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Kallikrein 6 is a mediator of K-RAS-dependent migration of colon carcinoma cells

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Abstract

Kallikrein 6 (KLK6) is a trypsin-like serine peptidase whose relevance in various types of cancers is currently being explored. Previous studies have shown that KLK6 mRNA is upregulated in colon and gastric cancers; however, the regulatory mechanisms and phenotypic consequences of this upregulation are largely unknown. Activating *K-RAS* mutations are common in colon cancer, occurring in approximately 50% of cases. We have recently reported the upregulation of KLK6 mRNA in Caco2 human colon cancer cells stably transfected with a mutant *K-RAS* allele (*K-RAS*^{G12V}). In this study we examined the pattern of K-RAS-dependent KLK6 expression and secretion in colon cancer cells. Using pharmacological inhibitors of pathways downstream of K-RAS, we could show that the PI3K and p42/44 MAPK pathways play an important role in the induction of KLK6 in mutant K-RAS-expressing colon cancer cells. Increased KLK6 expression enhanced colon cancer cell migration through laminin and Matrigel. Inhibition of KLK6 using small interference RNA treatment or a specific KLK6 antibody in Caco2 cells stably expressing the mutant K-RAS and in SW480 cells carrying a mutation in the *K-RAS* oncogene resulted in a reduction in invasiveness through cell culture inserts. These data support the oncogenic role of KLK6 in colorectal cancer.

Keywords

cell migration; colon cancer; kallikrein 6; *K-Ras* oncogene; Matrigel invasion

Introduction

K-RAS is a membrane-bound G protein that conveys extra- and intracellular mitogenic signals to several signaling cascades. Activating mutations in *RAS* proto-oncogenes occur in approximately 50% of colon cancers and can be an early or late mutation (Bos, 1989; Fearon and Vogelstein, 1990; Fang and Richardson, 2005; Pretlow and Pretlow, 2005; Shahrzad et al., 2005). In human cancers, the vast majority of these *RAS* mutations occur in the K-RAS isoform (Giehl, 2005). K-RAS is mutated in a variety of cancers, including pancreatic, lung and colon (Bos, 1989; Caron et al., 2005; Giehl, 2005). Once activated, K-RAS becomes GTP-bound and is able to activate downstream pathways such as the p42/44MAPK and PI3

kinase pathways (Fang and Richardson, 2005). Point mutations cause K-RAS to become insensitive to GTP-hydrolases, rendering it constitutively active (Adari et al., 1988). An activated K-RAS can contribute to many cancerous phenotypes, including increased proliferation, evasion of apoptosis, and increased migration and invasion (Schramm et al., 2000; Fang and Richardson, 2005; Giehl, 2005). Elucidating downstream effects of mutant K-RAS will help to gain insight into effective ways to detect and treat colon cancer.

Our laboratory has utilized an isogenic cell model system to find differences between cells with wild-type KRAS and those expressing a mutant K-RAS. Caco2 cells are highly differentiated colon cancer cells that express wild-type K-RAS (Shahrzad et al., 2005; Turck et al., 2005). Caco2 cells were stably transfected to constitutively express mutant K-RAS (Ignatenko et al., 2004a). RT-PCR and subsequent RFLP analysis confirmed that two Caco2 clones, Caco/Kras6 and Caco/KRAS26, express the K-RAS^{G12V} mutation (Ignatenko et al., 2004a). Microarray analysis was performed on these Caco/K-RAS clones and several changes in gene expression were observed compared to mock transfected Caco/Neo cells. One of the most striking changes was observed for kallikrein 6 (KLK6), a trypsin-like serine protease, for which expression was 11-fold higher in the Caco/K-RAS clones (Ignatenko et al., 2004b). RT-PCR confirmed this dramatic upregulation of KLK6 (Ignatenko et al., 2004b).

