Tumorigenesis and Neoplastic Progression

Kallikrein-Related Peptidase 14 Acts on Proteinase-Activated Receptor 2 to Induce Signaling Pathway in Colon Cancer Cells

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Serine proteinases participate in tumor growth and invasion by cleaving and activating proteinase-activated receptors (PARs). Recent studies have implicated PAR-1 and PAR-4 (activated by thrombin) and PAR-2 (activated by trypsin but not by thrombin) in human colon cancer growth. The endogenous activators of PARs in colon tumors, however, are still unknown. We hypothesize that the kallikrein-related peptidase (KLK) family member KLK14, a known tumor biomarker, is produced by colonic tumors and signals to human colon cancer cells by activating PARs. We found that i) KLK14 mRNA was present in 16 human colon cancer cell lines, ii) KLK14 protein was expressed and secreted in colon cancer cell lines, and iii) KLK14 (0.1 μmol/L) induced increases in intracellular calcium in HT29, a human colon cancer–derived cell line. KLK14-induced calcium flux was associated with internalization of KLK14-mediated activation of PAR-2. Furthermore, KLK14 induced significant extracellular signal–regulated kinases 1 and 2 (ERK1/2) phosphorylation and HT29 cell proliferation, presumably by activating PAR-2. A PAR-2 cleavage and activation–blocking antibody dramatically reduced KLK14-induced ERK1/2 signaling. Finally, ectopic expression of KLK14 in human colon adenocarcinomas and its absence in normal epithelia was demonstrated by IHC analysis. These results demonstrate, for the first time, the aberrant expression of KLK14 in colon cancer and its involvement in PAR-2 receptor signaling. Thus, KLK14 and its receptor, PAR-2, may represent therapeutic targets for colon tumorigenesis. (Am J Pathol 2011, 179:2625–2636; DOI: 10.1016/j.ajpath.2011.07.016)

The essential role of proteolytic enzymes, such as matrix metalloproteinases and various serine proteinases, in colon cancer progression and metastasis is well-known.1,2 However, the traditional view of the role of their contribution to cancer progression by degrading extracellular matrix proteins has significantly changed recently because it is now clear that a subclass of proteinases that serve as signaling molecules controls cell functions through specific membrane receptors called proteinase-activated receptors (PARs).3,4 PARs are tethered ligand receptors that are activated by cleavage of their extracellular amino-terminus by serine proteinases. Originally, PAR-1, PAR-3, and PAR-4 were described as mainly being activated by thrombin, whereas PAR-2 is activated by trypsin and other serine proteinases but not by thrombin.3,4 PARs are now known to be targeted by many serine proteinases, multiple enzyme families, and matrix metalloproteinase-1.4–6 Once activated, PARs trigger a cascade of downstream events leading to signal transduction resulting in stimulation of phosphoinositide breakdown, cytosolic and calcium mobilization,3,4 and diverse cellular responses in physiopathology, including gene transcription, cell proliferation, and tissue repair.5–9 Short synthetic peptides [activating peptides (APs)] corresponding to the newly exposed amino-terminus can activate a given PAR selectively and mimic the cellular effects of the proteinase.3,4,10

Supported by the Association pour la Recherche sur le Cancer (contract 3937) and Institut National de la Santé et de la Recherche Médicale, with ancillary support from the Canadian Institutes of Health Research (M.D.H. and E.P.D.).

Accepted for publication July 26, 2011.

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PARs and their activators are considered important contributors to the development of human colon cancer. Indeed, we previously demonstrated that in colonic tumors, trypsin acting through up-regulated PAR-2 and thrombin acting through aberrantly expressed PAR-1 and PAR-4 are very robust growth factors that control mitogen-activating protein kinase (MAPK) activation and subsequent cell proliferation and migration of human colon cancer cells.8,11–13 Furthermore, PARs participate in cell invasion and metastasis of many cancers.7,14,15 However, despite an intense research to discover novel PAR activators,4,5,16–18 the endogenous enzymes responsible for activating PARs in colon cancer remain unknown. Extensive literature exists demonstrating changes in PARs and kallikrein-related peptidases (KLKs) in the setting of various cancers, such as breast, lung, colon, pancreas, ovary, and prostate cancers.9,11,14,15,19,20 Recent pharmacologic approaches have implicated members of the KLK family (KLK4-6 and KLK14) as possible PAR activators in many cell systems.21–23 In prostate cancer cells, KLK2 and KLK4, initiated MAPK signaling.24 We demonstrated that KLK4 activates specifically aberrantly expressed PAR-1 signaling in colon cancer cells but not other PARs.25 KLK14 is a trypsin-like serine proteinase displaying arginine/lysine–specific proteinase activity26 similar to the known PAR activators.4,5 In this context, we explored the expression of KLK14 in colon tumors and tested the possibility that KLK14 can modulate PARs signaling in colon cancer cells. These results demonstrate, for the first time, the aberrant expression and secretion of KLK14 in colon tumor cells and its absence in normal colon. Furthermore, we show that KLK14 is a potent promoter of PAR-2 signaling leading to extracellular signal–regulated kinases 1 and 2 (ERK1/2) activation and colon cancer cell proliferation. Thus, we hypothesize that KLK14 is a potential endogenous activator of PAR-2 in colon tumors.

