sequence. Several members of the family are upregulated in tissues, ascites fluid, and serum of cancer patients and their levels have been correlated with the course of disease.

Kallikreins 4, 5, 6, 7, 8, 10, 11, 13, 14, and 15 are upregulated in the serum of ovarian cancer patients or in ovarian cancer tissues (Borgoño and Diamandis, 2004; Borgoño et al., 2004; Obiezu and Diamandis, 2005; Yousef et al., 2005). Among these KLKs, kallikrein-related peptidase 6 is the most widely studied family member, used as a biomarker of unfavorable prognosis in ovarian cancer. KLK6 levels can also be utilized for the purpose of disease diagnosis and monitoring. Notably, a serum analysis that combines measurements of KLK6 levels with those of CA125, a known ovarian cancer biomarker, has proved of greater utility than the measurement of either KLK6 or CA125 alone (Diamandis et al., 2003). Similar to KLK6, immunoreactive kallikrein-related peptidase 10 is overexpressed in ovarian carcinomas and has been correlated with unfavorable disease prognosis (Luo et al., 2003). In keeping with the combined analysis of KLK6 with CA125, combined measurements of both KLK10 and CA125 are of superior clinical value than measurements of either KLK10 or CA125 alone. Interestingly, upregulation of both kallikrein-related peptidase 6 and 10 genes has been observed in serous papillary ovarian tumors (Santin et al., 2004) and a correlation between the mRNA expression levels of the two enzymes in tumor cells disseminated in the ascites fluid of ovarian cancer patients has also been reported (Oikonomopoulou et al., 2006d). Several reports documenting the actions of KLKs *in vitro* have pointed to potential mechanisms whereby kallikrein-related peptidases can contribute to carcinogenesis. For instance, KLKs are able to degrade the extracellular matrix network and growth factor signaling components (Borgoño and Diamandis, 2004), suggesting their involvement in tissue remodeling and cell invasion, associated with tumor growth and metastasis. Another mechanism whereby KLKs can regulate cell function is by cleaving and activating proteinase-activated receptors (Oikonomopoulou et al., 2006a,b,c), which, via G-protein-coupled mechanisms, can trigger mitogenic and chemotactic signaling pathways. These pathways play roles in inflammation, carcinogenesis, invasion, metastasis, angioge-

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nesis, cell death, and survival (Steinhoff et al., 2005; Hansen et al., 2008). Despite the abundance of reports demonstrating the actions of KLKs *in vitro*, the exact roles that kallikrein-related peptidases play *in vivo* in settings, such as cancer, are still a matter for conjecture. This uncertainty is due in part to the lack of adequate methods of identifying specific KLKs that are enzymatically active in biological fluids and tumors. The regulation of kallikrein-related peptidase activity *in vivo* is achieved mainly through proteolytic activation and inactivation, either by autodigestion or degradation by other proteinases (Bayes et al., 2004; Borgoño and Diamandis, 2004). Another important mechanism for regulating kallikrein-related peptidase enzymatic activity is *via* interaction with endogenous inhibitors such as α2-antiplasmin, antithrombin III, and α1-antichymotrypsin. Serine proteinase inhibitors (e.g., serpins; Borgoño and Diamandis 2004; Borgoño et al., 2004; Luo and Jiang, 2006) can be a key determinant of the KLK pathophysiological roles. This kind of situation has been found in the setting of Netherton syndrome. In this syndrome, a defect in the serine proteinase inhibitor, Kazal type 5 (SPINK5), leads to a KLK-mediated ichthyosiform skin disease as a result of unchecked KLK enzyme activity (Deraison et al., 2007). Given that increases in KLK immunoreactivity have been documented in a variety of pathological settings, a key issue to consider is: what proportion of immunoreactive KLK represents active enzyme? Our previous study using immunofluorometric quantification of KLK6 coupled with activity-based probe analysis has pointed to the existence of very low KLK6 enzyme activity in biological fluids (Oikonomopoulou et al., 2008). In this preliminary report, we outline the principles of an approach for identifying KLK-proteinase activity in biological fluids, coupling the use of activity-based probes (ABPs) with proteomic analyses. In principle, this type of approach could be the basis of a high-throughput analysis of biological fluids for active proteinases and their potential inhibitors. With our method we have demonstrated for the first time that enzymatically active KLK10 can be found in ovarian cancer ascites. In contrast, other trypsin-like KLKs known to react with the ABP and known to be present in this fluid from immunoassay data were not detected with the ABP approach and are presumably enzymatically inactive. We hypothesize that the lack of enzyme activity could be due to the presence of multiple serine proteinase inhibitors that we have also identified in the ovarian ascites fluids with the use of our proteomic methods.

Results

Estimation of trypsin-like serine proteinase activity of ovarian cancer ascites

Proteinase enzymatic activity was estimated with the help of four different fluorogenic substrates: VPR-AMC, VLK-AMC, GPR-AMC, and GPK-AMC (Figure 1). The GPK-containing substrate mimicked the P2 and P1 position of the ABP used in our further analysis and was, therefore, used for comparison purposes. This analysis revealed that the overall enzymatic activity of trypsin-like serine proteinases in undiluted ovarian cancer ascites is low [average activity with VPR-AMC as substrate was approximately 6000 fluorescence (FU) units per min, whereas 5 nM of active trypsin exhibited 56 500 FU/min with the same substrate; data not shown]. The enzyme activity detected was variable, depending on the sequence of the tripeptide fluorogenic substrate, pointing to the presence of proteinases with different substrate specificities in our samples. Notably, one of the cancer ascites samples (no. 8) from a patient with advanced ovarian cancer revealed high overall trypsin-like activity for three out of the four substrates tested. In all of these three substrates, proline was present at the P2 position, an indication that the proteinases responsible for the substrate cleavage would exhibit high efficiency to react with the proline-lysine-containing ABP used in the following applications.

