

The cysteinylLT₁ receptor in human renal cell carcinoma

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Abstract. The metabolism of arachidonic acid (AA) by either cyclooxygenase or lipoxygenase is believed to play an important role in carcinogenesis. Leukotriene (LT) D₄ is a proinflammatory mediator derived from AA through various enzymatic steps, and 5-lipoxygenase is an important factor in generating LTD₄. We investigated LTD₄ receptor (cysteinylLT₁ receptor, CysLT₁R) expression in renal cell carcinoma (RCC), as well as the effect of the CysLT₁R antagonist on cell proliferation in the RCC cell line. CysLT₁R expression was detected by immunohistochemistry and examined in RCC patients and normal kidney (NK) tissues. The effect of the CysLT₁R antagonist on RCC cell growth was examined by MTT assay. Flow cytometry was used to determine whether or not the CysLT₁R antagonist induced apoptosis. Initially, only slight CysLT₁R expression was detected in NK tissues, and marked CysLT₁R expression in RCC tissues. CysLT₁R expression was higher in high-grade than in low-grade cancer. Furthermore, the CysLT₁R antagonist caused marked inhibition of RCC cells in a concentration- and time-dependent manner through early apoptosis. To conclude, CysLT₁R was induced in RCC and the results suggest that the CysLT₁R antagonist may mediate the potent anti-proliferative effects of RCC cells. Thus, CysLT₁R may become a new target therapy in the treatment of RCC.

Introduction

Recently, with increased routine medical check-ups and progress in diagnostic imaging techniques, diagnoses of renal cell carcinoma (RCC) have increased. The cause of RCC is

unknown. Compared to many other types of cancers, RCC does not generally respond well to chemotherapy, so surgery is currently the only therapeutic option.

The metabolism of arachidonic acid (AA) by either the cyclooxygenase (COX) or the lipoxygenase (LOX) pathway generates eicosanoids, which have been implicated in the pathogenesis of a variety of human diseases, including cancer, and are significantly involved in tumor promotion, progression and metastasis. Studying these pathways in specimens from patients with RCC, we demonstrated that COX-2 and 5-LOX were overexpressed in RCC tissues (1,2).

Leukotriene (LT) belongs to an important group of pro-inflammatory mediators and is synthesized by AA via the 5-LOX pathway. The activity of 5-LOX leads to the formation of unstable LTA₄, which can be converted into either LTB₄ or cysteinyl (Cys) LTs (LTC₄, LTD₄ and LTE₄). LTD₄ is the most important component of the CysLTs (CysLT₁, CysLT₂), and CysLT₁ receptor (CysLT₁R) is specific for LTD₄ (3). Generally, CysLTs are important mediators of human bronchial asthma, and many CysLT receptor antagonists are clinically used in the treatment of human bronchial asthma. A few reports have addressed the relationship between CysLT₁R and colon cancer (4,5). To date, no report has addressed the relationship between CysLT₁R and human RCC. Our team has already demonstrated that 5-LOX is overexpressed in human urological cancer (2,6-8).

With this background, the purpose of our study was to examine the expression of CysLT₁R in human RCC tissues and in an RCC cell line, to evaluate the inhibitory effect of the CysLT₁R antagonist on a human RCC cell line, and to determine whether or not the CysLT₁R antagonist induces apoptosis in these RCC cells.

Materials and methods

Tumor specimens. Specimens were obtained from 58 patients with RCC and 20 patients with normal kidney (NK) tissues (average age 62.1±10.4) who underwent total nephroureterectomy due to ureteral cancer. Tumor tissues, non-tumor tissues, vascular endothelium and interstitial tissues from the subjects were preserved in 10% formalin, embedded in paraffin and serially sectioned onto microscope slides at a thickness of