Materials and Methods

Reagents

Reagents were obtained from the following sources: the APs TFLLR-NH₂ (AP1, a PAR-1 agonist) and SLIGKV-NH₂ (AP2, a PAR-2 agonist) or SFLLRN-NH₂ (the thrombin receptor agonist peptide (TRAP) that activates PAR-1 and PAR-2) and 2-furoyl-LIGRLO-NH₂ (a potent PAR-2 agonist)27 from NeoMPS (Strasbourg, France); purified recombinant KLK14 (2 nmol/L of KLK14 equivalent to 1 U/mL of trypsin-like activity) has been previously described.28 Highly purified α-thrombin (3000 U/mg) from Kordia Laboratory Supplies (Leiden, the Netherlands); trypsin (16,000 U/mg) and Alexa Fluor 488 dye–conjugated goat anti-mouse antibody from Life Technology Inc. (Cergy Pontoise, France); and Fura-2/AM from Molecular Probes (Leiden). Antibodies were purchased from the following vendors: phosho-specific antibodies to ERK1/2 from Cell Signaling Technologies (Beverly, MA) and polyclonal anti-ERK1/2 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal and polyclonal KLK14 antibodies were produced as described.29 The human PAR-2–targeted monoclonal antibody (mAb 13-8) was a gift from Dr. Duke Virca and colleagues (Amgen Inc., Seattle, WA). This antibody has been verified to target PAR-2 in calcium signaling assays in vitro (M.D. Hollenberg and G.D. Virca, unpublished data). All other chemicals were purchased from Interchim (Asnière, France).

Cell Culture

The human colon cancer cell lines HT29, SW480, HCT116, Caco-2, HCT-8, LoVo, LS-174T, SW620, and T84 were obtained from American Type Culture Collection (Rockville, MD). The CI.19A cell line was a gift from Dr. Christian Laboisse (EA 4273 Biométadys University of Nantes, Nantes, France), and the HT29-D4 cell line was a gift from Dr. Jacques Marvald (Université d’Aix-Marseille, Marseille, France). Cells were routinely cultured in 25-cm² plastic flasks (Costar, Cambridge, MA) as recommended by American Type Culture Collection. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/air in Dulbecco’s modified Eagle’s medium containing 4.5 g/L of glucose supplemented with 10% fetal calf serum (FCS), except for Caco-2 cells, which were maintained in 20% fetal calf serum (FCS) and 1% nonessential amino acids. T84 cells were maintained in Dulbecco’s modified Eagle’s medium–Ham’s F-12 (1:1) supplemented with 10% FCS. PC-3, a prostate cancer cell line, and MDA-MB-468, a breast cancer cell line, both from American Type Culture Collection, were cultured in RPMI 1640 medium supplemented with 10% FCS.

Human tissues were processed according to French guidelines for research on human tissues.30 Fresh normal human colons with no digestive disease were obtained in the past from France-Transplant according to French bioethics law.51 Colonic epithelial cells were isolated by manual shaking in a dispersing solution containing EDTA (2.5 mmol/L) as previously described.32 Cells were stored in guanidium isothiocyanate at −70°C until isolation of total RNA.

RT-PCR

Total RNA (4 μg) was reverse transcribed using oligo (dT) primer. Amplifications were conducted using the resulting cDNAs. Twenty-five percent of the cDNA mixture was amplified using human KLK14 sense primer 5’-CAGTGCAGCCGCCCCGATC-3’ and antisense primer 5’-GGCCAGGCAGCGCGTCC-3’. Glyceraldehyde-3-phosphate dehydrogenase cDNA amplification was used as an internal control with sense primer 5’-TCGGAGTCAACGGATTGGTGCTGA-3’ and antisense primer 5’-AGCCTTCTCCATGTTGTTGAAG-3’. Each of the 30 cycles of amplification was performed as follows: 94°C for 40 seconds, 59°C for 40 seconds, and 72°C for 40 seconds. PCR products were identified by electrophoresis in 2% agarose gel followed by SYBR Safe staining (Invitrogen, Carlsbad, CA).
Tissue IHC Analysis
Immunohistochemical (IHC) analysis was performed on archival formalin-fixed, paraffin-embedded tissue samples from two cases with normal colonic mucosa and 11 cases of colonic adenocarcinomas (Pathology Department of Bichat-Claude Bernard Hospital, Paris, France), of which 7 adenocarcinomas were located in the right colon and 4 in the left colon. Twenty-nine samples of TMA (along with their matched normal tissue controls) (CliniSciences, Montrouge, France) were also analyzed. Tissues were used in accordance with the requirement of the Human Research Committee of the Bichat-Claude Bernard Hospital and according to French bioethics law. Tumors were staged according to the TNM classification. Staging was performed according to the pathologic records. De-waxed sections were overlaid overnight with the KLK14 antibody diluted in PBS 1:400. Specific binding was detected by the streptavidin-biotin-peroxidase method (Universal immunostaining kit; Immunotech, Marseille). Sections were counterstained with Mayer’s hemalum. In control experiments, the primary antibody was replaced with the antibody diluent. KLK14 immunostaining was performed according to the pathologic records. First, the percentage of immunostained epithelial cancer cells was evaluated and second, the staining intensity was scored on a scale from 0 to 4 where 0 represents no staining; 1, weak; 2, moderate; 3, strong; and 4, intense staining.