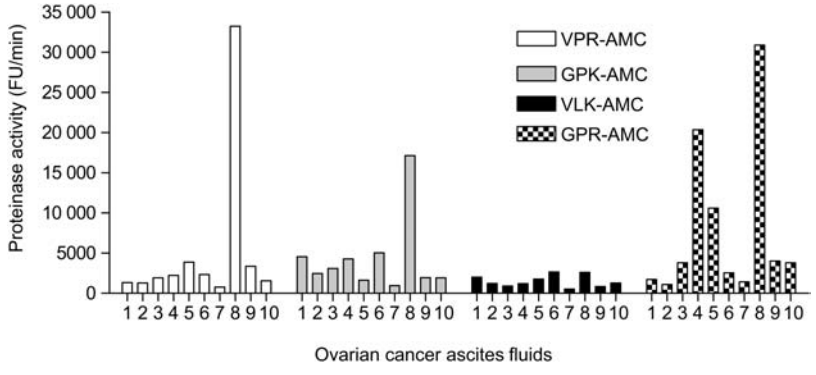

Figure 1 Trypsin-like serine proteinase activity of ovarian cancer ascites.
Enzymatic activity was estimated using VPR-, VLK-, GPR-, and GPK-AMC. Incubation of the enzyme with each substrate (1 mM) was performed in 50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% Tween-20, pH 7.6, at 37°C. Fluorescence was monitored for 20 min on a Wallac Victor fluorometer (355 nm for excitation and 460 nm for emission) and the background fluorescence was subtracted from each value. Slopes of the fluorescence curves (fluorescence units/min; FU/min), used to quantify the proteinase activity, were estimated by nonlinear regression analysis taking into account the fluorescence units released per minute of reaction.

Detection of active trypsin-like proteinases in ovarian cancer ascites fluid using ABP proteomics

The ABP proteomics protocol, involving the analysis of protein sequences specifically adsorbed to the streptavidin beads, unequivocally identified the KLK6, which was added to the ascites fluid prior to ABP incubation and served as our positive control (data not shown). Furthermore, other endogenous proteinases and proteinase-related proteins were identified within the ovarian cancer ascites fluid proteome. To control for enzymes that were adsorbed to the streptavidin beads non-specifically, we excluded peptide signals that were generated by mass spectrometry for 'control' ascites samples that had not been reacted with the ABP. Only those proteins identified as ABP/streptavidin binding-specific are recorded in Table 1. Our list of ABP-labeled enzymes included trypsin-like proteinases of the complement system (C1s), thrombin, coagulation factors F11 and F12, kallikrein-related peptidase 10 (KLK10), and plasma kallikrein-related peptidase (KLKB1), as well as complement-related proteins such as C1q and C4 (which can bind to C1s). More specifically, KLK10 was identified in two of these samples, whereas all of C1s, F11, F12, thrombin, KLKB1, C1q, C4 were found as a 'cluster' in one sample and individually in other samples. We thus concluded that all of the enzymes listed in Table 1 could in principle be identified by our approach. The ovarian cancer ascites fluid sample previously mentioned (Figure 1, no. 8), which had exhibited relatively high trypsin-like proteinase activity (cleavage of the VPR-fluorogenic substrate at a rate comparable to the activity of approximately 5 nM trypsin, using the same tripeptide-AMC substrate), was shown with the ABP-proteomic approach to contain complement components and KLK10. Despite the variability of the proteinases in each individual ovarian cancer ascites fluid sample that might be able to react with the activity-based probe used in this study, all of the proteinases identified belonged to three proteinase families: the kallikrein-related peptidases, the enzymes of the coagulation system, and the proteinases of the classical pathway of the complement cascade. All three families have been previously associated either directly or indirectly with carcinogenesis.

Table 1 Trypsin-like proteinases identified in ovarian cancer ascites fluid samples.

Protein name	+ABP	-ABP
Trypsin-like proteinase identities and number of unique peptides identified by mass spectrometric analysis		
Complement component C1s	4	0
Coagulation factor XI	9	0
Coagulation factor XII	2	0
Thrombin	12	0
Plasma kallikrein (KLKB1)	2	0
Kallikrein-related peptidase 10 (KLK10)	2	0
Proteinase-bound protein identities and number of unique peptides identified by mass spectrometric analysis		
Complement component C1q	4	0
Complement component C4	5	0

The Table presents selected identities from four ovarian cancer ascites fluid samples and shows the number of unique peptides assigned to each protein hit. '+ABP' indicates the samples treated with the activity-based probe, whereas '-ABP' denotes the negative control treatments omitting the activity-based probe.

Active KLK6 forms enzymatically inactive high-molecular mass complexes with components of ovarian cancer-derived ascites fluid

SDS-PAGE separation and Western blot analysis of ascites fluid did not yield a signal for KLK6 at the expected 28–30 kDa region of the gel. This result was similar to a lack of Western blot signal at the 28–30 kDa position for cell supernatant from breast tumor-derived MDA-MB-468 cells (negative control). In contrast, a good Western blot signal was observed in the 28–30 kDa region of the gel with the cerebrospinal fluid (CSF) sample, so as to serve as our positive control (white arrowhead, Figure 2A). The results of the Western blot analyses can be rationalized in terms of the relatively low levels of KLK6 detected by immunoassay in some of these samples (MDA-MB-468: 13 μg/l, ovarian cancer ascites: 42 μg/l), compared with the higher levels in the CSF sample (567 μg/l). The KLK6 levels in the ovarian cancer ascites and MDA-MB-468 cell supernatants lie below the detection limit of our Western blot approach. However, in the MDA-MB-468 and ovarian cancer ascites samples, the KLK6 Western blot analysis did reveal higher molecular mass crossreactive components migrating in the region of 58–188 kDa (upper bands, Figure 2A), particularly for the breast cancer cell supernatant. Because the KLK6 immunoreactivity in these higher molecular mass regions of the gel failed to react with the ABP (no biotin signal detected), the ABP data implied that the trypsin-like proteinases migrating with these higher molecular weight bands under reducing conditions were not enzymatically active in the original sample (data not shown). Trypsin fragmentation and mass spectral analysis of protein in samples obtained from the 50 to 62 kDa region of the gel were found to contain high levels of albumin. Thus, potentially KLK6 might have been able to co-migrate with albumin, even under SDS/reducing conditions. However, proteomic analysis of this area of the gel was difficult to interpret in terms of its content of KLK6 and inhibitors and was, therefore, excluded from further analysis. The proteomic analysis of components that migrated in the higher molecular mass region of the gel (>62 kDa) revealed sequence information that identified proteinase inhibitors such as α2-macroglobulin (α2M), α1-antitrypsin, and inter-α-trypsin inhibitor (ITI). These inhibitors were able to form covalent complexes with exogenously added active KLK6 (Figure 2B). Of note is that for the CSF, MDA-MB-468 cell supernatant, and the ovarian cancer ascites samples supplemented with KLK6, the molecular mass range of the gel in which proteomic analysis identified α2M-derived sequences did not correspond to the expected mass of an intact KLK6-α2M complex. In theory, the α2M-KLK6 complex should exhibit a molecular mass of approximately 210 kDa, according to the α2M monomer size (185 kDa) reported by Zhang et al. (2006b). This result points to the possible presence of a truncated form of the