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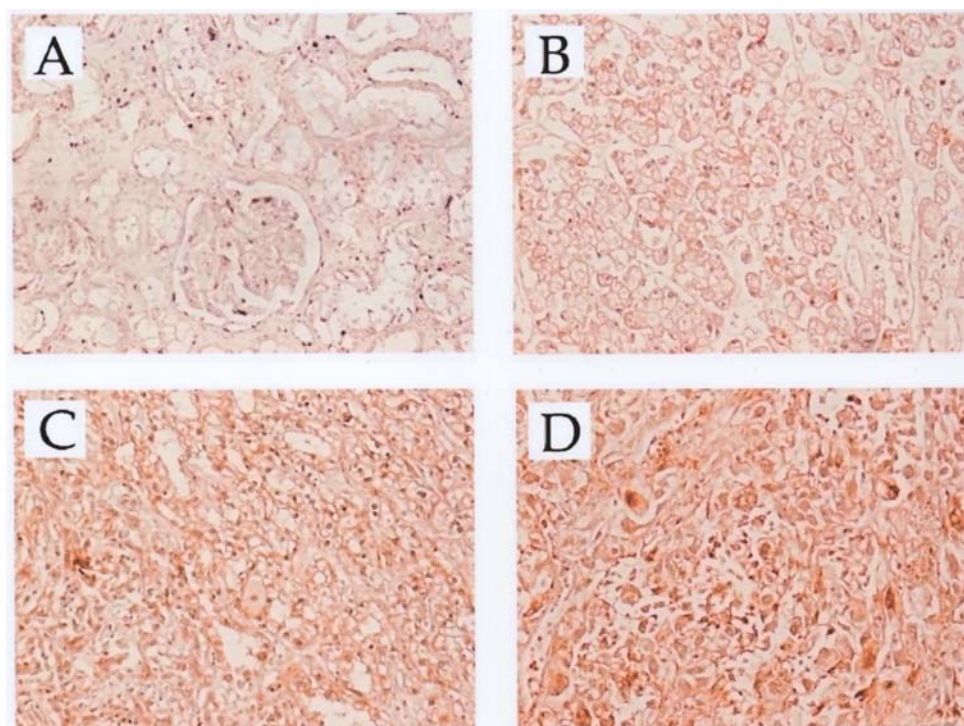


Figure 1. Representative CysLT₁R expression in kidney tissues. Very weak CysLT₁R expression was found in NK cases (A). In contrast, significantly strong CysLT₁R expression was observed in cancer tissues, including the nuclei and cytoplasm in all RCC groups: G1 (B), G2 (C), and G3 (D).

4 μ m. Concerning pathological grade, there were 20 G1 cases (average age 61.3 ± 10.4), 23 G2 cases (average age 62.0 ± 9.9) and 15 G3 cases (average age 59.0 ± 13.9).

Immunohistochemistry and patient samples. RCC and NK tissue specimens were preserved in 10% formalin, embedded in paraffin and serially sectioned onto microscope slides at a thickness of 4 μ m, then deparaffinized. Immunohistochemical staining was performed with the VectaStain avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA) as previously described (9). Primary antibodies against goat CysLT₁R (Abcam, Cambridge, UK) were diluted 1:50 with 1% bovine serum albumin in phosphate-buffered saline (PBS), and allowed to react with the sample for 1 h at room temperature. Similar staining with non-immune goat serum was performed as a negative control.

Immunohistochemical analysis. For each tissue specimen, the extent and intensity of staining with anti-CysLT₁R antibody were graded on a scale of 0-4 by two observers in a blinded manner on two separate occasions using coded slides, and an average score was calculated. Staining was classified into five grades from 0 to 4 according to the intensity of staining and the number of positive cells (9). Observers assessed all tissues to assign the score. Grade 4 indicated that all staining was maximally intense throughout the specimen, while grade 0 indicated that staining was absent throughout the specimen. The microanatomical sites of staining were also recorded. In order to quantify the expression of CysLT₁R, the same two pathologists made assessments throughout the study, staining control specimens simultaneously so as to increase the credibility of data. In addition, all specimens were re-assessed, which also contributed to excluding subjective variability.

Reagents and materials. RPMI-1640 was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) and a penicillin-streptomycin mixture were from Biowhitteker (Walkersville, MD, USA). Trypsin/EDTA was from Gibco-BRL (Rockville, MD, USA). Montelukast is a selective CysLT₁R antagonist and was obtained from LKT Laboratories Inc. (MN, USA).

Cell cultures. The human RCC cell line Caki-1 and normal proximal tubular endothelial cells (PRTEC) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were grown in a culture flask (Nunc, Roskilde, Denmark) in RPMI-1640 supplemented with 10% FBS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin in a humidified 5% CO₂ atmosphere at 37°C. The media were changed every 3 days, and cells were separated via trypsinization using Trypsin/EDTA when they reached subconfluence.