The kallikrein protein family consists of 15 serine pro-teases, each of which has a unique pattern of activation, expression and set of substrates (Borgono and Diamandis, 2004; Obiezu and Diamandis, 2005; Yoon et al., 2007). The most widely utilized kallikrein is KLK3, also known as prostate-specific antigen (PSA). Owing to their nature as secreted proteins, kallikreins are currently under investigation as potential biomarkers. KLK6 is being examined as a marker for certain types of ovarian and uterine cancers (Diamandis et al., 2003; Santin et al., 2005). KLK6 mRNA and secreted protein were found to be significantly upregulated in uterine serous papillary cancer (USPC) as compared to benign tumor and endometrial carcinoma patients (Santin et al., 2005). Patients with ovarian carcinoma also had significantly higher levels of serum KLK6, about twice the concentration of normal or benign tumor patients (Diamandis et al., 2003). In both gastric and colon cancer, KLK6 mRNA was observed to be more highly expressed as compared to normal mucosa (Nagahara et al., 2005; Ogawa et al., 2005). In each previously mentioned study, above average expression of KLK6 correlated with a poor prognosis (Diamandis et al., 2003; Nagahara et al., 2005; Ogawa et al., 2005; Santin et al., 2005).

KLK6 may have a role in cancer progression. KLK6 has been implicated in angiogenesis, migration and invasion via mechanisms involving ECM degradation (Borgono and Diamandis, 2004). Substrates for KLK6 include collagen I and IV, fibrinogen, fibronectin and laminin (Bernett et al., 2002; Blaber et al., 2002; Borgono and Diamandis, 2004). Prezas et al. (2006) demonstrated that ovarian cells stably transfected to express KLK4, 5, 6 and 7 were significantly more invasive *in vitro* and formed larger tumors in mice. KLK6 overexpression has also recently been shown to increase skin cell migration and invasion (Klucky et al., 2007). Another study demonstrated that a synthetic kallikrein inhibitor, FE999024, attenuated breast cancer cell invasion by 39% through a Matrigel substrate (Wolf et al., 2001).

The objectives of this study were to evaluate K-RAS-dependent regulation of KLK6 and to characterize patterns of mRNA and intracellular protein expression and protein secretion. Finally, we investigated the consequences of KLK6 inhibition on cell migration through laminin and invasion through Matrigel.

Results

Kallikrein 6 is regulated in a K-RAS-dependent manner

It was determined through microarray analysis that KLK6 mRNA is significantly elevated in the Caco/Kras clones (Ignatenko et al., 2004b). Using semi-quantitative RT-PCR, we confirmed that, over time, KLK6 mRNA levels were indeed higher in Caco/Kras6 cells compared to Caco2 parental and Caco/Neo3 cells (Figure 1A, upper panel). The RT-PCR results were quantified using densitometric analysis (Figure 1B). The cell lines showed no appreciable difference in KLK6 expression after 24 h. After 48 h, Caco/Kras6 cells had 2.1- and 1.6-fold higher KLK6 expression compared to Caco2 and Caco/Neo3 cells, respectively. After 72 h, KLK6 levels were 2.5- and 1.6-fold higher than in Caco2 and Caco/Neo3 cells, respectively. Finally, after 96 h, the difference was 1.2- and 2.0-fold higher, respectively. K-RAS transcript levels, which were measured at the same time points, remained unchanged, which implies that the effect of the mutant K-RAS gene on KLK6 transcription is not due to an increase in intracellular K-RAS levels, but to the presence of the mutation (Figure 1A, lower panel). Next, we investigated whether this increase in mRNA corresponded to intracellular protein levels, protein secretion, or both. We found that intracellular levels of KLK6 were not significantly different over time (Figure 1C). Protein secretion, however, was significantly higher at all time points in Caco/Kras6 compared to Caco2 and Caco/Neo3 cells (Figure 1D).

KLK6 upregulation in Caco/Kras6 cells is regulated by the PI3K and p42/44 MAPK pathways