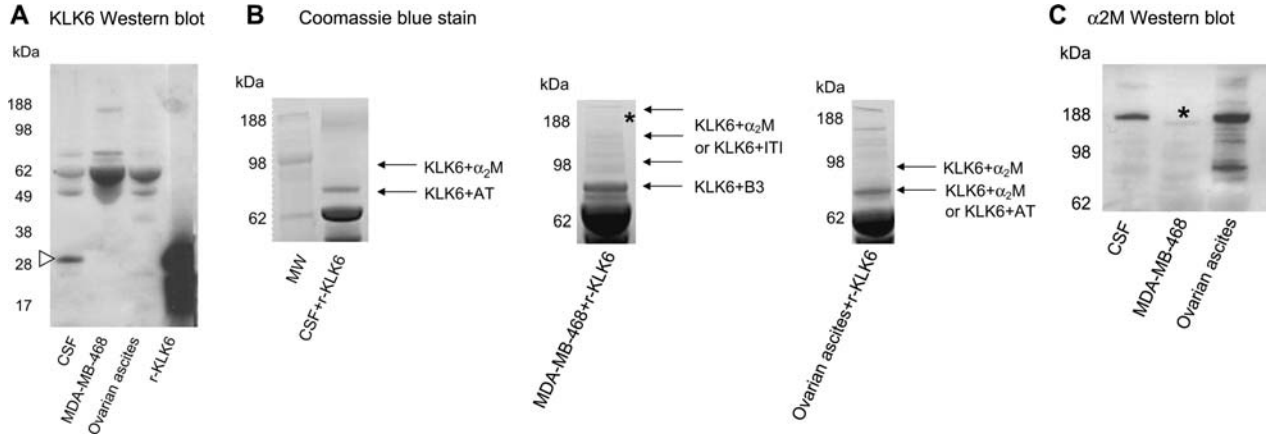

Figure 2 Western blot of kallikrein-related peptidase 6 in biological fluids.
Samples of CSF, MDA-MB-468 cell culture supernatant and ascites fluid were analyzed by polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred to Hybond-C Extra nitrocellulose membrane. (A) KLK6 immunoreactivity was detected using a rabbit anti-serum for KLK6 produced in-house (1:3000 dilution) and a goat anti-rabbit ALP-conjugated secondary antibody (1:5000 dilution). The open arrowhead denotes the position in the gels of active recombinant KLK6. The positions of the molecular mass markers (kDa) are shown on the left of the gels. (B) The CSF, MDA-MB-468 cell supernatant, and ovarian cancer-derived ascites fluid samples were supplemented with 20 μg/ml recombinant enzymatically active KLK6 (r-KLK6) and were subjected to SDS-PAGE separation under reducing conditions followed by detection of protein by Coomassie stain. Coomassie Blue-stained proteins were excised from the gel for proteomic analysis. Gel samples were subjected to trypsin digestion and mass spectrometric identification of the tryptic fragments. The arrows to the right of the Coomassie-stained gels in panel (B) show the high-molecular weight components identified by mass spectrometric analysis at each position in addition to a signal for KLK6: α2M=α2-macroglobulin; AT=α1-antitrypsin; ITI=inter-α-trypsin inhibitor; B3=serpin B3 or squamous cell carcinoma antigen 1. The asterisk denotes positions at which the same components were identified from the gel slices for samples either supplemented or not with recombinant KLK6. (C) α2-Macroglobulin-immunoreactive species were identified using a goat α2-macroglobulin anti-serum (1:2000 dilution) and a bovine anti-goat ALP-conjugated secondary antibody (1:5000 dilution). The Western blot data in panel (C) show the higher molecular mass forms of α2-macroglobulin-immunoreactive species in relation to the gel positions of the Western blot signals for KLK6 (panel A). The positions of the molecular mass markers (kDa), visualized separately, are shown on the left.

inhibitor bound to KLK6. The sequences of α2M identified in our samples by mass spectrometry indicated that the human protein was present, with its unique sequence (DMYSFL**E**DMGLK: the glutamic acid residue confirming the presence of the human sequence is shown in a box). This result proved that at least a portion of the α2M detected in the MDA-MB-468 cell supernatants did not come from the bovine serum used to grow the cells. Western blot analysis of the samples verified the presence of these inhibitors (α2M and α1-antitrypsin) in all three samples (data shown only for α2M in Figure 2C: the α2M species discussed above is indicated with an asterisk).