Immunofluorescence and Confocal Microscopy
Immunofluorescence detection was performed on HT29 cells grown on glass coverslips. The polyclonal KLK14 antibodies, previously characterized, were used to detect KLK14 in HT29 cells. We also performed PAR-2 immunofluorescence studies as described after incubation of HT29 cells for various times (5, 10, 15, 30, and 60 minutes) at 37°C with either trypsin (0.01 μmol/L) or KLK14 (0.1 μmol/L). Cells were washed three times in PBS before being fixed in 2% paraformaldehyde or with cold acetone for 30 seconds for PAR-2 internalization studies, were washed three times in PBS, and then were incubated with PBS containing 2% bovine serum albumin for 15 minutes before application of the primary anti–PAR-2 mAb (mAb 13-8) for 2 hours at room temperature. The cells were then washed again in PBS containing 1% bovine serum albumin and secondary antibody, Alexa Fluor 488 dye–conjugated goat anti-mouse antibody, was applied for 45 minutes at room temperature. The cells were washed again in PBS containing 1% bovine serum albumin and finally in PBS. Negative controls were obtained by omitting primary antibodies. The cells were then mounted in Vectashield medium (Vector Laboratories, Peterborough, UK). Images were examined using a fluorescence microscope (Leica DM IRB; Leica Microsystems, Wetzlar, Germany) (original magnification, ×630) and by confocal analysis (Zeiss LSM 510; Carl Zeiss Microlmaging GmbH, Jena, Germany) (original magnification, ×630).

ELISA for KLK14
HT29 cells were seeded at 500,000 cells per flask. At confluence, cells were counted, and the conditioned medium was collected for measurement of KLKs. Enzyme-linked immunosorbent assay (ELISA) for KLK14 was performed using a noncompetitive immunoassay, as previously described. Briefly, the KLK14-specific mAb was first immobilized on a 96-well white polystyrene plate (500 ng per well) by incubating in coating buffer (50 mmol/L Tris, 0.05% sodium azide, pH 7.8) overnight at room temperature. The plate was then washed three times with washing buffer [50 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween 20 (pH 7.8)] (Sigma-Aldrich, St Louis, MO). KLK14 standards or samples were then put into each well (100 μL per well), diluted 1:1 in assay buffer [50 mmol/L Tris, 6% bovine serum albumin, 10% goat IgG, 2% mouse IgG, 1% bovine IgG, 0.5 mol/L KCl, 0.05% sodium azide (pH 7.8)], incubated for 2 hours with shaking, and then washed six times, as previously herein. Subsequently, 100 μL of rabbit anti-KLK14 polyclonal sera diluted 1000-fold in assay buffer was added and incubated for 1 hour. After incubation, the plate was washed as described previously herein; alkaline phosphatase–conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), diluted 3000-fold in assay buffer, was applied; and plates were incubated for 45 minutes. After washing as described previously herein, the alkaline phosphatase substrate diflunisal phosphate (100 μL of a 1-mmol/L solution) in substrate buffer [0.1 mol/L Tris (pH 9.1), 0.1 mol/L NaCl, and 1 mmol/L MgCl2] was added to each well and incubated for 10 minutes followed by the addition of developing solution (100 μL containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L TbCl3, and 3 mmol/L EDTA) for 1 minute. The resultant fluorescence was measured by time-resolved fluorometry using the Cyberfluor 615 immunoanalyzer (MDS Nordion, Kanata, ON, Canada).

Intracellular Calcium Measurement
Intracellular calcium concentration was measured using Fura-2/AM. HT29 cells were seeded onto the center of glass coverslips and cultured in Dulbecco’s modified Eagle’s medium to 80% confluence. Coverslips were then loaded with 5 μmol/L Fura-2/AM in Na-HEPES–buffered saline, pH 7.4, containing 135 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L MgCl2, 11 mmol/L HEPES, 11 mmol/L glucose, and 0.01% pluronic acid with 1.5 mmol/L CaCl2 for 45 to 60 minutes at 37°C. They were then washed in Na-HEPES buffer and placed at 37°C in a fluorimeter. Cells were treated with KLK14, thrombin, or human trypsin or the PARs agonists peptides TRAP (SFLLR-NH2), AP1 (TFFLR-NH2), AP2 (SLIGKV-NH2), and 2-furoyl-LIGRLO-NH2, and changes in intracellular Ca2+ were monitored. Fluorescence was measured using a dual-wavelength excitation fluorimeter at 340 and 380 nm for excitation and at 510 nm for emission.
Western Blot Analysis