There are several pathways downstream of K-RAS that could potentially influence KLK6 expression. Using various small-molecule inhibitors, we disrupted these pathways to determine which of them might affect KLK6 expression in Caco2 cells expressing mutant K-RAS. The pathways downstream of K-RAS include PI3 kinase, p42/44MAPK, p38MAPK and JNK (Giehl, 2005; Fang and Richardson, 2005). The following inhibitors were used: LY294002 for PI3K, PD98059 for p42/44MAPK, SB202190 for p38MAPK, and SP600125 for JNK. Cells were treated for 48 h at a concentration of 50 μ M for both PD98059 and LY294002. Using quantitative real-time PCR, we demonstrated that LY294002 dramatically reduced KLK6 mRNA expression by 81% in Caco/Kras6 cells and by 87% in Caco/Neo3 cells. PD98059 also significantly reduced mRNA levels by 55% in the Caco/Kras6 clone and by 63% in the Caco/Neo3 clone (Figure 2A). KLK6 protein secretion closely reflected the mRNA expression. LY294002 and PD98059 both dramatically reduced KLK6 secretion by 100% and 69%, respectively. The inhibitors used to disrupt the p38 and JNK pathways caused no significant changes in KLK6 mRNA levels (Figure 2C). Intracellular levels of KLK6 did not change significantly with any drug treatment (data not shown). These data indicate a major role for the PI3K and p42/44MAPK pathways in K-RAS-mediated transcriptional regulation of KLK6.

Migration through laminin is decreased by KLK6 siRNA and by KLK6 antibody treatment

We were able to successfully knock down KLK6 mRNA expression in Caco/Kras6 cells using KLK6 siRNA (Figure 3A). Cells were transfected with 75 nM KLK6 siRNA for a period of 48 h, after which mRNA and conditioned media were collected. As with other treatments, intracellular levels of KLK6 were not greatly affected (data not shown). The siRNA also significantly inhibited KLK6 protein secretion 48 h after transfection (Figure 3B). Because KLK6 is a secreted protease, we wanted to observe what effects KLK6 inhibition would have on *in vitro* cell migration. We chose laminin as a substrate because it is a known substrate for KLK6 and because Caco2 cells are able to adhere and proliferate on it (Orian-Rousseau et al., 1998; Bennett et al., 2002; Blaber et al., 2002; Turck et al., 2005). Under normal conditions, Caco2 and Caco/Neo3 cells do not migrate efficiently across

laminin. However, Caco/Kras6 cells do migrate across laminin (Ignatenko et al., 2004b). First, Caco/Neo3 and Caco/Kras6 cells were plated onto laminin-coated migration filters following pretreatment with either KLK6 antibody or a control mouse IgG. Treatment with the KLK6 antibody caused a 61% reduction in migration of the Caco/Kras6 cells (Figure 3C). Next, we inhibited KLK6 using siRNA. As shown previously, KLK6 siRNA inhibits protein secretion (Figure 3B). Caco/Neo3 or Caco/Kras6 cells transfected for 48 h with either KLK6 or negative control siRNA were plated onto laminin-coated filters and allowed to migrate for another 48 h. The migration of Caco/Kras6 cells transfected with KLK6 siRNA was decreased by approximately 50% (Figure 3D). The level of migration of Caco/Kras6 cells after treatment with both the antibody and siRNA was slightly higher, but not significantly different than the level of migration observed for Caco/Neo3 cells.

Invasion through Matrigel is decreased by KLK6 siRNA

In addition to examining the effect of KLK6 on migration through the singular substrate of laminin, the effect of KLK6 on cell invasion through the basement membrane-like substrate Matrigel was also analyzed. Cells were transiently transfected for 48 h with KLK6 or control siRNA, and then seeded onto Matrigel-coated filters. Once on the filters, cells were allowed to invade for 72 h. A longer time point was used for the Matrigel assay than for the laminin assay because cellular invasion into Matrigel was slower overall and almost no invasion was observed after 48 h. Similarly to migration through laminin, there was no significant difference observed in Matrigel invasion for Caco/Neo3 cells transfected with either control or KLK6 siRNA. However, there was a 50% reduction in cellular invasion observed in Caco/Kras6 cells transfected with KLK6 siRNA compared to control transfected cells (Figure 3E).