Immunoextraction of KLKs from ovarian cancer ascites fluid together with proteinase inhibitors

The proteins captured by the microtiter plate approach using the KLK monoclonal antibodies and fragmented by tryptic digestion were subjected to mass spectrometry analysis to identify potential inhibitors that had been adsorbed on the KLK-antibody-coated plates along with the KLKs (Figure 3; left panel). Our approach successfully identified KLKs 5, 6, 8, and 10 in two ovarian cancer ascites fluids (Figure 3; right panel). One of these fluids (Figure 1, no. 8) was the one that also exhibited relatively high enzymatic activity using the fluorogenic substrates and also demonstrated reactivity with the ABP, resulting in the identification of KLK10 using the ABP proteomic approach (Table 1). For the other KLKs, a fragment indicative of the proteinase could not be identified with this protocol. Of note, tryptic supernatants recovered from wells coated with the KLKs 5, 6, 8, and 10 antibodies were found to contain serine proteinase inhibitors, namely α1-antitrypsin and ITI (Figure 3; right panel). That said, the same inhibitors were also detected, but in smaller amounts (lower mass spectrometry signal), after trypsinization of wells that were exposed to the ascites samples but which had either (a) been precoated with antibodies targeted to KLKs observed by immunoassay in preliminary work (e.g., KLKs 2 and 3) to be either low or absent from the ascites fluid (i.e., the 'control' antibody wells) or (b) had not been exposed at all to antibody ('non-coated control' wells). Despite the limitation of this protocol, our data verified the findings of our Western blot approach followed by the in-gel trypsin-based proteomics, demonstrating the presence of the proteinase inhibitors in the ovarian ascites samples. No doubt, these inhibitors are capable of forming complexes with the kallikrein-related peptidases present in the same flu-

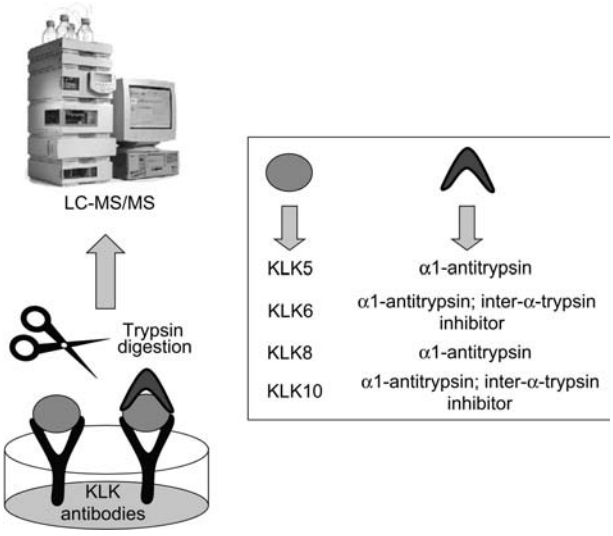

Figure 3 Inhibitors identified together with KLKs 5, 6, 8, and 10 in ovarian cancer ascites fluid.
96-well microtiter plates were coated with 500 ng/well mouse monoclonal antibodies against KLKs using 50 mM Tris buffer pH 7.8. The plates were washed five times with 0.9% NaCl solution, samples were loaded in duplicates, incubated for 1 h at room temperature, washed, prepared for protein digestion and identified by mass spectrometry. The left-hand scheme depicts the capture, trypsin fragmentation, and mass spectrometric identification of the proteins. The right-hand box shows the kallikrein-related peptidases (gray sphere) and proteinase inhibitors (gray arrowhead) identified from the sequences. Control experiments were done in which the plates were either not coated with antibody or were coated with anti-KLK antibodies for KLKs other than KLKs 5, 6, 8, and 10 and known not to be present in the fluids by immunoassay measurements.

ids. Whether or not the KLKs were present as covalent complexes with the inhibitors remains to be determined by a more stringent biochemical analysis.

Gel filtration chromatography of highly active ovarian cancer ascites

The ovarian cancer ascites sample (no. 8) that showed a relatively high trypsin-like proteinase activity and KLK reactivity (immunoassay and ABP reactivity for KLK10) using our previous methods was fractionated via size-exclusion chromatography. Eight fractions were collected and analyzed for trypsin-like proteinase activity and protein immunoreactivity (Figure 4A). Fractions 4–7 contained enzymes that were found to cleave VPR-AMC with higher efficiency (Figure 4B, white bars), with the peak of enzyme activity detected in fraction 5 (slope of the fluorescence curves, expressed as fluorescence units (FU)/min, of 3320 FU/min). ELISA quantification indicated the presence of KLK6 in the same fractions, but the concentration of this enzyme detected by immunoassay in fraction 5 (less than approximately 0.5 μg/l) was much lower than the KLK6 levels (average of approximately 12 μg/l) in the fractions that eluted later in the chromatogram (fractions 6 and 7; Figure 4B, gray bars). Furthermore, immunofluorometric quantification of KLK10 also confirmed the presence of KLK10 (mainly in fractions 5 and 6: average of approximately 4 μg/l; Figure 4B, black bars). Western blot analysis and ABP analysis of the same fractions further confirmed the ABP-reactive trypsin-like activity of fraction 5 (ABP-biotin labeling detected with streptavidin), whereas KLK10 immunoreactivity was also localized in fractions 5 and 6 (Figure 4C). This analysis also showed an overlap of Western blot reactivity of the KLK10 and KLK6 polyclonal antibodies in the eluted samples (data not shown). Nevertheless, the appearance of enzyme activity was clearly out of step with the elution of immunoreactive KLKs and, therefore, no correlation could be made between the levels of KLKs 6 or 10 and the overall abundance of trypsin-like enzymatic activity in the chromatographic fractions. This lack of correlation between enzyme activity and KLK immunoreactivity points to the presence of proteinases other than the KLK enzymes in the fluid, in addition to the active KLK10 that was verified to be present as an active species by the ABP/proteomic analysis.

