An Increased Expression of Cysteinyl Leukotriene 2 Receptor in Colorectal Adenocarcinomas Correlates with High Differentiation

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Abstract

Increased levels of inflammatory mediators such as cysteinyl leukotrienes (CysLT) have been found in and around tumors. These data, along with our previous observation that the G-protein–coupled receptor CysLT1R, which signals survival and proliferation, is up-regulated in colon cancer, suggest an important role for CysLT1R in tumor development. The objective of this study was to examine the expression and function of the low-affinity CysLT2 receptor (CysLT2R) in colon cancer. We found lower expression levels of CysLT2R compared with CysLT1R in cancer cell lines as well as clinical tumor material. Interestingly, CysLT2R, like CysLT1R, was found to be one of few G-protein–coupled receptors that are located both at the plasma membrane and the nuclear membrane. No effect of CysLT2R signaling on cell proliferation was observed, nor was there a correlation between CysLT2R and different proliferation markers such as Ki-67 and cyclin-D1 in the tumor material. Instead, we found that activation of this receptor in colon cancer cells led to cellular differentiation similar to the effects of butyrate treatment. In accordance with this finding, we found that reduced expression of CysLT2R in colon cancer was associated with poor prognosis. We report the novel finding that CysLT2R signaling leads to terminal differentiation of colon carcinoma cells and growth inhibition, and that its expression is relatively high in less malignant forms of colon cancer. These data suggest that the balance between these two receptors is important for tumor progression and disease outcome. [Cancer Res 2007;67(19):9190–8]

Introduction

Cysteinyl leukotrienes (CysLT), LTC4, LTD4, and LTE4 are proinflammatory mediators derived from arachidonic acid through the activation of the 5-lipoxygenase (5-LO) pathway (1). CysLTs mediate their effects through at least two different membrane-bound receptors, CysLT1R (2) and CysLT2R (3). CysLT1R is the higher-affinity CysLT receptor and has a higher affinity for LTD4 than LTC4 (2), whereas CysLT2R has lower overall affinity but equal for LTD4 and LTE4 (3). The CysLT receptors belong to the G-protein–coupled receptor family that is the largest family of cell-surface receptors. G-protein–coupled receptors have almost exclusively been considered to be plasma membrane–located receptors that initiate intracellular signaling through an interaction with heterotrimeric G-proteins consisting of α and β/γ subunits (4). Recently, it has been appreciated that plasma membrane G-protein–coupled receptors may translocate and establish ligand-induced responses in other cellular structures such as the nucleus, and these may involve diverse pathways for signaling (5). Research into the G-protein–coupled receptor family for peptide and lipid mediators has shown expression of some of these ligands in both the nuclear and plasma membranes (6). We have recently shown that CysLT1R can be located at the nuclear membrane as a signaling-competent receptor (7). Comparatively little is known about CysLT2R with regard to its cellular localization, signaling properties, and functions. This receptor was originally detected in pulmonary vein preparations by pharmacologic assays and has since been identified in numerous tissues (8) including placenta, spleen, heart, peripheral blood leukocytes, brain, and epithelial and endothelial cells (9–12). The only known antagonist for CysLT2R is the LTE4 analogue Bay-u9773. However, Bay-u9773 is also an antagonist for CysLT1R and, at higher concentrations, it can act as an agonist for CysLT2R (13).

Leukotrienes (LT) are well characterized with regard to their roles in the inflammatory process. They influence various cellular functions including smooth muscle contraction, mucus production, and chemotaxis (14). LTD4 has also been linked to the inflammatory disorders, asthma, and inflammatory bowel disease (1). Elevated levels of LTs have been found in the intestinal tract of inflammatory bowel disease patients (15).