Evaluation of KLK6 expression, secretion and migration in SW480 colon cancer cells

The above-described effects of mutant K-RAS on KLK6 expression were performed in Caco/K-RAS isogenic cell lines in which a mutant *K-RAS* allele was introduced via transfection and selection. We also wanted to evaluate KLK6 expression and its effects on cell migration in colon cells with endogenous expression of mutant *K-RAS*. We chose to use SW480 colon cancer cells, which are homo-zygous for the K-RAS^{G12V} mutation (Caron et al., 2005). The levels of KLK6 RNA were increased in SW480 cells compared to both parental Caco2 cells and mock transfected Caco/Neo3 cells (Figure 4A, upper panel). The K-RAS transcript levels were similar in these cell lines (Figure 4A, middle panel). RFLP analysis for mutant *K-RAS* in Caco2 parental cells and clones and SW480 cells confirmed the expression of one mutant *K-RAS* allele in the Kras6 clone and of both mutant *K-RAS* alleles in SW480 cells (Figure 4A, lower panel). SW480 cells secreted significantly higher amounts of KLK6 protein compared to not only Caco2 cells, but also the mutant K-RAS-expressing Caco/Kras6 cells, probably due to the higher level of mutant K-RAS transcription and activity.

Similarly to the Caco/Kras cell model system, we successfully suppressed KLK6 transcription in SW480 cells and subsequently observed a statistically significant decrease in KLK6 protein in media (Figure 5A). Treatment of SW480 cells with KLK6 siRNA inhibited cell migration across laminin-coated cell culture inserts (Figure 5B). These data demonstrate that mutant K-RAS-mediated induction of KLK6 is a common feature of colon cancers with mutation in the *K-RAS* gene.

Discussion

The goals of this study were to confirm the previously observed link between expression of activated K-RAS and KLK6 upregulation, and to identify possible regulatory mechanisms

involving K-RAS-mediated KLK6 expression. In addition, we explored the effects of KLK6 upregulation on cancer cell phenotype. We successfully demonstrated that KLK6 mRNA and secreted KLK6 protein are positively influenced by the presence of mutated *K-RAS* in both Caco/Kras6 and SW480 cell lines, whereas intracellular levels of KLK6 protein were relatively unchanged. Two pathways downstream of K-RAS that appear to have an effect on KLK6 expression are the PI3K and p42/44 MAPK pathways. Again, interference with these pathways led to a decrease in mRNA and secreted protein levels, but intracellular KLK6 was unaffected. Finally, using KLK6 antibodies and siRNA, we showed that KLK6 enhances cell migration and invasion. These data clearly indicate that in Caco2 cells stably expressing mutant K-RAS, as well as in SW480 cells with endogenous expression of mutant K-RAS, KLK6 mRNA and secreted protein levels were elevated. Intracellular KLK6 protein levels were rarely altered due to K-RAS status, drug treatment, or siRNA transfection. Protein processing, trafficking and degradation are a few potential contributors to the disparity between mRNA and intracellular protein levels.

Regardless of the mechanism of KLK6 upregulation, the implications of its over-expression in colon cancer may be far-reaching. Several kallikreins, including KLK6, are either already in clinical use or are currently under investigation as serum biomarkers for cancer (Diamandis et al., 2003; Obiezu and Diamandis, 2005; Santin et al., 2005). K-RAS is often found to be an early mutation in colon cancer (Pretlow and Pretlow, 2005), and therefore a serum marker associated with mutant K-RAS could prove to be valuable. A study by Wan et al. (2004) demonstrated that K-RAS mutations could be identified in fecal samples from approximately 57% of patients with colon cancer, with a small but non-significant difference between early- and late-stage disease. Other studies have demonstrated the usefulness of identifying mutant K-RAS in DNA isolated from fecal and serum samples (Hibi et al., 1998; Dong et al., 2001). Discovery of a serum protein indicative of mutant K-RAS would allow simpler testing for this genetic abnormality. In addition to its potential role as a biomarker, KLK6 may play a role in cancer cell invasion. We explored this possibility by investigating *in vitro* cell migration and invasion.