Discussion

One main result of this preliminary study was to demonstrate, in principle, that in an objective way, it is possible with our ABP-proteomic approach to identify a number of active trypsin-like proteinases in clinical samples such as ovarian ascites (Table 1). That said, only a very low level of enzyme activity relative to the total amount of immunoreactive KLKs was present in the ovarian cancer ascites fluids samples. The low levels of KLK enzyme activity are most likely due to the presence of serine proteinase inhibitors that we were also able to identify by our proteomic approach using the same samples. The inhibitors that we found could in principle neutralize both the KLKs and other serine proteinases very effectively. Whether or not our data unequivocally identified covalent complexes between the KLKs and these inhibitors remains to be verified by further analytical work. Our conclusion that only a low proportion of immunoreactive KLKs represents enzymatically active proteins is supported by the following: (a) we detected only minimal trypsin-like activity in the ascites samples with the fluorogenic substrate cleavage assay using a variety of tripeptide substrates, (b) the activity-based proteomics approach failed to detect enzymatically active endogenous KLKs, other than KLK10 (Table 1), despite the identification of KLKs 5, 6, and 8 by the immunocapture protocol, (c) the detection in analytical gels of KLK6 immunoreactivity that was present in the higher molecular mass regions of the analytical gels, co-migrating with proteins having sequences of known proteinase inhibitors. These inhibitors were identified by our proteomic analysis of components recovered from the higher molecular mass regions of the gels, and (d) the immunological isolation from the ascites of KLKs 5, 6, 8, and 10 along with serine proteinase inhibitors identified by proteomics (Figure 3).

In particular for KLK6, we have previously reported (a) the low abundance of active enzyme relative to immuno-

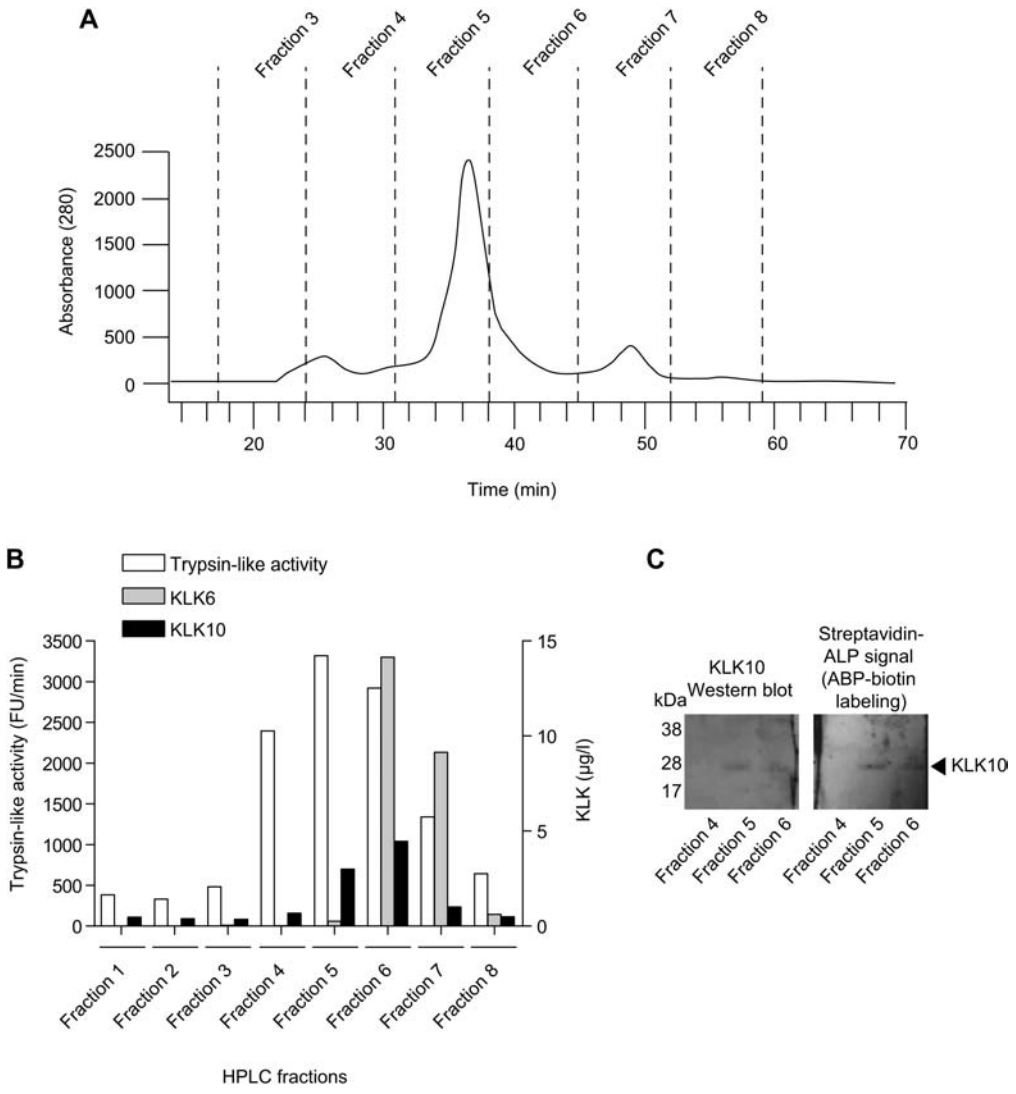

Figure 4 Fractionation of ovarian cancer ascites fluid by size-exclusion chromatography.
(A) Chromatography was performed in an ovarian cancer ascites fluid sample (100 µl) through a gel filtration column; proteins were eluted with 0.1 M NaH_2PO_4/0.1 M Na_2SO_4, pH 6.8 at a flow rate of 0.25 ml/min for 60 min and eight fractions were collected. The markings over the x-axis represent the HPLC fraction numbers. (B) Fractions were monitored for KLK6 (gray bars) and KLK10 immunoreactivity (black bars) (fractions 1–8, analysis groups for each fraction are indicated by a solid line on the x-axis). Overall trypsin-like enzyme activity was estimated using the VPR-AMC substrate (white bars) and compared with the concentration of KLK6 (gray bars) and KLK10 (black bars). (C) Proteins in the gels were also detected by either Western blotting or by using the streptavidin-alkaline phosphatase (ALP) reagent to visualize the biotin-tagged enzymes bound to the Bio-PK ABP. The position of immunoreactive KLK10 at which the ABP-biotin label was also detected is denoted by the black arrowhead.