For ERK phosphorylation assays, cells were grown in six cluster wells (Costar) to 70% confluence and then were serum deprived for 48 hours. Quiescent cells were treated with test substances for various periods as indicated. In some experiments, HT29 cells were preincubated with anti–PAR-2 (mAb 13-8) (200 nmol/L) for 2 hours and then were challenged for 5 minutes with trypsin (10 nmol/L) or KLK14 (2 nmol/L). Cells were lysed with RIPA assay buffer [PBS, 1% NP-40 (Thermo Fisher Scientific, Illkirch Cedex, France), 0.5% sodium deoxycholate, and 0.1% SDS] containing proteinase inhibitor cocktail (Sigma-Aldrich) and 1 mmol/L sodium orthovanadate for 30 minutes at 4°C, and lysates were centrifuged at 12,000 × g for 15 minutes. Equal amounts of extracts (50 µg) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were incubated in blocking Tris-buffered saline buffer (20 mM Tris, 50 mM NaCl) containing 5% (w/v) low-fat milk and 0.1% (v/v) Tween 20 and then were probed with phospho-specific antibodies to ERK1/2 (1:2000) overnight at 4°C. Subsequently, blots were washed and incubated with the anti-IgG-peroxidase-linked secondary antibody for 1 hour at room temperature before detection using a chemiluminescent detection kit (NEN Life Science, Paris) and exposure to X-rays. Membranes were reprobed using a polyclonal anti-ERK1/2 antibody (1:1000) that recognizes total ERK1/2 regardless of its phosphorylation state and served as loading controls.

Proliferation Assay

Determination of cellular proliferation was accomplished by direct cell count as previously described. Colon cancer cells were seeded sparsely (5000 cells per well) in 96 cluster wells (Costar) and allowed to adhere and grow for 3 days. The medium was removed, and adherent cells were rinsed twice with serum-free medium. Cells were then grown in 200 µL of culture medium without FCS for 48 hours, after which 200 µL of a fresh serum-free medium was added with or without trypsin, SLIGKV-NH₂, or KKLK14. After designated times in culture, cells were detached from triplicate wells by trypsin (0.25% w/v)-EDTA (0.02% w/v) and counted in a hemacytometer. Cell death was evaluated using trypan blue. No significant cell death was observed after treatment with the enzymes or the AP.

Results

Human KKL14 Expression in Human Colon Cancer Cell Lines in Vitro

The expression of KKL14 transcripts was investigated in 15 human colon cancer cell lines by RT-PCR analysis. KKL14 mRNA seemed to be present in all human colon cancer cells analyzed. A strong KKL14 mRNA signal was detected at the predicted band size of 485 bp in HT29, HT29-D4, SW480, LS174T, HCT8, SW620, SW48, LoVo, HCT116, T84 cells, and WiDr cells (Figure 1). In contrast, weak expression of KKL14 was detected in Caco-2, HT29-Cl.19A, Colo 205, and Colo-HSR cells. The human breast cancer cell line MDA-MB-468 and the human prostate cancer cell line PC-3 were used as positive controls and, as expected, showed high levels of KKL14 mRNA (Figure 1A). Under the experimental conditions, KKL14 mRNA was faint or absent in epithelial cells isolated from normal human colon (Figure 1B).

To provide further evidence of KKL14 expression at the protein level, IHC analysis detection was performed on paraffin-fixed HT29 and MDA-MB-468 cells. Cell blocks were prepared from HT29 cells and MDA-MB-468 cells that were grown in culture. As shown in Figure 2A, strong cytoplasmic staining was detected in HT29 cells, similar to that observed in MDA-MB-468 cells used as positive control. Conversely, cells were negative when KKL14 primary antibody was omitted.

To confirm the cytoplasmic localization of KKL14, HT29 cells were stained by the immunofluorescence method using a polyclonal antibody directed against human KKL14. Staining was localized all over the cytoplasmic region of HT29 cells (Figure 2B). Immunostaining was also detected in close proximity to the plasma membrane and in the perinuclear region of HT29 cells.

Figure 1. Expression of KKL14 in human colon cancer cell lines (A) and in HT29 cells versus human normal colonic epithelial cells (B). Total RNA (4 µg) was reverse transcribed and PCR amplified with KKL14 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. A: A single PCR-amplified product of the predicted size (485 bp) for KKL14 was visualized after electrophoresis on 2% agarose gel. The breast cancer cell line (MDA-MB-468) and the prostate cancer cell line (PC-3) were used as positive controls.
contrast, no fluorescence could be detected when the antibody was omitted, showing the specificity of the antibody. Confocal laser scanning microscopy confirmed that immunoreactivity was localized in a perinuclear region of HT29 cells (Figure 2B). These data are consistent with the RT-PCR detection of KLK14 in HT29 cells and show that colon cancer cells express high intracellular levels of KLK14 protein.

Calcium Signaling Triggered by KLK14 in HT29 Cells Is Mediated by PARs

Because it has been reported that some KLK members have the ability to activate PARs,21,22 we investigated whether KLK14 can trigger calcium signaling in HT29 cells that constitutively express high levels of PAR-1 and PAR-2.8,9 As we previously reported, receptor selective peptide agonists for PAR-1 (TFFLR-NH2) and PAR-2 (SLIGKV-NH2) induced changes in Ca2+ mobilization in HT29 cells, confirming that HT29-expressed PAR-1 and PAR-2 are functional (data not shown).8,9 As shown in Figure 3A, HT29 cell challenge with KLK14 induced intracellular Ca2+ mobilization in the concentration range of 30 to 150 nmol/L. At a low concentration (30 nmol/L), KLK14 caused only a small Ca2+ response, whereas HT29 cell challenge with 0.1 μmol/L or 0.15 μmol/L of the enzyme resulted in significant but comparable Ca2+ responses, suggesting that the plateau of the Ca2+ response lies at this range of KLK14 levels. Therefore, subsequent experiments were performed with 0.1 μmol/L of KLK14. The data suggest that KLK14 signals to HT29 cells by causing an increase in calcium concentrations.