We first examined cell migration through a single substrate of laminin. Laminin expression is observed significantly more often in colon tumor tissue than in normal colonic epithelia (Lenander et al., 2001). Expression of laminin is also correlated with poor differentiation and increased tumor size (Lenander et al., 2001). Within tumor tissues, laminin expression is most often observed at the invasive front of budding tumors, and rarely within established tumor tissue, indicating an important role in invasiveness (Pyke et al., 1995). Whereas Caco2 cells adhere and proliferate on laminin, only the Caco/K-RAS clones effectively migrate through this substrate, implying a K-RAS-dependent mechanism for migration (Orian-Rousseau et al., 1998; Ignatenko et al., 2004b). Our study demonstrates that KLK6 may indeed play a role in K-RAS-dependent migration. Both the KLK6 antibody and anti-KLK6 siRNA significantly reduced the migration of Caco/Kras6 and SW480 cells through laminin. The effect of KLK6 on cellular invasion through the basement membrane-like substrate Matrigel was also investigated. For Caco/Kras6 cells transfected with KLK6 siRNA, we observed a decrease in invasion through Matrigel similar to the decrease observed in the laminin migration assay. Matrigel is a more complex mixture of substrates and consists primarily of collagen IV, laminin and entactin (Albini et al., 2004). These data demonstrating decreased migratory and invasive potential are in agreement with a study by Ghosh et al. (2004) in HTB19 breast cancer cell, in which knock-down of KLK6 activity with monoclonal antibody treatment led to inhibition of migration on various substrates in Boyden chambers. A study performed in HEK293 cells demonstrated increases in cell proliferation, wound healing and cell invasion when cells were transfected with KLK6 expression plasmids, and a loss of cell-cell adhesion was attributed to a decrease in membrane-associated E-cadherin and subsequent nuclear localization of β -catenin (Klucky

et al., 2007). Taking into account the potentially important role of KLK6 as a serum biomarker for colon cancer and its contribution to cancer progression, elucidation of the mechanisms of KLK6 regulation is vital.

Materials and methods

Antibodies and reagents

All cell culture reagents and Lipofectamine 2000 were purchased from Invitrogen Corp. (Carlsbad, CA, USA). KLK6 monoclonal antibody was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Normal mouse IgG and goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LY294002, SB202190 and SP600125 were purchased from Calbiochem (La Jolla, CA, USA). PD98059 was purchased from Alexis® Chemicals (San Diego, CA, USA). Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich, Life Science Research (St. Louis, MO, USA). The RNeasy Kit and siRNA sequences were purchased from Qiagen Inc. (Valencia, CA, USA). The Reverse Transcription Kit was purchased from Promega Corp. (Madison, WI, USA).

Cell culture

Caco2 cells were maintained in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. Caco2 cells were transfected using the calcium phosphate transfection technique with a pcDNA3.0 vector (Invitrogen Corp.) expressing a Ki-RAS^{Val12} oncogene under a cytomegalovirus (CMV) promoter. Transfectants were maintained in MEM supplemented with 350 µg/ml G418. Caco2 parental cells and Caco2 clonal cells stably transfected with the pcDNA3.0 empty vector (Caco/Neo3) or with pcDNA-K-RAS plasmid (Caco/Kras6) were used in this study. The SW480 colon cancer cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). SW480 cells were grown in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin.

Drug treatments

For drug treatments using SB202190, SP600125, PD98059 and LY294002, the same protocol was used. The compounds were all suspended in DMSO. PD98059 and LY294002 were both used at a final working concentration of 50 µM. SB202190 and SP600125 were used at final working concentrations of 10 and 20 µM, respectively. Cells were plated at a concentration of 5.0×10^5 cells per 60-mm plate, and were treated with the drug or vehicle control at the time of plating. The media and drug were removed 24 h later and replaced with fresh media and drug. Cell lysates, conditioned media and RNA were collected 48 h after initial plating. All treatments were performed in triplicate. The media used was MEM with 10% fetal bovine serum and 1% penicillin/streptomycin.

Semi-quantitative RT-PCR

Total cellular RNA was extracted from Caco2, Caco/Neo3, Caco/KRAS6, and SW480 cells according to a protocol supplied with the Qiagen RNeasy Kit. Reverse transcription was performed using 1 µg of total RNA with random primers and a Reverse Transcription System kit (Promega) according to the manufacturer's protocol. PCR was performed on identical amounts of cDNA (100 ng) in a 25-µl reaction with puReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences Corp., Piscataway, NJ, USA). Primers used were as follows: KLK6, sense 5'-GCC CAG CCA AAC TCT CTG-3' and antisense 5'-TGT TAC CCC ATG ACA CAA GG-3'; and *GADPH* sense 5'-TGG TAT CGT GGA AGG ACT CAT GAC-3' and antisense 5'-AGT CCA GTG AGC TTC CCG TTC AGC-3'. The annealing temperature used was 59°C for 35 cycles. After PCR, samples were run on 2%

agarose gel. PCR products were visualized by ethidium bromide staining and photographed using a Gel Logic 200 Imaging System and Kodak 1D 3.6 software. The expression was quantified by densitometric analysis (Scion Imaging Quantification Software, Scion Image for Windows, Beta 3b, Scion Corp, Frederick, MD, USA). Expression of the KLK6 gene was normalized to that of the housekeeping gene *GADPH*.