reactive protein in cancer-related biological fluids (<5% of the total immunoreactive KLK6) and (b) the low recovery of active KLK6 upon the addition of recombinant active enzyme to the tumor-derived fluids (Oikonomopoulou et al., 2008). It was presumed that the active KLK6 enzyme added to the fluids was efficiently neutralized by proteinase inhibitors like the ones we have detected in this new study. As opposed to our previous research that specifically targeted KLK6, our new approach offers an opportunity to study simultaneously, in a non-prejudiced way, other trypsin-like proteinases, apart from KLK6, that can be identified with the ABP approach used in this study. This approach, using other proteinase-targeted ABPs (e.g., for cysteine proteinases, as well as for other families of serine proteinases), might be of general applicability to complement the immunoassay-based analysis of proteinases in a variety of clinically obtained biological fluids.

Based on our previous research with KLK6, we hypothesized that other trypsin-like serine proteinases could be active *in vivo* in cancer-derived fluids but that they are, like KLK6, rendered inactive by inhibitor species that are also present in the fluids. To test this hypothesis, we used a pro-

teomic approach to identify the proteins that migrated together with the exogenously added KLK6 immunoreactivity in the higher molecular mass regions of the gels (Figure 2A). Our proteomic approach identified α1-antitrypsin, α2M, and ITI. Our data coming from the immunoisolation-proteomics protocol also documented the co-existence of KLK6 with α1-antitrypsin and ITI in ovarian cancer ascites, in accordance with the gel-derived proteomic data. The presence of these inhibitors in the ascites fluid provides a likely explanation of the low reactivity of the ABP with immunoreactive KLK6. The reactivity with the biotinylated trypsin-like serine proteinase probe forms the basis of our ABP-ELISA developed previously for the detection of active KLK6 in patient samples (Oikonomopoulou et al., 2008). Furthermore, our data suggest that complexes could also be potentially formed by KLKs 5 and 8 with α1-antitrypsin similar to the one formed with KLK6. Given that we also found proteomic evidence for the presence of lower levels of the same proteinase inhibitors using the 'control' KLK capture plates or plates lacking the anti-KLK antibodies (i.e., non-specific adsorption of the inhibitors to the plastic or to antibodies for KLKs known not to be low or absent from ovarian cancer ascites fluid), we suggest that the inhibitors can be present in high abundance either as enzyme complexes or in an enzyme-free state. This issue merits further study. Our data are in keeping with the previous identification of complexes of KLK5 with inhibitors, such as α1-antitrypsin, in ascites fluid of ovarian cancer patients (Yousef et al., 2003).

Our finding of active KLK10 with the use of our objective ABP proteomic approach could be of particular significance. KLK10 has been predicted to have trypsin-like enzymatic activity (Liu et al., 1996). However, early attempts to activate the KLK10 zymogen were unsuccessful (Zhang et al., 2006a), and the presence of active KLK10 in ovarian cancer ascites could not be experimentally confirmed (Luo et al., 2006). Nevertheless, a study by Debela and colleagues has reported the characteristics of enzymatically active KLK10 and has profiled its substrate specificity (Debela et al., 2006). Thus, our detection of KLK10 as an ABP-biotinylated protein partly confirms the above reports and provides evidence that this KLK family member can indeed be present as a catalytically active enzyme *in vivo*. Additionally, our findings point to a potential complex between KLK10 and α1-antitrypsin or ITI in ovarian cancer ascites fluids. Because KLK10 has not been readily available as an active enzyme for study, it is not yet possible to predict its potential physiological functions or its potential susceptibility to naturally occurring inhibitors. To the best of our knowledge, this is the first report of the presence of active KLK10 activity in biological fluids. Our data thus suggest that this enzyme could play a functional role in ovarian cancer.

There are already several studies that have identified the presence of proteinase inhibitors in clinical fluid samples, such as ascites fluid, where several of the kallikrein-related peptidases such as KLK6 are expressed. This proteinase is expressed in tumors (Yousef et al., 2004; Nagahara et al., 2005) where α1-antitrypsin and ITI can also be detected (Kuramitsu and Nakamura, 2006). Moreover, α1-antitrypsin and α2M have been identified in CSF, along with KLK6 (Dumont et al., 2004; Yuan and Desiderio, 2005). In previous research, using a hybrid antibody approach (selective anti-KLK antibody capture of KLK6 followed by detection of KLK-bound inhibitors with polyclonal antibodies against the inhibitors), it has been possible to identify a small proportion of KLK6 as a complex with α1-antichymotrypsin in fractionated human milk and ascites fluid from ovarian cancer patients (Hutchinson et al., 2003). However, other inhibitors (e.g., α2M or α1-antitrypsin) were not detected in the fractionated samples. Our proteomic detection in the samples of inhibitors such as α1-antitrypsin, α2M, and ITI suggests that complexes with these inhibitors are also possible. This hypothesis merits further testing with more rigorous biochemical methods along with the hybrid antibody approach, which have been utilized in previous studies (Hutchinson et al., 2003).

A possible complex of the general proteinase inhibitor α2M with KLK6 could be of special interest, as this high-molecular weight inhibitor, in addition to promoting the clearance of KLK6 by macrophages, can protect the proteinase from degradation. By binding to the proteinase and blocking its enzymatic activity, but preserving the integrity of its active site, the proteinase in principle might subsequently be released in an active form when needed. This situation has been described for another serine proteinase, tissue plasminogen activator, which also forms a complex with α2M in patients with acute myocardial infarction (Ieko et al., 1997). Although not reactive with the ABP, and possibly undetectable by the KLK6 monoclonal antibody, the KLK6-α2M complex might be fully functional in the ovarian cancer ascites fluid and able to cleave selected small peptides but not intact protein substrates. This type of restricted reactivity against small substrates has been reported for the complex formed between another kallikrein-related peptidase, KLK3 (also known as prostate-specific antigen, PSA) and α2M (Christensson et al., 1990). Furthermore, a recent study has shown that the α2M-urokinase plasminogen activator complex has the ability to retain enzyme activity to cleave plasminogen, while at the same time being resistant to inhibition by the plasminogen activator inhibitor PAI-1 (Komissarov et al., 2009).