The specificity of the response via PAR-1 and PAR-2 was demonstrated by cross desensitization studies using specific agonist peptides and enzymes.10 As shown in Figure 3B, a challenge of the cells with TRAP (SLFLR-NH2) (100 μmol/L), which is known to desensitize PAR-1 and PAR-2 but not other PARs,10 abrogated subsequent AP1 and AP2 responses, the known agonists of PAR-1 and PAR-2, respectively. KLK14-induced Ca2+ mobilization was also abrogated after PAR-1 and PAR-2 desensitization with TRAP (Figure 3B). These experiments suggest that KLK14 signals mainly via PAR-1 and/or PAR-2.

To determine which PAR is activated by KLK14, first we used thrombin (10 nmol/L) to desensitize PAR-1. Indeed, KLK14–induced Ca2+ mobilization was not affected by a first challenge of the cells with thrombin (Figure 4A). In concordance with these data, KLK14-induced calcium mobilization did not abrogate the calcium responses induced by AP1 (TFFLR-NH2, the PAR-1–specific APs) (100 μmol/L) (Figure 4B). Conversely, desensitization of PAR-2 by the PAR-2–specific peptide 2-furoyl-LIGRLO-NH2 abrogated the KLK14-induced Ca2+ elevation, whereas subsequent challenge of HT29 cells with AP1 (TFFLR-NH2) (100 μmol/L) still induced Ca2+ elevation (Figure 4C). These results suggest that KLK14 preferentially activates PAR-2 in HT29 cells.

Figure 2. Immunodetection of KLK14 in human colon cancer cells. A: Immunodetection of KLK14 in paraffin sections from HT29 (left upper panel) and MDA-MB-468 cells (left lower panel). No immunoreactivity was detected when the primary antibody was omitted (right panels). B: Immunofluorescence detection of KLK14. HT29 cells were fixed using 2% paraformaldehyde. Left: KLK14 protein was evident in the cytoplasm of HT29 cells. Right: No immunofluorescence was observed when the anti-KLK14 antibody was omitted. Original magnification, ×630. The inset shows confocal microscopic immunocytochemical localization of KLK14 in HT29 cells. Original magnification, ×630; zoom, ×4. Arrows show perinuclear staining of KLK14.

Figure 3. KLK14 induces calcium mobilization in HT29 cells via PARs. HT29 cells were loaded for 60 minutes at 37°C using Fura-2/AM. A: Cells were challenged with the indicated concentrations of KLK14. B: Cells were challenged by the addition of the TRAP that coactivates PAR-1 and PAR-2, followed by sequential challenges with AP1, AP2, and KLK14 (0.1 μmol/L). Addition of the agonists is indicated by arrows. These results are representative of three independent experiments.
KLK14 Induces Internalization of PAR-2 in HT29 Cells with Disappearance of Receptor from the Cell Surface

By using immunofluorescence microscopy and an antibody directed against the N-terminal domain of PAR-2 that recognizes the uncleaved and cleaved receptor, we examined the loss of PAR-2 immunoreactivity at the cell surface of KLK14-treated cells. As shown in Figure 5A, PAR-2 was readily detected at the plasma membrane of unstimulated HT29 cells (control). In control experiments, as expected, trypsin treatment caused loss of PAR-2 from the cell surface, and thrombin treatment did not affect PAR-2 staining (Figure 5A). Stimulation of HT29 cells with KLK14 (0.1 μmol/L) for 15 minutes resulted in a significant decrease in PAR-2 immunoreactivity at the cell surface (Figure 5A). These data indicate that KLK14 mediates N-terminal cleavage of PAR-2 and concomitant loss of receptor staining from HT29 cell surface.

Activated PAR-2 is rapidly desensitized and internalized.3,35 Because PAR internalization requires proteolytic cleavage at defined activation sites, we analyzed PAR-2 intracellular localization after KLK14 challenge by confocal microscopy. As shown in Figure 5B, trypsin treatment induced PAR-2 internalization, as indicated by the appearance of diffuse intracellular staining, compared with the membrane staining in control unstimulated cells. As expected, thrombin did not affect the membrane localization of PAR-2. In contrast, treatment with KLK14 induced rapid (15 minutes) internalization of PAR-2 and diffuse localization in the cytosol of HT29 cells. These data indicate that in HT29 cells, KLK14-induced calcium signaling is mediated by a proteolytic cleavage of PAR-2 at a specific activation site that induces its internalization.