Rudolf Virchow suggested that there was a connection between chronic inflammation and the development of cancer as early as 1863, when he discovered the presence of leukocytes in neoplastic tissue (16, 17). It has been estimated that more than 15% of all cancers can be accredited to infectious agents and the chronic inflammation that follows (16). The most well-established connection exists between inflammatory bowel disease and colon cancer (18, 19); however, other chronic inflammatory conditions in the intestinal tract have also been implicated in dysplasia and colon carcinogenesis (20, 21). Patients with ulcerative colitis suffer a 30% to 50% increased risk of developing colon cancer compared with persons without an inflammatory disorder (19). It is not currently well understood if and how soluble inflammatory mediators are involved in colon carcinogenesis. However, it has been shown that LTD4 mediates increased cell survival, proliferation, and migration of intestinal epithelial cells (22–24), indicating a possible link between inflammation and cancer through CysLT receptor signaling. Interestingly, increased expression levels of CysLT1R in cancer tissue has been shown to be a strong indicator of poor prognosis for colon cancer patients (25). We have recently shown the existence of constitutive autocrine CysLT1R signaling that mediates both survival and proliferation in intestinal cells (26). We have also shown that LTD4 similar to Wnt-1 signaling, results in an increased level of free β-catenin and...
elevated T-cell factor/lymphoid enhancer factor promoter activity (27). This finding that the inflammatory mediator LTD₄ regulates key events via an increased accumulation of β-catenin constitutes a significant contribution to the understanding of potential mechanisms in carcinogenesis under inflammatory conditions.

Numerous signaling pathways regulate normal cell function and tissue homeostasis by controlling cellular processes such as proliferation, differentiation, and apoptosis. Deregulation of these control systems is involved in the development of cancer (28). Dietary factors such as ingested fat have been shown to act as tumor promoters, whereas fibers have been reported to exert antitumor activity. Short-chain fatty acids such as butyrate are one of the principal products from fiber fermentation in the gastrointestinal tract, and have been shown to induce growth inhibition and increase apoptosis and terminal differentiation in many different human colon cancer cell lines (29, 30).

Currently, little is known about the role, if any, for CysLT₂R in colon cancer. Therefore, in the present study, we sought to examine the effects of CysLT₂R activation on cell growth, differentiation, and apoptosis. We investigated the expression levels and location of CysLT₂R in nontransformed intestinal and colon cancer cell lines as well as in colorectal adenocarcinomas.

Materials and Methods

Materials

The rabbit polyclonal anti-human CysLT₂R antibody (diluted 1:250 for immunofluorescence, 1:500 for Western blotting, and 1:600 for immunohistochemistry) was purchased from Innovagen. The rabbit polyclonal anti-human CysLT₂R antibody (diluted 1:250 for immunofluorescence, 1:500 for Western blotting, and 1:300 for immunohistochemistry) and the ligands LTD₄ and LTC₄ were from Cayman Chemicals Co. The mouse anti-actin antibody (diluted 1:2,000 for Western blotting) was purchased from ICN. The monoclonal mouse anti–Ki-67 (diluted 1:100 for immunohistochemistry) and the ligands LTD₄ and LTC₄ were from DAKO. The conjugated secondary antibody Alexa 488 was obtained from Molecular Probes, Inc. The inhibitor Bay-u9773 was from BIOMOL. [methyl-³H]Thymidine, the enhanced chemiluminescence (ECL) reagents, and Western blot detection reagents and hyperfilm were from Amersham International. The caspase-3 fluorometric substrate peptide Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) was from Upstate Biotechnology. Alanine-p-nitroanilide (H-Ala-PNA) was from Bachem. Unless otherwise stated, all other reagents were of analytic grade and purchased from Sigma Chemicals Co. or ICN.

Cell Culture

Nontransformed human intestinal epithelial cells (Int 407), which exhibit typical epithelial morphology and growth, were cultured as a monolayer for 5 days in Eagle’s basal medium supplemented with 15% newborn calf serum. Two different colon cancer cell lines were also used, Caco-2 and SW480. Caco-2 is derived from a moderately well-differentiated primary colon adenocarcinoma that is less tumorigenic and invasive than SW480, which is moderately differentiated with high tumorigenic and invasive properties. Caco-2 was grown in DMEM supplemented with 10% fetal bovine serum. The SW480 cell line was grown in RPMI 1640 with the same supplements as Caco-2 cells. To all media, 55 IU/mL penicillin and 55 µg/mL streptomycin were added. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Cell Fractionation

Total cell lysates. Cells were washed with PBS and lysed with ice-cold lysis buffer (23) for 30 min on ice. The lysates were centrifuged at 200 × g for 5 min.