In summary, we have demonstrated the feasibility of using an objective combined proteomic-ABP labeling approach to identify trypsin-like proteinases and their inhibitors in a cancer-derived biological fluid. Our approach can potentially be adapted for a high-throughput procedure, which can in the future be used for the identification of other classes of enzymes in various types of tumors. Our preliminary analysis of ovarian cancer ascites suggests that the fluid contains an excess of proteinase inhibitors, such that the active KLKs and other proteinases can potentially be neutralized by the ambient levels of these inhibitors. However, when active recombinant KLK6 was added to the ascites, these inhibitors were not sufficient to block the activity of the total amount of added enzyme entirely, such that it could still be visualized by ABP labeling (data not shown). Thus, as for the balanced relationship between matrix metalloproteinases and their

tissue inhibitors (the TIMPs), the biological actions of the KLKs in the environment of tumors will depend on the balance between enzyme activation and inhibitor abundance. The potential ability of KLKs to form enzymatically inactive complexes with a variety of non-selective proteinase inhibitors (e.g., α2M, α1-antitrypsin, and ITI) suggests that the microenvironment in which the KLKs are produced and activated could be balanced in favor of strictly limiting the duration and extent of action of the enzymes. Thus, measurements of the abundance of those inhibitors capable of attenuating KLK action in the setting of cancer or other pathologies should be seen as the essential 'other side of the coin' in understanding the pathophysiology of the KLK family of enzymes.

Materials and methods

Biological samples

For our analysis we selected ovarian cancer ascites fluid, which is a representative type of biological fluid with high KLK6 and/or KLK10 expression. All ascites fluids were collected from ovarian cancer patients at Princess Margaret Hospital or Toronto General Hospital (Toronto, ON, Canada), according to procedures previously approved by the Ethics Committee, and clarified by centrifugation to remove any containing cells. We also used CSF samples, as control biological fluids, collected at Mount Sinai Hospital (Toronto, ON, Canada), and supernatants from the breast cancer cell line HTB-132 (MDA-MB-468). The cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA), supplemented with fetal bovine serum (10%), in 75 cm^2 flasks. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere until the cell monolayers became confluent and supernatants were collected. All samples were stored at -20°C prior to analysis.

Estimation of trypsin-like serine proteinase activity

The enzymatic activity of trypsin-like serine proteinases of ovarian cancer ascites fluid was measured using fluorogenic *t*-butoxycarbonyl-Tri-peptide-7-amino-4-methylcoumarin (AMC) synthetic substrates (e.g., VPR-AMC, GPR-AMC, GPK-AMC, VLK-AMC; Bachem Bioscience, Inc., King of Prussia, PA, USA) (Magklara et al., 2003). The overall enzyme activity measured in the ascites fluid was compared relative to the activity of 5 nM trypsin (Sigma, St. Louis, MO, USA). The enzymatic reaction was performed in 50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% (v/v) Tween-20, pH 7.6, at 37°C, conditions which resemble the neutral pH of ovarian cancer ascites. Fluorescence was monitored for 20 min on a Wallac Victor fluorometer (PerkinElmer Life Sciences, Wellesley, MA, USA) set at 355 nm for excitation and 460 nm for emission and the fluorescence of the enzyme-free reactions was subtracted from each value. Kinetic analysis was done by nonlinear regression analysis using the Enzyme Kinetics Module 1.1 (Sigma Plot, SSPS, Chicago, IL, USA). Enzyme activity was quantified by monitoring the rates of substrate hydrolysis (slopes of the fluorescence curves expressed as FU/min). Enzyme activity was also monitored using a biotinylated ABP, biotin-linker-Pro-Lys-diphenylphosphonate (Bio-PK-DPP 4), kindly provided by Dr. Amos Baruch (KAI Pharmaceuticals, South San Francisco, CA, USA). The probe was designed to specifically target the active site of serine proteinases with trypsin-like activity (Pan et al., 2006). In brief, the biological samples were incubated with 5 nM of the biotin-linker-Pro-Lys-diphenylphosphonate probe for 8 h, at room temperature and separated with SDS-PAGE gel electrophoresis. Biotinylated proteins were transferred to a Hybond-C Extra (Amersham Biosciences, Pittsburgh, PA, USA) nitrocellulose membrane, treated with streptavidin-alkaline phosphatase (SA-ALP; 50 ng/ml SA-ALP, diluted in 1% casein-TBST: 20 mM Tris, 137 mM NaCl, 0.1% v/v Tween-20, pH 7.6), and visualized with Immulite Chemiluminescent substrate treatment for 10 min (Diagnostic Products Corp., Los Angeles, CA, USA). The concept behind this blotting of probe-proteinase complexes has been previously described (Oikonomopoulou et al., 2008).