Cell-proliferative studies. Approximately 1.0×10^4 cells placed on 8x8-mm diameter multichamber slides (Nunc, Copenhagen, Denmark) were treated with the CysLT₁R antagonist dissolved in ethanol. The final concentration of ethanol was <0.05%. Cell viability was measured at day 1 by a microplate reader using a modified 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (WST-1 assay; Dojindo, Kumamoto, Japan) and presented as the percentage of control culture conditions.

Flow cytometry

Annexin V and propidium iodide staining. The effect of CysLT₁R antagonist on RCC cells was determined by dual staining with Annexin V-FITC and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit I (Biosciences

	Epithelium	Blood vessels
Renal cell carcinoma (RCC)		
Grade 1	1.9±0.7 ^a	1.5±0.5
Grade 2	2.4±1.0 ^a	1.6±0.6
Grade 3	2.6±0.7 ^a	1.4±0.5
Normal kidney (NK)	1.3±0.6	1.4±0.6

A grade based on a scale of 0-4 was assigned to the coded sections by two observers in a blinded manner: 0, no staining; 4, maximum intensity. Statistical analysis was performed using ANOVA (p-value). CysLT₁R expression was significantly more extensive and intense in RCC than in NK tissues in the epithelium only. However, CysLT₁R expression showed no significant differences in blood vessels between RCC and NK tissues. *P<0.05.

Table II. Comparison of CysLT₁R expression in every grade of renal cell carcinoma (RCC) epithelium.

RCC	p-value (ANOVA)		
	Grade 1	Grade 2	Grade 3
Grade 1		0.0235 ^a	0.005 ^a
Grade 2	0.0235 ^a		NS
Grade 3	0.0050 ^a	NS	

Grade 0-4 on coded sections by two observers in a blinded manner. Statistical analysis was performed using the analysis of variance. NS, not significant. ^ap≥0.05.

Pharmingen). Annexin V-FITC and PI were added to the cellular suspension as per the manufacturer's instructions, and a sample fluorescence of 10,000 cells was analyzed by flow cytometry conducted with FACScan (Becton Dickinson, Germany). Cells which were Annexin V-FITC-positive and PI-negative were identified as early apoptotic. Cells which were Annexin V-FITC-positive and PI-positive were identified as late apoptotic or necrotic.

Identification of DNA fragmentation. The assay was performed using the TdT-mediated dUTP Nick End Labelling (TUNEL) method using the Apo-Direct™ Kit (Becton Dickinson). Following the experiments, RCC cells in suspension (1×10⁶/ml) were fixed with 1% PBS, washed in PBS and suspended in 70% (v/v) ice-cold ethanol. The cells were stored in ethanol at -20°C until use. The positive and negative controls and the sample were stained with FITC-dUTP by incubation in terminal deoxynucleotidyl transferase buffer as per the manufacturer's instructions, and the sample fluorescence of 10,000 cells was analyzed by flow cytometry (Becton Dickinson). Results were expressed as the percentage (%) of TUNEL-positive cells.

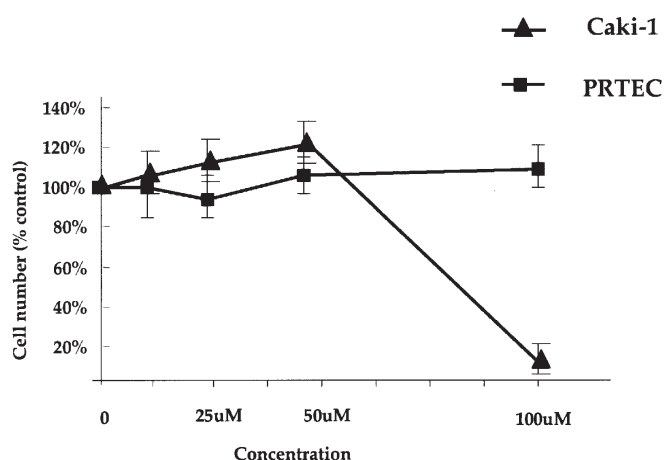


Figure 2. Effects of the CysLT₁R antagonist in a concentration-dependent manner. The CysLT₁R antagonist induced the reduction of cell viability with half-maximal concentration of growth inhibition of RCC cells in the 12.5-100 μM range. The CysLT₁R antagonist halted the growth of RCC cells.