KLK14 Activates ERK1/2 in Human Colon Cancer Cells

Because we have previously shown that PAR-2 activation plays a pivotal role in ERK1/2-induced activity in colon cancer,12 we next investigated the effect of KLK14 on ERK1/2 phosphorylation. Addition of KLK14 (0.1 μmol/L) to quiescent HT29 cells for various times induced a rapid time-dependent phosphorylation of p42/p44, reaching a maximum within 5 to 10 minutes and persisting for 40 minutes (Figure 6A). KLK14 induces ERK1/2 phosphorylation in the concentration range of 2 to 50 nmol/L (Figure 6B). Significant ERK1/2 phosphorylation was obtained with KLK14 concentrations as low as 2 nmol/L KLK14. These experiments suggest that KLK14 activates the MAPK pathway in colon cancer cells at concentrations equivalent to those at which trypsin activates PAR-2 signaling.12

To clearly show the involvement of PAR-2 in KLK14 signaling, we used mAbs directed against the sequence spanning the protease cleavage/activation site of PAR-2 (mAb 13-8).25 As shown in Figure 6C, blocking cleavage of PAR-2 with the mAb antagonist mAb 13-8 inhibited KLK14-induced ERK1/2 phosphorylation. As expected, PAR-2 mAbs also inhibited trypsin-induced ERK1/2 phosphorylation. This result shows that KLK14 acts at the cleavage site of PAR-2 to induce cell signaling in colon cancer cells.

KLK14 Stimulates Proliferation of Human Colon Cancer Cells in Vitro

PAR-2–induced ERK activation plays a pivotal role in colon cancer cell proliferation.8,12 Next, we evaluated the effect of KLK14 on HT29 cell growth. Stimulation of HT29 cells with KLK14 (1 nmol/L) significantly increased HT29 cell numbers at 96 hours (Figure 7). This stimulation was comparable with that induced by 1 nmol/L trypsin or 100 μmol/L AP2. All these data suggest that low concentrations of recombinantly expressed KLK14 can stimulate
colon cancer cell proliferation in vitro, presumably through PAR-2.

Identification of KLK14 in Human Colonic Cancer Cell Supernatants

The relevance of KLK14’s effect on cell proliferation was investigated by measuring KLK14 secretion into the conditioned media from human colon cancer cells in vitro. The levels of KLK14 in various cell line supernatants were quantified using an immunoassay (see Materials and Methods). KLK14 protein is secreted by many human colon cancer cell lines (Figure 8). The amount of KLK14 in these supernatants was corrected for the total cell number. The highest KLK14 levels (mean ± SEM: 90 ± 17.3 ng/L; ∼3 pmol/L) were observed in the conditioned media from the SW480 cell line, followed by the T84, SW48, and LoVo cell lines. Lower levels were seen in the HT29 and LS174T cell lines. KLK14 immunoreactivity in the other cell lines (SW620, Caco-2, HCT-116, and HT29-16E) was very low or undetectable. These data suggest that colon cancer cell lines express and secrete KLK14 extracellularly.

KLK14 Is Expressed in Colon Cancer Tumors in Vivo

We next examined the potential pathologic relevance of our observations by performing IHC analysis of KLK14 in colorectal adenocarcinomas and normal colonic tissues. In normal human colonic mucosa from control subjects without colonic cancer, no staining for KLK14 was observed in epithelial cells (Figure 9A). However, immunostaining was seen in the stromal cells and the submucosa. Similarly, in patients with colon cancer, immunoreactivity was absent in the “normal” mucosa, far from the neoplastic tissue (Figure 9B). In contrast, KLK14 expression was clearly seen in the contiguous dysplastic mucosa (Figure 9C). Twenty-nine patient adenocarcinomas expressed KLK14 in the cancerous epithelium regardless of the site of the tumor in the colon and regardless of the type of tumor, tumor stages, or degree of tumor differentiation (Table 1). The intensity of staining, however, varied from case to case (Figure 9, C–E). Staining was localized in the cytoplasmic compartment and appeared strong at the apical part of the cells. These observations thus show, for the first time, that human colonic adenocarcinomas aberrantly express high levels of KLK14, in contrast to normal mucosa, where expression is nondetectable. Note that these observations are in agreement with the mRNA analysis of colon cancer versus normal colonic cells (Figure 1).

Discussion

The main finding is that there is a marked up-regulation of KLK14 in colon cancer–derived glandular epithelial cells, relative to noncancer-derived tissues, and that KLK14 in the nanomolar range can activate cell signaling via
PAR-2. We previously showed that activation of PAR-2 induces a signaling pathway that leads to colon cancer cell proliferation.\textsuperscript{8,12} However, the endogenous proteases that can activate this member of the PAR family in colonic tumors are still unknown. Thus, KLK14 can be put forward as a likely aberrantly expressed colon cancer–produced proteinase that, via PAR-2 signaling, can play a role in the colon oncogenic process. Thus, KLK14-
triggered PAR-2 activation can now be added to KLK4-stimulated PAR-1 activation\textsuperscript{25} as a potentially key element in the tumorigenic process. In this regard, members of the KLK family can be seen as likely endogenous-derived regulators of PAR-1 and PAR-2.