RT-PCR analysis of K-RAS

RT-PCR analysis of codon 12 of the *K-RAS* gene was performed as previously described (Ignatenko et al., 2004a).

Real-time PCR

Reverse transcription was completed using a TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA, USA). A sample of 1 µg of total RNA was transcribed into cDNA in a 50-µl reaction with random hexamers under the thermal conditions recommended by the protocol. Real-time PCR amplification was performed on an ABI PRISM 7700 SDS instrument (Applied Biosystems) under the universal thermal cycling conditions recommended by the Assay-on-Demand products protocol. Each 50-µl real-time PCR reaction included 25 µl of TaqMan Universal PCR master mix, 10 µl of the resulting cDNA from the RT step, and 15 µl of diluted primer and probe mixes from Assay-on-Demand products (Applied Biosystems). No template controls were included in each plate to monitor the potential PCR contamination. Each cell line was tested in triplicate and each reaction was run in duplicate. To determine the relative expression level of each target gene, the comparative C_T method was used. The C_T value of the target gene was normalized to that of the endogenous reference ($\Delta C_T = C_{T(\text{target})} - C_{T(\text{GAPDH})}$) and compared with a calibrator, in our case control RNA ($\Delta\Delta C_T = \Delta C_{T(\text{target})} - \Delta C_{T(\text{calibrator})}$). The relative expression of each target gene was calculated from the equation $2^{-\Delta\Delta C_T}$.

Western blotting

Caco2, Caco/Neo3 and/or Caco/Kras6 cells were collected and lysed on ice in radioimmunoprecipitation assay buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 µg/ml aprotinin, 100 mM sodium orthovanadate, 10 mg/ml phenylmethylsulfonyl fluoride). Samples were kept on ice for 30 min, followed by centrifugation at 13 000 *g* for 10 min. Supernatants were collected and protein concentrations were determined using a Bio-Rad DC protein assay. Samples of 60 µg of cell lysate were loaded per lane and run on a 12.5% SDS-PAGE gel. The proteins were transferred electrophoretically to a Hybond-C nitrocellulose membrane (Amersham) overnight. Blots were blocked in Blotto A [5% w/v non-fat dry milk, 0.1% Tween 20, and Tris-buffered saline (TBS) consisting of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl] for 1 h at room temperature. A mouse anti-kallikrein 6 monoclonal antibody (R&D Systems) was used at a dilution of 1:100 and incubated for 2 h at room temperature. The blots were washed in TBS/0.1% Tween-20. The primary antibodies were detected with an anti-mouse immunoglobulin G antibody conjugated to horse-radish peroxidase for 1 h at room temperature. Blots were washed as described above and protein was detected using enhanced chemiluminescence detection reagent (Amersham). Western blotting was repeated three times and representative blots are shown.

ELISA for secreted KLK6

An ELISA kit for the detection of human KLK6 was obtained from Ibex™ (Quebec, Canada). The assay was performed according to the manufacturer's protocol. Briefly, standards were prepared at concentrations of 0, 0.2, 0.5, 2.0, 5.0, 10.0 and 20.0 ng/ml and used to plot a standard concentration curve. Samples of 50 µl of either a standard or

conditioned media were added to precoated wells and incubated for 2 h. The wells were washed six times with the wash buffer provided. Then 100 μ l of KLK6 Antibody-Biotin was added to all wells and incubated for 1 h, followed by six washes. Streptavidin-HRP (100 μ l) was added and incubated for 30 min, followed by six washes. TMB substrate (100 μ l) was added and incubated for 30 min, followed by addition of 100 μ l of Stop Solution. All incubations were carried out on a high-speed plate shaker at room temperature. Within 10 min of addition of the Stop Solution, the plate was read at 490 nm on an EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