Membrane and cytosol fractions. Int 407 cells were either left unstimulated or incubated with 1 µmol/L Bay-u9773 for 15 min before stimulation with 100 nmol/L LTD₄ or LTC₄ for 30 min. Cells were washed with 1× PBS, covered with buffer A (7), and placed for 30 min on ice. The cells were homogenized in a Dounce homogenizer for 25 strokes and thereafter centrifuged for 10 min at 500 × g. The supernatant was centrifuged for 10 min at 10,000 × g, and the resulting supernatant was separated into plasma membrane and cytosol by 1-h centrifugation at 200,000 × g.

Nuclear fractions. After washing with PBS, cells were covered in a homogenization buffer (0.32 mol/L sucrose, 3 mol/L MgCl₂, 10 mmol/L Tris-HCl [pH 7.4], 4 µg/mL leupeptin, 60 µg/mL phenylmethylsulfonyl, 5 mmol/L NaVO₄) and placed on ice. The cells were scraped and homogenized in a Dounce homogenizer for 25 strokes, followed by 10-min centrifugation at 700 × g. The pellet was washed with homogenization buffer. The pellet containing the crude nuclei was dissolved in homogenization buffer containing a higher sucrose concentration (2.4 mol/L) and centrifuged for 1 h at 50,000 × g. The precipitate was dissolved in the homogenization buffer containing 0.25 mol/L sucrose and nuclei were obtained by centrifugation at 700 × g for 10 min.

Gel Electrophoresis and Immunoblotting

To ensure equal loading, all samples were evaluated and compensated for protein concentration using the Coomassie blue protein assay. Cell samples were solubilized by boiling in sample buffer (23) for 10 min. The samples were run on a 10% polyacrylamide gel in the presence of SDS and were subjected to electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in PBS for 1 h, followed by overnight incubation with primary antibody at 4°C. Washing of the membranes was followed by 1-h incubation with a horseradish peroxidase–linked goat anti-rabbit or goat anti-mouse antibody at room temperature, with additional washing. The membranes were developed by ECL and exposed to hyperfilm.

Immunofluorescence

Cells were grown on coverslips for 5 days. The cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min, then washed with PBS and permeabilized in 0.5% Triton X-100/PBS for 5 min. After repeated washings, the cells were incubated at room temperature in a 3% bovine serum albumin/PBS solution for 30 min, and thereafter for 1 h with antibody against either CysLT₂R or CysLT₁R (both diluted 1:250). The coverslips were washed with 0.05% Tween/PBS and incubated at room temperature for 1 h with Alexa 488 goat anti-rabbit secondary antibody. The coverslips were subsequently washed and mounted in fluorescent mounting medium. The samples were photographed with a Nikon Eclipse 800 microscope using a PlanApo 60× objective.

Electron Microscopy

Electron microscopy was done on intact Int 407 and Caco-2 cells incubated with CysLT₂R antibody as previously described by Nielsen et al. (7).

Patient Samples

Formalin-fixed and paraffin-embedded colon cancer and control colon specimens from colorectal cancer patients were obtained from the archives of the Department of Pathology at Malmö University Hospital. Tissues from 77 patients with varying grades and stages of disease were included. Staging of the tumors was done using Dukes’ classification (31). For clinical pathologic features, see Table 2 in ref. 25. The matched control samples of normal colon tissue in this investigation were taken from the borders of the surgical specimens. Fresh biopsies of human colon cancer tissue and normal tissue from the border of the tumor were obtained for CysLT₂R analysis. The samples were immediately frozen by submersion in liquid nitrogen.

Tissue Arraying and Immunohistochemistry

For histologic assessment, the archival paraffin-embedded colorectal cancer and normal mucosa specimens were cut into 1-µm sections, dried, deparaffinized, rehydrated, and stained with H&E, as described in ref. 25. A
DAKO automatic slide stainer was used according to the manufacturer's instructions for all immunohistochemical procedures. More detailed methods are described in ref. 25.