Statistical analysis. All results were presented as the mean ± SD. Analysis of data was performed using the analysis of variance (ANOVA) (10).

Results

CysLT₁R expression of kidney tissues. To examine the expression and localization of CysLT₁R in RCC and NK tissues, immunohistochemistry was performed. Very weak CysLT₁R expression was found in the NK cases (Fig. 1A). In contrast, significantly strong CysLT₁R expression was observed in cancer tissues, including the nuclei and cytoplasm, in all RCC groups (Fig. 1B, G1; C, G2; and D, G3). Immunostaining with PBS was negative in all subjects (data not shown).

Statistical analysis of CysLT₁R expression. The extent and intensity of staining of RCC tissues using the CysLT₁R antibody were graded on a scale of 0-4 by two observers in a blinded manner. In the epithelium, CysLT₁R expression was significantly more extensive and intense in all the RCC groups (G1, 1.9±0.7; G2, 2.4±1.0 and G3, 2.6±0.7) than in the NK tissues (1.3±0.6). However, CysLT₁R expression showed no significant differences in blood vessels between RCC and NK tissues (Table I). Furthermore, CysLT₁R expression was higher in grade 2 and 3 than in grade 1 cancers. There were no significant differences between grade 2 and 3 (Table II).

CysLT₁R antagonist-induced growth inhibition in RCC cells by MTT assay. To investigate the effects of the CysLT₁R antagonist on RCC cell proliferation, we analyzed cell viability *in vitro* by modified MTT assay. As shown in Fig. 2, although the CysLT₁R antagonist had no effect on PRTEC proliferation, it induced a reduction in cell viability with the half-maximal concentration of growth inhibition of RCC cells (Fig. 2) in the range of 12.5-100 μM, and halted the growth of RCC cells.

CysLT₁R antagonist-induced apoptosis shown by flow cytometry. To evaluate whether or not the cell death induced by the CysLT₁R antagonist was achieved through apoptosis,

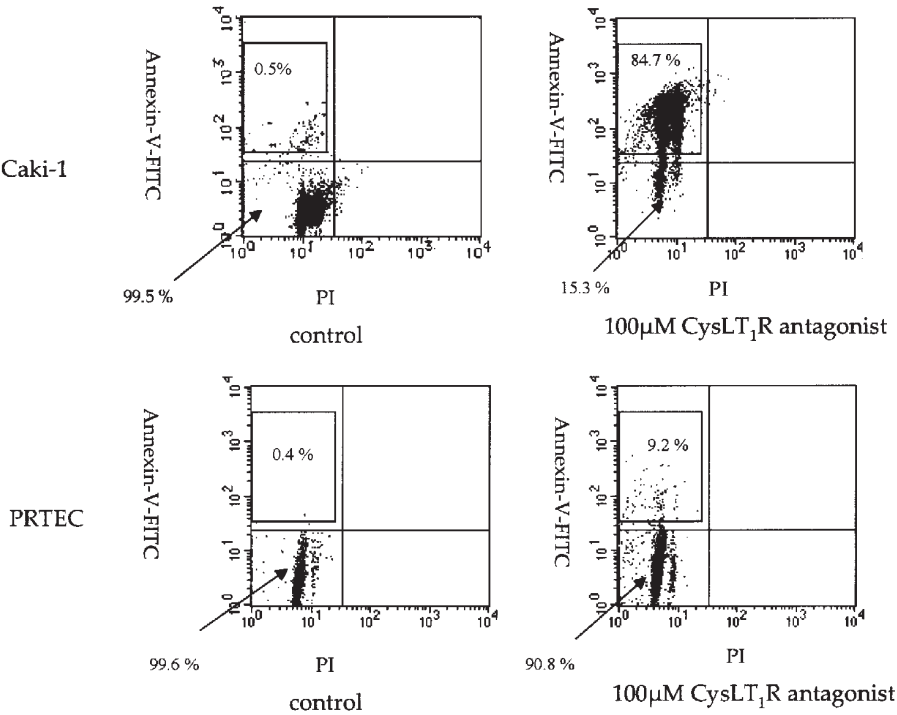


Figure 3. Effects of the CysLT₁R antagonist on early and late apoptosis as shown by flow cytometry. Treatment with 100 µM CysLT₁R antagonist induced early apoptosis in almost the total percentage of RCC cells. However, treatment with 100 µM CysLT₁R antagonist did not induce apoptosis in PRTEC. The top left quadrants represent early apoptosis (Annexin V-FITC-positive cells and PI-negative cells). The top right quadrants represent late necrosis and necrosis (Annexin V-FITC-positive cells and PI-positive cells). Diagrams of FITC-Annexin V/PI flow cytometry in a representative experiment are presented.