siRNA transfection

Qiagen anti-kallikrein 6 siRNA and negative control siRNA (catalog nos. SI00043071 and 1022076, respectively) were prepared as follows. The lyophilized sequences were resuspended in the manufacturer's siRNA Suspension Buffer to a concentration of 20 μ M. The suspension was incubated at 90°C for 1 min, then at 37°C for 60 min to disrupt aggregates formed during lyophilization. Both siRNA sequences were used at a final concentration of 75 nM during cell transfection. At 16–18 h preceding transfection, 2.0×10^5 cells/well were plated in 6-well plates in MEM with 10% fetal bovine serum and no antibiotics. Just prior to transfection, cells were rinsed three times with saline. Cells were then incubated in Opti-MEM with siRNA and HiPerfect (12 μ l/well) for 6 h, after which an equal volume of Opti-MEM plus 10% fetal bovine serum was added to each well. Cell lysates, conditioned media and RNA were collected 48 h after transfection. All experiments were carried out in triplicate and repeated at least twice.

Laminin migration assay

For analysis of the effect of KLK6 antibodies on migration, cells were pre-treated with 2.5 μ g/ml KLK6 mAb or 2.5 μ g/ml normal mouse IgG for 20 min at 4°C with agitation. At 24 h after plating, 2.5 μ g/ml additional antibody or mouse IgG was added to each well. For analysis of the effect of KLK6 siRNA on migration, cells were transfected with siRNA as outlined previously. At 48 h after transfection, cells were harvested and plated onto migration assay filters.

Cell migration assays were performed using BIOCOAT® control cell culture inserts (BD Biosciences Discovery Labware, Bedford, MA, USA). Inserts were coated with 2 μ g of laminin (Sigma-Aldrich) per well. Cell suspensions of Caco/Neo3 and Caco/Kras6 were prepared at a concentration of 5.0×10^5 cells/ml in serum-free MEM. Samples of 200 μ l of the cell suspensions were plated in the inner chamber of the coated insert in a 24-well tissue culture plate. Then 500 μ l of MEM containing 10% fetal bovine serum, 1% penicillin/streptomycin and 350 μ g/ml G418 was added to the outer chamber of the insert. At 48 h after plating, cells that migrated through the surface of the membrane were stained with 100 μ l/well of staining solution (0.5% crystal violet, 20% methanol, 80% water) for 1 min. Stained cells were dissolved in 200 μ l of 0.1 M citric acid in a 96-well plate. The plate was placed on a high-speed plate shaker for 5 min and then 150 μ l of the supernatant was transferred to a new well and read at 562 nm on an EL800 Universal Microplate Reader (Bio-Tek Instruments). Six replicate migration experiments were carried out and were repeated three times.

Matrigel invasion assay

Matrigel cell invasion assays were performed on prepared 24-well-plate-sized Matrigel inserts (BD Biosciences Discovery Labware), which were shipped frozen and prepared for use in the laboratory. Briefly, the inserts were thawed to room temperature and then 500 μ l of serum-free MEM was added to both the insert and its corresponding well to rehydrate both the top and bottom of the insert. Inserts were rehydrated for 2 h at 37°C in 5% CO₂.

Meanwhile, cells were prepared for plating. Cells previously transfected with KLK6 siRNA for 48 h were trypsinized and suspended to a concentration of 5.0×10^5 cells/ml in serum-free MEM. After the Matrigel rehydration period, the medium was removed from the inserts and wells, and 500 μ l of the cell suspension was seeded onto each insert. To the bottom of the well, 750 μ l of MEM with 10% FBS was added. At 72 h after plating, excess medium was removed and cells were stained with crystal violet and analyzed as outlined for the laminin migration assay.

Statistics

Statistical analysis of experimental data was performed using a paired *t*-test (Microsoft Excel, Microsoft Corp., Redmond, WA, USA).

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Figure 1.

Kallikrein 6 mRNA and secreted protein are upregulated in Caco/Kras6 cells compared to Caco2 parental and Caco/Neo3 cells.