**Thymidine Incorporation**
Caco-2 cells were grown in 12-well plates for 3 days, medium was changed to serum-free, and cells were stimulated with LTD4 (80 nmol/L) or LTC4 (80 nmol/L) for 24 h. Cellular DNA synthesis was assayed by adding 0.5 μCi of[^3H]thymidine during the final 18 h of stimulation. The assay was done as described in ref. 23. Radioactive thymidine incorporation was measured with a β-liquid scintillation counter (LKB Rack Beta, Wallac).

**Caspase-3 Activity**
Caco-2 cells were cultured in six-well plates for 4 days. Growth medium was then changed to medium containing 1.5% serum, and cells were stimulated for 48 h with 5 mmol/L sodium butyrate (NaBT), 40 nmol/L LTD4, or LTC4. The assay was done as described in ref. 27.

**Alkaline Phosphatase Activity**
Alkaline phosphatase activity was measured using disodium p-nitrophenyl phosphate as substrate. Caco-2 cells were seeded in Petri dishes and incubated for 24 h at 37°C. NaBT (2 mmol/L) and/or LTC4 (40 nmol/L) was added and incubated for 72 h at 37°C. New LTC4 was added every 24 h. The assay was done as described in ref. 32. The alkaline phosphatase activity was estimated by measuring the absorbance at 405 nm due to formation of p-nitrophenol.

**Aminopeptidase N Activity**
Ala-PNA was used as a substrate to measure aminopeptidase N activity. Cells were cultured and stimulated as for the alkaline phosphatase activity assay. Cells were incubated for 30 min at 37°C with constant shaking with 50 mmol/L PBS (pH 7.0) and 6 mmol/L Ala-PNA. After incubation, the reaction was terminated by placing samples on ice. Cells were centrifuged at 400 g for 5 min. The supernatants were applied in triplicates to a 96-well plate and aminopeptidase N activity was estimated by measuring the absorbance at 405 nm due to hydrolyzed para-nitroanilin.

**Statistical Analysis**
Pearson's r test was used when comparing different sets of immunostaining. All tests were two sided. Univariate survival analysis was done according to Kaplan-Meier. Differences between groups were assessed by the log-rank test. Survival was corrected for cancer-related deaths, counting metastatic disease at the time of death as specific and counting time from the initial date of surgery. Patients who died of other causes were censored at the time of death, whereas healthy surviving patients were censored at the time of clinical discharge. SPSS software 12.0.1 (SPSS, Inc.) was used at the time of death, whereas healthy surviving patients were censored at the time of clinical discharge. SPSS software 12.0.1 (SPSS, Inc.) was used.

**Results**

Expression patterns of CysLT1R and CysLT2R. We have previously shown that CysLT1R is up-regulated in colon cancer tissue compared with normal tissue (7, 25). Little is known about the expression pattern of CysLT2R in intestinal epithelial cells. We examined fresh biopsies of normal and colon cancer tissues from patients and observed an up-regulation of CysLT2R in whole smashed cancer tissue preparations, which includes inflammatory cells from the microenvironment (Fig. 1A). However, this up-regulation was not as high as that observed earlier for CysLT1R, with a >7-fold increase reported in cancer compared with normal tissue (25). Examination of different cell lines revealed that CysLT2R was up-regulated in the colon cancer cell lines Caco-2 and SW480 compared with the nontransformed intestinal epithelial cell line Int 407, consistent with previous findings, whereas CysLT1R was down-regulated in the colon cancer cell lines compared with the nontransformed human epithelial cells (see Fig. 1B). Immunofluorescent data revealed that CysLT1R is present at both the plasma and nuclear membranes, which is consistent with earlier findings (7). The expression of CysLT1R at the nuclear membrane is of particular interest because G-protein–coupled receptors have generally been considered to be principally plasma membrane receptors. The immunofluorescence data revealed a similar expression pattern for CysLT2R (Fig. 2A), as did electron microscopy (data not shown). Western blots of fractionated Int 407 cells and Caco-2 cells (Fig. 2B) exhibited a similar pattern, with CysLT2R expressed at both the plasma membrane and in the nuclear fraction. An increase of endogenous CysLT2R at the plasma membrane could be detected after 30 min of LTC4 (100 nmol/L) stimulation in serum-free medium and also, to a lesser extent, after LTD4 (100 nmol/L) stimulation. This increase of CysLT2R at the plasma membrane could be blocked by the CysLT2R antagonist Bay-u9773 (1 μmol/L) when administered 15 min before LTC4/LTD4 stimulation (Fig. 2C and D). These results show that both CysLT1R and CysLT2R have nuclear membrane expression. However, the two receptors seem to have different expression patterns in tumor...
versus nontransformed cell lines, given that CysLT2R is downregulated in colon cancer cell lines compared with a nontransformed intestinal cell line, whereas CysLT1R is upregulated in cancer. It is conceivable that CysLT2R may have a more protective role in colon cancer. It was therefore of interest to further investigate the CysLT2R expression pattern in adenocarcinomas.