Although originally known for its biomarker value in breast, ovarian, and prostate cancer,\textsuperscript{20} to our knowledge, this is the first evidence of KLK14 detection in colon cancer and its absence in normal epithelia of human colon and its link to PAR-2 receptor signaling in colonic tumors. This study shows that KLK14 is expressed in many colon tumors tested in vivo and in colon cancer cell lines in culture. In vivo, we demonstrated by IHC analysis that KLK14 is present in cancer mucosal tissues but is expressed at a very low level, if any, in the adjacent normal mucosa (Figure 9). Normal mucosa from control colonic tissues does not stain with the KLK14 antibody. This is in line with the low detection of KLK14 mRNA by RT-PCR in isolated cells from normal human colon. The expression of KLK14 does not correlate with the type of tumor, the tumor stage, or the degree of tumor differentiation (Table 1). The data regarding KLK14 expression in normal colon and colonic tumors have been controversial. The present finding that KLK14 is absent in normal colonic tissues is in agreement with another study that showed, by ELISA and RT-PCR, the absence of KLK14 in adult colon.\textsuperscript{33} However, in another study, ELISA analysis measuring the expression of a panel of KLKs (KLK5-8, KLK10, KLK11, and KLK13-15) did not show significant differences in average KLK14 levels between normal mucosa and colonic tumors. Nevertheless, using a multivariate statistical analysis, elevated KLK14 levels were found to be associated with an unfavorable survival prognosis in patients with colon cancer.\textsuperscript{36} Other studies using an in silico analysis\textsuperscript{19} and real-time PCR quantification\textsuperscript{37–39} did not report differences in KLK14 expression between colonic tumors and their paired normal mucosa. This discrepancy in KLK14 expression between previously published data and the present findings is most likely due to specimen selection and to differences in the KLK14 detection methods used (eg, in silico, quantitative RT-PCR, and ELISA rather than IHC analysis). Indeed, the previously listed studies used samples comprising intact resected colonic tissues that would unavoidably contain a large amount of immune cells and stromal cells in addition to glandular mucosal cells. In contrast, herein we evaluated KLK14 expression by IHC analysis, which can localize specifically KLK14 expression in a selected population of cells in the tissue (Figure 9). This cell-targeted approach showing KLK14 expression in stromal cells in the mucosa but not in glandular epithelial cells in normal tissues and a marked up-regulation of KLK14 in the epithelial cells of adenocarcinoma tissues leads to a more precise evaluation of the sites of the proteinase up-regulation and might explain failure to detect significant cancer-associated changes of KLK14 levels in others studies. Furthermore, some KLKs are highly expressed at sites of inflammation\textsuperscript{40} and might, therefore, also be elevated in the immune cells and in the glandular epithelial cells in non–cancer-bearing tissues obtained from individuals with inflammatory bowel disease. Further studies are needed to evaluate the KLK14 expression in inflammatory bowel diseases.

Although several studies indicating that KLK expression in many cancers, including breast, ovarian, prostate, and lung cancers, is under multiple regulatory mechanisms, such as hormonal and epigenetic mechanisms,\textsuperscript{41} the mechanism whereby the KLK14 gene is switched on in colon cancer is unknown. It is likely that this ectopic expression can probably be controlled by common regulatory mechanisms, as in the other epithelial cancers. In colon cancer, expression of KLK6, another member of the kallikrein family, has been shown to be modulated by the proto-oncogene (Ki-ras), which is frequently mutated in colon cancers.\textsuperscript{42} Further studies on KLK14 regulation in colon cancer cells are, thus, warranted to evaluate a possible role of Ki-ras or other signaling mechanisms.

Whether endogenously released KLK14 contributes to colon cancer development in vivo is not known but certainly warrants further investigation. KLK14 is synthesized and secreted as a zymogen that needs cleavage of a short prodomain by trypsin-like proteinases for activation.\textsuperscript{43} Thus, it remains to be determined how the endogenously released KLK14 zymogen might be activated in the setting of colon cancer so as to contribute to colon tumorigenesis. Because trypsin-like serine proteinases other than KLK14 can be detected in the colorectal cancers,\textsuperscript{2,44} it is possible that such enzymes might activate...
the KLK14 zymogen. Alternatively, many studies have reported that KLKs participate in an enzymatic cascade to activate each other. Indeed, in vitro biochemical studies showed that pro-KLK14 can be processed by KLK5. It will, therefore, be important to establish whether colon cancer cell lines express and secrete active KLK5. In addition, several peptidases in the thrombostasis axis, including plasmin, tissue plasminogen activator, urokinase-type plasminogen activator, Factor Xa, and thrombin, can efficiently activate selective pro-KLKs, including pro-KLK14. It is possible that activation of pro-KLK14 may result from activation of serine proteinases of the coagulation pathway, which is known to be dysregulated in many cancers, including colon cancer.

We previously showed that trypsin, acting via PAR-2, induces a signaling pathway that leads to colon cancer cell proliferation. However, endogenous proteases that can activate either this receptor or other members of the PAR family in colonic tumors are still unknown. A variety of studies have linked proteolytic enzymes, including KLKs, with unfavorable prognoses of many cancers, including colon cancer. Herein, KLK14 can be seen to induce efficient calcium mobilization via PAR-2 activation and to promote efficient cell growth in the colon cancer–derived cell line HT29, mimicking the effect of trypsin or AP2-driven PAR-2 activation. Therefore, it is plausible that KLK14 becomes a potential endogenous PAR-2 activator in colon cancer.