(A) Semi-quantitative RT-PCR for *KLK6* and *K-RAS* RNA levels at cells harvested 24, 48, 72 and 96 h post-plating. (B) RT-PCR results were quantified by densitometric analysis using Scion Imaging Quantification software. (C) Western blot analysis of *KLK6* intracellular protein levels in Caco2 parental, Caco/Neo3 mock-transfected cells and Caco/Kras6 cells. (D) *KLK6* ELISA performed on Caco2 parental, Caco/Neo3 and Caco/Kras6 cells. * $p < 0.02$.

Figure 2.

Inhibition of the PI3-K/AKT and p42/44 MAPK pathways suppresses KLK6 mRNA expression and protein secretion in Caco/Kras6 cells, whereas inhibition of p38 and JNK pathways does not inhibit mRNA expression.

(A) Quantitative real-time PCR was performed on Caco/Neo3 (Neo3) and Caco/Kras6 (Kras6) cells treated with 50 μ M PD98059 and LY294002 for 48 h. * $p < 0.01$. (B) KLK6 ELISA was performed using conditioned media from Caco/Kras6 cells treated either with vehicle control or with PD98059 and LY294002 as described in the materials and methods section. * $p < 0.01$. (C) Semi-quantitative RT-PCR was performed on Caco/Kras6 cells treated with 10 μ M SB202190 or 20 μ M SP600125 for 48 h. Compared to the vehicle (DMSO)-treated control, there is no significant decrease in KLK6 mRNA.

Figure 3.

KLK6 mRNA expression and protein secretion are inhibited by anti-KLK6 siRNA, and inhibition of KLK6 abrogates Caco/Kras6 migration across laminin.

(A) KLK6 mRNA expression was inhibited by anti-KLK6 siRNA. Cells were harvested 48 h after subculture and processed for RNA isolation as described in materials and methods.

(B) KLK6 ELISA performed with samples transfected with anti-KLK6 siRNA. Cells were transfected with KLK6 siRNA as described in materials and methods, and conditioned media were collected 48 h after transfection. * $p < 0.05$. (C) The anti-KLK6 antibody inhibits migration of Caco/Kras6 cells (Kras6) through laminin. Cells were pretreated with anti-KLK6 antibody or normal mouse IgG for 20 min prior to seeding on laminin-coated migration filters and allowed to migrate for 48 h. * $p < 0.05$. (D) Migration through laminin of Caco/Kras6 cells transfected with KLK6 siRNA was inhibited. Cells were transfected with KLK6 siRNA for 48 h as described in the materials and methods section, and were then seeded on laminin-coated migration filters. Cells were allowed to migrate for another 48 h before staining and analysis. * $p < 0.01$. (E) Caco/Kras6 invasion through Matrigel was inhibited in cells transfected with KLK6 siRNA. Caco/Neo3 and Caco/Kras6 cells were transfected with either KLK6 or control siRNA for 48 h and were then seeded on Matrigel-coated filters. Cells were allowed to invade for 72 h before staining and analysis was performed. * $p < 0.002$.

Figure 4.

Kallikrein 6 expression and secretion in SW480 colon cancer cells.

(A) Semi-quantitative RT-PCR analysis of *KLK6* RNA (upper panel) and *K-RAS* transcript levels (middle panel) in SW480 cells in relation to Caco/Kras clones. *GAPDH* RNA transcript levels were used as a loading control. RFLP analysis for the presence of the mutant *K-RAS* allele (lower panel). Cells were harvested for RNA isolation 48 h after subculture and RT-PCR and RFLP analyses were performed as described in materials and methods. (B) Levels of KLK6 secretion in SW480 cells in relation to Caco/Kras cells. Media samples were collected 48 h after subculture and analyzed for KLK6 secretion using ELISA as described in materials and methods. * $p < 0.05$, ** $p < 0.001$.

Figure 5.

Migration of SW480 colon cancer cells across laminin is KLK6-dependent.

(A) KLK6 expression and secretion are inhibited by KLK6 siRNA in SW480 cells. siRNA experiments were performed as described in the materials and methods section. (B)

Inhibition of KLK6 by KLK6 siRNA abrogates SW480 cell migration across laminin.

* $p < 0.02$, ** $p < 0.001$.