**CysLT2R immunoreactivity in colorectal adenocarcinomas.**

As mentioned above, we detected different expression levels of CysLT1R and CysLT2R in cell lines, whereas the result from the whole tumors (adenocarcinomas and immune cells) in Fig. LA showed an up-regulation of both CysLT receptors in colon cancer tissue. We further examined the expression of these receptors in a human colon cancer array and found that CysLT1R could be seen at the nuclei as well as at the plasma membrane. These data supported the *in vitro* findings (Fig. 2A and B), where CysLT1R was detected both at the plasma membrane and, more extensively, at the nuclear membrane. The staining of CysLT2R in the colon cancer array was distributed as follows: cytosol/plasma membrane, +/− 15.6%, + 37.7%, ++ 37.7%, +++ 9.1%; nucleus, +/− 31.2%, + 46.8%, ++ 19.5%, and +++ 2.6% (Table 1). A number of patients did not exhibit any nuclear staining while still exhibiting high total staining for CysLT2R (Fig. 3A). We did not identify any correlation between the nuclear staining and various potential viability and carcinogenic factors. The accuracy of the primary antibody is shown in the titration of the CysLT2R antibody and with preimmune rabbit serum in sections taken from the same piece of paraffin-embedded tissue as used in this study (Fig. 3B). Less than half of the total number of colon carcinomas we examined had high CysLT2R staining, whereas the remaining number exhibited low or no staining at all. Fourteen pairs of matched tumor versus normal tissue from colon cancer patients were then examined. We identified a significantly lower CysLT2R expression in the tumor tissue (Fig. 3C and D). Approximately 75% of this cohort had higher staining for CysLT2R in the normal compared with the tumor area, and there was a predominance of Dukes’ B and C patients in this group (Fig. 3C). A tendency for lower CysLT2R staining with

**Figure 2.** Subcellular localization of CysLT2R in intestinal epithelial cells. A, Int 407 cells and Caco-2 cells were grown on coverslips until ~75% confluence, fixed with 4% paraformaldehyde, and immunostained for either CysLT1R or CysLT2R antibodies. The coverslips were mounted on glass slides and images taken with a Nikon Eclipse 80i microscope using a PlanApo 60× objective. B, Int 407 and Caco-2 cells were fractionated into membrane (M), cytosol (C), and nuclear fractions (N) as described in Materials and Methods. The samples were then subjected to SDS-PAGE and immunoblotted with a specific CysLT2R antibody (diluted 1:500). C, translocation of CysLT2R to the plasma membrane after LTD4 or LTC4 stimulation and/or pretreatment with the CysLT2R antagonist Bay-u9773. D, immunofluorescence showing translocation of endogenous CysLT2R to the plasma membrane in Int 407 cells after LTC4 stimulation and/or pretreatment with Bay-u9773.

* P < 0.05; ** P < 0.01, treated versus untreated cells (unpaired Student’s *t* test). Representative blots from at least three independent experiments.
increasing severity of colon cancer and shorter survival could be seen (Fig. 4A and B). We also found that in the patient group with the highest expression of CysLT1R, the loss of CysLT2R expression in the tumors was associated with a shorter survival after 5 to 10 years (Fig. 4B). These results indicate that CysLT2R can have a repressive role in carcinogenesis because it is down-regulated in colon tumor tissue.