Activity-based probe proteomics

Ascites fluid (1 ml) from ovarian cancer patients was incubated with 5 μM of the Bio-PK-DPP 4 ABP for 3 h at room temperature. The incubation was followed by treatment of the probe-protein biotinylated complex with streptavidin-coated magnetic beads for 30 min according to the manufacturer's instructions (Dynabeads® MyOne™ Streptavidin T1; Invitrogen Canada Inc., Burlington, ON, Canada). The beads were extensively washed with phosphate-buffered isotonic saline, pH 7.4 and were resuspended in 8 M urea (322 μl; Sigma) for protein denaturation. Subsequently 50 μl of 200 mM dithiothreitol (DTT) (Sigma) and 50 μl of 1 M NH_4HCO_3 were added and samples were reduced at 50°C for 30 min. Finally, the reduced proteins were alkylated by incubation with 125 μl of 500 mM iodoacetamide (Sigma) for 1 h at room temperature. Bead-captured proteins were magnetically separated from the supernatants and digested overnight (16 h) at 37°C, using 250 μl of 50 mM NH_4HCO_3, 100 μl of methanol, 150 μl of water, and 40 μg/ml trypsin (Promega, Madison, WI, USA). The digest was collected, clarified by passage through C_{18} extraction ($ZipTipC_{18}$ pipette tip, Millipore, Billerica, MA, USA) and eluted in 4 μl of 68% acetonitrile (ACN) made up of 3 parts of Buffer A (95% water, 0.1% formic acid, 5% ACN, 0.02% v/v trifluoroacetic acid) and 7 parts of Buffer B (90% ACN, 0.1% formic acid, 10% water, 0.02% TFA). Finally, 80 μl of Buffer A was added to dilute the samples and the peptide/protein content was analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS; LTQ-Orbitrap XL; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (Gunawardana et al., 2009; Kuk et al., 2009). Raw data were assigned to proteins included within the human protein database Swiss-Prot using the Mascot algorithm (Matrix Science Inc., Boston, MA, USA) and probabilities were further evaluated with Scaffold (Proteome Software Inc., Portland, OR, USA). Peptide identification was accepted if established at greater than 95% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Similarly, protein identification was accepted if established at greater than 80% probability as verified by the high probability identification of at least one 'signature' peptide. Protein probabilities were appointed by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Identified enzymes were assigned to molecular and biological pathways using Scaffold.

Identification of proteins by Western blot analysis and by gel-excised protein hydrolysis and mass spectrometry

Western blot analysis Samples were resolved by electrophoresis using SDS-containing polyacrylamide gels (SDS-PAGE) under reducing conditions (NuPage 4–12% Bis-Tris gels; Invitrogen, Burlington, ON, Canada). Proteins were transferred to a Hybond-C Extra (Amersham Biosciences) nitrocellulose membrane. Membrane-

bound proteins were reacted with KLK or inhibitor-specific antibodies (rabbit KLK6 and KLK10 polyclonal antibodies were produced in-house; goat α2M anti-serum was obtained from R&D Systems, Minneapolis, MN, USA) followed by detection with an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit and bovine anti-goat ALP-conjugated antibody from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and were visualized with Immulite Chemiluminescent substrate (Diagnostic Products Corp.).

Gel elution, hydrolysis, and proteomic analysis Representative samples of ovarian cancer ascites fluid, CSF, and a cancer cell line (MDA-MB-468) supernatant were incubated at room temperature for 8 h (to allow examination of possible protein degradation), analyzed by SDS-PAGE and stained with SimplyBlue™ SafeStain (Invitrogen Canada Inc., Burlington, ON, Canada). Visible gel bands were excised and subjected to in-gel trypsin digestion. In brief, the gel was shrunk with 100 μl pure acetonitrile (Sigma) for 10 min at room temperature and the bands were reduced with 100 μl of 10 mM DTT (Sigma) for 10 min at 60°C, followed by 20 min incubation at room temperature. Gel bands were subsequently alkylated with 100 μl of 100 mM iodoacetamide (Sigma) for 15 min at room temperature and the gel was shrunk once more with pure acetonitrile for 10 min. Finally, proteins were digested with 5 μg/ml trypsin (Promega) in 100 μl of 50 mM NH_4HCO_3. After a 16-h trypsin digestion at 37°C, samples were subjected to LC-MS/MS analysis (LTQ Thermo-Finnigan; Thermo Fisher Scientific, Inc.), as previously described (Kulasingam and Diamandis, 2007). This process allowed identification of proteins/peptides in each sample, corresponding to the selected molecular masses visualized by SDS-PAGE.

Immunoextraction of KLKs from ovarian cancer ascites

Mouse monoclonal antibodies against KLKs 2, 3, 4, 5, 6, 7, 8, 10, 11, 13, and 14 (500 ng/well) were used to coat 96-well microtiter plates (200 μl) overnight using 50 mM Tris buffer pH 7.8. The plates, either antibody-coated or not (blank plate controls), were then washed five times with 0.9% NaCl solution, followed by loading of the ovarian ascites fluid samples in duplicates (50 μl sample and 50 μl of water) and incubation for 1 h at room temperature. The plates were washed 10 times with 0.9% (w/v) NaCl and allowed to incubate with 48 μl of 200 mM NH_4HCO_3, 10 μl of methanol, 40 μl of water, and 2 μl of 20 mM DTT (Sigma) at room temperature for 30 min. Subsequently, 2 μl of 100 mM iodoacetamide (Sigma) was reacted with the mixture for 1 h at room temperature in the dark. Finally, 2.5 μg/ml trypsin (Promega) was added to each well and incubated overnight (16 h) at 37°C. The tryptic digest was prepared for mass spectrometry analysis by LC-MS/MS (LTQ-Orbitrap XL; Thermo Fisher Scientific, Inc.), as previously reported (Gunawardana et al., 2009; Kuk et al., 2009).

Gel filtration chromatography

To gain insight into the active proteinases contained in the ovarian cancer ascites fluid, we fractionated one ascites fluid sample with high overall trypsin-like activity and an increased amount of KLKs, using size-exclusion high performance liquid chromatography (HPLC) as previously described (Hutchinson et al., 2003). In brief, 100 μl of sample was injected to the gel filtration column TSK-GEL G3000SWxl [5 μm, 30 cm×7.8 mm (i.d.); Tosoh Bioscience LLC, Montgomeryville, PA, USA] and eluted with 0.1 M NaH_2PO_4/0.1 M Na_2SO_4, pH 6.8 and a flow rate of 0.25 ml/min for 60 min. Eight fractions were collected and were subjected to: (a) activity analysis using the Bio-PK-DPP 4 ABP and the VPR-AMC substrate as described in the previous sections, (b) protein identification by blotting with antisera targeting KLKs 6 and 10, and (c) quantification of KLKs 6 and 10 using in-house immunofluorometric assays, as previously described (Luo et al., 2006).

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