Several lines of evidence indicate that KLK14 signals in HT29 cells only through PAR-2 and not via PAR-1: i) HT29 cells challenged with thrombin, the PAR-1 agonist, did not attenuate the KLK14-induced Ca^{2+} flux; ii) desensitization of PAR-2 with 2-furoyl-LIGRLO-NH₂, a potent PAR-2–specific AP, abrogated all KLK4-induced calcium transients while the response to PAR-1–specific peptide remained unaffected; and iii) KLK14 specifically induced PAR-2 internalization without any effect on PAR-1 surface localization. These results are in agreement with those of two others studies showing that KLK14, KLK5, and KLK6 are strong activators of PAR-2 in cells recombinantly expressing these receptors.

Further analysis of receptor staining in HT29 cells has yielded important insights into the ability of KLK14 to induce signaling via PAR-2. The present data from microscopic analysis clearly showed a loss of PAR-2 from the cell surface of HT29 cells after KLK14 incubation. The specificity of the cleavage in the activating site was confirmed by confocal microscopy analysis, which showed that KLK14-mediated cleavage of PAR-2 is accompanied by receptor internalization. The observation that KLK14 treatment of HT29 cells did not significantly affect PAR-1 cell surface location or thrombin-induced calcium influx suggests that KLK14 has low or no efficacy on PAR-1 at the concentrations used in the present studies or that KLK14 cleavage, if it occurs at all, is upstream of the activation site of PAR-1 and, thus, has no further consequences on the functionality of the receptor. The present

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**Table 1.** Estimation of KLK14 Expression in Colon Carcinoma Epithelial Cells

<table>
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<tr>
<th>Case no.</th>
<th>Histologic type</th>
<th>Tumor stage</th>
<th>Differentiation grade*</th>
<th>Stained cells (%)</th>
<th>Staining intensity†</th>
<th>Score‡</th>
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<td>80</td>
<td>2</td>
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</tbody>
</table>

*Differentiation grade was scored as follows: 1, well; 2, moderately; and 3, poorly differentiated.
†Staining intensity was often variable from place to place and was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong; and 4, intense.
‡Scores were obtained by multiplying the percentage of cells staining positive by the staining intensity (maximum possible score = 400).
observations are in agreement with those of two other studies that reported that KLK14 signals via PAR-2 in recombinant KNKRK cells and HEK cells overexpressing PAR-2.21,22 However, this enzyme can also target PAR-1 in a complex way, disarming the receptor at low concentrations and activating PAR-1 at high concentrations.21 The net effect of KLK14 would depend on the levels of KLK14 production in the tumor microenvironment and on the expression levels of PARs on the tumor. Indeed, PAR-1 is expressed at levels lower than PAR-2 in HT29 cells (D. Darmoul, unpublished data). Recent data analyzing Ca^2+ mobilization showed that in the HT29 cell line expressing PAR-1 and PAR-2, KLK4 is a related enzyme that is also a trypsin-like serine proteinase displaying arginine/lysine-specific proteinase activity and is able to activate specifically PAR-1 and not PAR-2.23 Although, the precise role played by these KLKs in the setting of colon cancer depends on the absolute amount of enzyme activity and the levels of PAR expression, these findings underline the potential role of KLKs with their target PARs in colon cancer.

In the present study, we demonstrated that colon cancer cells produce a significant amount of KLK14 in the supernatant. In a restricted environment, the enzyme concentration produced by colonic tumors would be sufficient to induce cell signaling, making it likely that tumor-secreted KLK14 can act in an autocrine loop in the colonic tumor. KLK14-induced ERK1/2 phosphorylation was initiated with a lower KLK14 concentration than that needed for induction of calcium mobilization. It is possible that PAR-2–dependent ERK1/2 activation by KLK4 might also be initiated via a calcium-independent pathway. Indeed, although PAR-2 signaling is known to couple to G_{q/11}, which induces calcium mobilization, coupling to PAR-2 has also been reported.4 It has also been shown that PAR-2 signals via a G-protein–independent signaling network or “biased” protease signaling that affects protein phosphorylation.92,93 These results demonstrate for the first time aberrant expression of KLK14 in colon cancer and its involvement, via PAR-2, in colonic cancer signaling and cell growth. Thus, KLK4 may be a promising new biomarker in colonic tumors but also can be considered a crucial contributor to the development of human colon cancer. Whether KLK14 signaling through PAR-2 in colon cancer can directly influence tumor development in vivo deserves further investigation. However, concomitant expression of KLK14 with its receptor, PAR-2, in colonic tumors would suggest that KLK14-mediated PAR-2 activation can, indeed, play an important role in colon tumorigenesis.

**Acknowledgments**

We thank Antoninus Soosaipillai (Mount Sinai Hospital) for confirming the immunoassay data for KLK14, Samira Benadda (Institut Fédératif de Recherche 02, Paris) for confocal technical assistance, and Nathalie Beaufort (INSERM U698) for helpful discussions.

**References**