**Potential viability factors in relation to CysLT2R.** We then investigated if there was a correlation between the expression of CysLT2R and different proteins of interest for colon carcinogenesis. No significant correlation between the proliferation marker Ki-67 and the expression of CysLT2R was found. CysLT2R immunohistochemical staining was also compared with cyclooxygenase-2 (COX-2), 5-LO, the survival protein Bcl-xL, and apoptotic protein Bax (25). There was a significant correlation between CysLT2R and the expression of survival protein Bcl-xL and the enzyme 5-LO (see Table 1). To further investigate whether CysLT2R was involved in cell proliferation, we carried out a cell proliferation assay in Caco-2 cells.

**CysLT2R is not involved in proliferation.** It has been shown that CysLT1R can increase the cell proliferation of intestinal epithelial cells (23, 33). To examine whether or not CysLT2R was also involved in cell proliferation, we measured the incorporation of radioactively labeled thymidine. In agreement with earlier results (23), 24-h stimulation with 80 nmol/L LTD4, which stimulates the high-affinity receptor CysLT1, resulted in increased cell proliferation. However, stimulation with 80 nmol/L LTC4, which mainly activates CysLT1R, did not induce proliferation in Caco-2 cells. These results indicate that CysLT1R, and not CysLT2R, is involved in intestinal epithelial cell proliferation.

**CysLT2R effect on apoptosis.** To investigate a possible influence of CysLT2R on apoptosis, we investigated its effect on intracellular caspase-3 activity after 48-h treatment of Caco-2 cells with 40 nmol/L of LTC4 or LTD4 (Fig. 5B). In contrast to LTC4, 40 nmol/L of LTD4 did not cause any significant increase in caspase-3 activity (Fig. 5B). Sodium butyrate significantly increased apoptosis, which was further enhanced by the addition of LTC4 (Fig. 5B).

**CysLT2R is up-regulated in the G0 phase.** Assuming from our earlier data that CysLT2R was not involved in cell proliferation in epithelial cells, we were interested to investigate if the expression

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<th>Table 1. CysLT2R immunoreactivity versus potential viability factors</th>
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NOTE: Staining (immunoreactivity) indices for 5-LO, Bcl-xL, Bax, and COX-2 in 77 (Ki-67 in 60) colorectal carcinomas are shown in relation to immunostaining for CysLT2R.
levels of CysLT₂R were changed during the cell cycle. In the absence of proliferating stimuli, cells are kept in a resting state, denoted phase gap 0 (G₀), and reenter the cell cycle when subjected to growth factors. To force the Int 407 cells to cell cycle arrest and to enter the G₀ state, cells were serum starved for 24 h. To allow cells to reenter the cell cycle and the G₁ phase, they were subjected to growth factor stimulation with the readdition of complete medium (with serum). All cells were grown in complete medium for the same total amount of time. Western blot analyses of cell lysates from the different time points revealed that the expression level of CysLT₂R in the G₀ state was significantly higher than for cells that had entered the different phases of the cell cycle: G₁, where cell growth takes place, and G₂, where the cells prepare for cell division (Fig. 5C). As seen in Fig. 5C, we used cyclin A2 as a

Figure 3. Representative CysLT₂R immunohistochemical staining of normal human colon tissue and colorectal adenocarcinomas. A, top, staining of normal control tissue and the degree of CysLT₂R staining of the carcinomas (+/- to +++; 10x/40x objective). Bottom, an enlargement of the area inside the boxes (40x). B, a series of sections from the same paraffin block (note that sections are not in order), showing three dilutions of CysLT₂R antibody and rabbit preimmune serum. C, representative pairs of control and tumor immunostaining from Dukes’ A, B, and C patients stained with CysLT₂R antibody (diluted 1:300). D, distribution of CysLT₂R in normal versus tumor tissue in different stages of colon cancer. Group 1 represents patients with higher staining for CysLT₂R in normal compared with tumor colon tissue. Group 2 represents patients with equal staining for CysLT₂R in normal and tumor colon tissue. Group 3 represents patients with lower CysLT₂R staining in normal compared with tumor colon tissue.
marker for G1-S phase (34). After 24 h of serum starvation, the cyclin A2 levels were dramatically reduced, which confirmed the G0 state, and, as expected, the cyclin A2 levels increased again after serum reintroduction. These results support the hypothesis that CysLT2R might be involved in differentiation.

**LTC4 induced differentiation in Caco-2 cells.** The human colon cancer cell line Caco-2 is known to undergo spontaneous enterocytic differentiation while in postconfluent state in culture (35). Within the luminal brush border, a number of different hydrolases involved in the final stages of digestion are expressed, including intestinal alkaline phosphatase and aminopeptidase N (36). These brush border proteins can be used as differentiation markers for Caco-2 cells. We have used NaBT as a positive control for differentiation because butyrate is a known inducer of terminal differentiation of colon cancer cells (37). Intestinal alkaline phosphatase and aminopeptidase N are digestive brush border enzymes, which are highly up-regulated during small intestinal epithelial cell differentiation (38). We therefore used the activity of these enzymes to measure the induction of cell differentiation. LTC4 (40 nmol/L) was able to induce a consistent increase of alkaline phosphatase activity in Caco-2 cells after 72 h of stimulation compared with unstimulated cells, indicating that LTC4 via CysLT2R is involved in differentiation of Caco-2 cells (Fig. 5D). LTC4 at 40 nmol/L for 72 h could not, by itself, induce increased aminopeptidase N activity, but was able to significantly enhance the effects of NaBT (Fig. 5D). These data suggest that CysLT1R may have a role in the differentiation of Caco-2 cells.

**Discussion**

G-protein–coupled receptors are usually located at the plasma membrane from where they trigger their intracellular signaling

![Figure 4](image-url)  
**Figure 4.** A, distribution of high and low staining intensities in tumors in Dukes’ stages A, B, and C, based on the tumors from 77 patients included in the study. Samples were assessed according to total CysLT2R staining. B, Kaplan-Meier analysis of overall 10-y survival of patients diagnosed with colon cancer and with high expression of CysLT1R primary tumors stratified by expression of CysLT2R.

![Figure 5](image-url)  
**Figure 5.** The CysLT2R is not involved in proliferation but is involved in differentiation of Caco-2 cells. A, Caco-2 cells were incubated with LTD4 or LTC4 for 24 h in serum-free medium. To enable determination of proliferation by thymidine uptake, [methyl-3H]thymidine (0.5 µCi/well) was added to the wells during the stimulation. The lysates were mixed with scintillation liquid and a LKB Wallac 1209 Rack Beta counter was used to determine the radioactivity. B, cells were stimulated for 48 h with 5 mmol/L NaBT, 40 nmol/L LTD4, or 40 nmol/L LTC4. Caspase-3 activity was assessed by measuring the fluorescence at excitation 390 nm and emission 460 nm after the addition of fluorometric substrate (Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin). C, cells were incubated for 24 h in serum-free medium, followed by 0, 18, or 24 h in complete medium. The cell lysates were separated by SDS-PAGE and immunoblotted with specific CysLT2R, cyclin A2, or actin antibodies. D, alkaline phosphatase and aminopeptidase N activity assays were used to determine differentiation of Caco-2 cells. Cells were treated with 2 mmol/L NaBT and/or 40 nmol/L LTC4 for 72 h. The alkaline phosphatase activity was determined by measuring the absorbance at 405 nm due to formation of p-nitrophenol. Aminopeptidase N activity was measured by absorbance at 405 nm due to hydrolyzed para-nitroanilin. Representative blots from at least three independent experiments in C. * , P < 0.05; **, P < 0.001, between untreated cells and treated cells using unpaired Student’s t test.
cascade. However, CysLT1R has a bipartite nuclear localization sequence that also enables it to be located at the nuclear membrane (39). Despite the lack of a nuclear localization sequence domain in CysLT2R, we made the novel finding that CysLT2R is also expressed at the nuclear membrane. This conclusion is based on findings from both microscopic analyses and analyses of nuclei preparations by Western blot. Indeed, CysLT2R seems to be even more concentrated at the nuclear membrane than CysLT1R, and in contrast to CysLT2R, agonist stimulation leads to an increased accumulation of CysLT2R at the plasma membrane. Although lacking a nuclear localization sequence domain, CysLT2R does possess an IFN regulatory factor 7 (IRF7) site. The presence of this site could indicate that CysLT2R is a downstream target for IFNs and possibly also an interacting partner to IRF7. If there is a direct interaction between CysLT2R and an IRF transcription factor, the presence of a nuclear localization sequence domain in the latter (40) could possibly explain the current observation of nuclear localization of CysLT2R. IRF functional activities have been closely related to the induction of apoptosis and antiproliferative effects in several different tumor types (41–44).

In agreement with a functional response of IRF, we found no correlation between the expression of CysLT2 and the proliferation markers Ki-67 and COX-2. These findings are in contrast to the previously observed correlation between CysLT1R expression and different proliferation markers (25). Based on the latter finding, we felt it necessary to directly test if CysLT2R signaling in a colon cancer cell line would cause an increased proliferation response. The results indicated that in contrast to CysLT1R signaling, activation of CysLT2R did not result in increased proliferation. In agreement with its lack of proliferation signaling, we observed that cells forced to leave the cell cycle and enter the G0 phase expressed much more CysLT2R than proliferating cells, indicating that CysLT2R might signal for differentiation in colon cancer cells. It is established that colon cancer cell lines can be stimulated to undergo typical enterocytic differentiation involving the expression of at least three different brush border membrane hydrolases. The expression levels of such hydrolases are good markers for differentiation of colon cancer cells (45). The human colon cancer cell line Caco-2 is a useful tool when investigating differentiation (35). Butyrate is an often used experimental substance because it induces a clear differentiation response in Caco-2 cells (37). In the present study, we found that LTC4 was able to induce differentiation of Caco-2 cells, although not to the extent that butyrate could. These data indicate that CysLT2R signaling is part of a differentiation signaling cascade and support the idea that high expression of CysLT2R can be used as a marker of less invasive tumors. At present, we cannot explain the observed correlation between CysLT2R and the antiapoptotic protein Bcl-xL. This correlation could either indicate that CysLT2R promotes cell survival or reflects a survival feedback mechanism following a CysLT2R-induced apoptotic response. The expression of 5-LO, which produces the ligand to the receptor, strongly correlated with CysLT2R expression, and is also associated with a prosurvival effect (46).

As discussed above, we believe that CysLT2R signaling is involved in epithelial cell differentiation. Although colorectal cancer is the third most common cancer in the Western world, there is a current lack of good prognostic markers in clinical use despite different markers being suggested in the literature (47, 48). In addition to our present finding that CysLT2R can induce differentiation, we also show in the present study that the expression of CysLT2R is reduced in colon cancer cell lines compared with nontransformed intestinal epithelial cell lines. Of further interest is our finding that in 11 of 14 colon cancer patients, we observed a clearly reduced expression of CysLT2R in colon cancer tissue compared with the normal tissue in the same patient. The three patients that did not show this expression pattern had equal staining for CysLT2R in their tumor tissue and the surrounding normal tissue. Our finding that loss of CysLT2 expression in tumors was associated with a shorter overall survival after 5 to 10 years supports these data. This was further supported by our Western blot data that showed increased expression levels of CysLT2R in the nontransformed cell line Int 407, compared with colon cancer cells. Normal intestinal epithelial cells have high expression levels of CysLT2R and low expression levels of CysLT1R, whereas tumor and colon cancer cells have high expression levels of CysLT1R, which promotes cell proliferation and survival, and low expression levels of CysLT2R, which promotes redifferentiation of cancer cells.

Several inflammatory cells are known to express CysLT2R (12) and infiltrates of inflammatory cells are frequently observed in cancer tissues. The apparent discrepancy of the result in Fig. 1A between the Western blot with cell lines and the immuno-histochemical staining can possibly be explained by a contamination of infiltrating inflammatory cells in the isolated cancer tissue from whole tumors.

In conclusion, we have found that activation of CysLT2R does not induce cell proliferation in colon cancer cells and its expression does not correlate with the proliferation marker Ki-67 in colorectal adenocarcinomas. Instead, the expression of CysLT2R is decreased in colorectal adenocarcinomas compared with surrounding normal tissue and is involved in terminal differentiation of colon cancer cells. These data suggest that CysLT2R could exert a more antitumorigenic activity compared with CysLT1R in intestinal epithelial cells, and that the balance between the two receptors is important for the outcome of tumor progression.

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