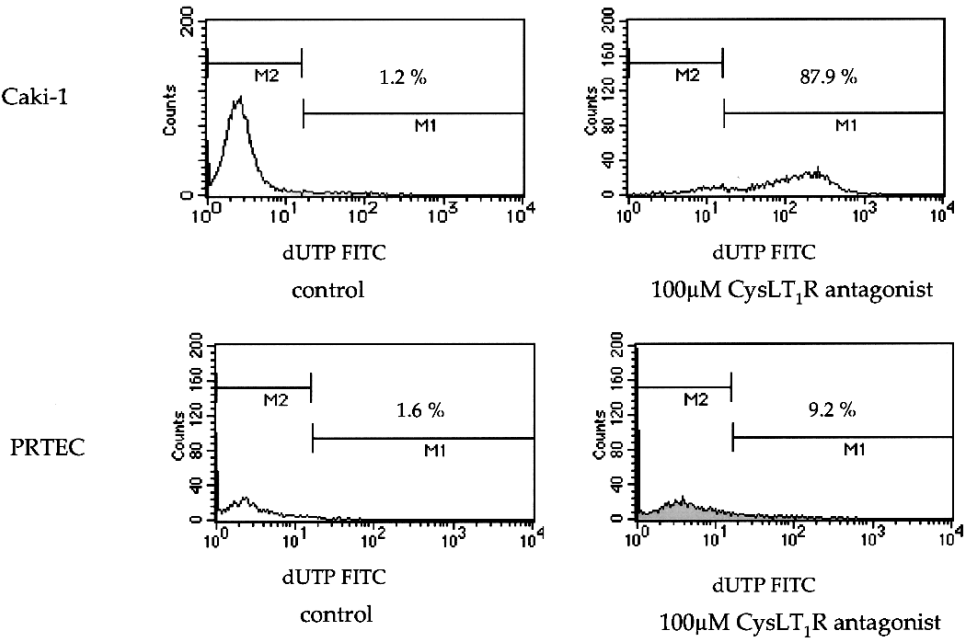


Figure 4. CysLT₁R antagonist induced DNA fragmentation in human RCC cells. The CysLT₁R (100 µM) antagonist induced DNA fragmentation in RCC cells. However, 100 µM CysLT₁R antagonist did not induce DNA fragmentation in PRTEC. Typical flow cytometry analysis histograms in a representative experiment are presented.

we used flow cytometry. As shown in Fig. 3, the top left quadrants represent early apoptosis (Annexin V-FITC-positive cells and PI-negative cells). The top right quadrants represent late necrosis and necrosis (Annexin V-FITC-positive cells and PI-positive cells). Treatment with 100 µM CysLT₁R antagonist induced early apoptosis in almost the

total percentage of RCC cells. However, treatment with 100 µM CysLT₁R antagonist did not induce apoptosis in PRTEC. Furthermore, 100 µM CysLT₁R antagonist induced DNA fragmentation in RCC cells. However, 100 µM CysLT₁R antagonist did not induce DNA fragmentation in PRTEC (Fig. 4).



Leukotrienes (LTs) are biologically-active fatty acids derived from the oxidative metabolism of arachidonic acid (AA) (11,12) via the 5-LOX pathway. The activity of 5-LOX leads to the formation of unstable LTA₄, which can be converted into either LTB₄ or CysLTs (LTC₄, LTD₄ and LTE₄). CysLTs are components of a slow-reacting substance of anaphylaxis. LTD₄ plays the most important role in CysLTs (CysLT₁, CysLT₂) and CysLT₁ is specific for LTD₄. LTs are potent biochemical mediators that are released from mast cells, eosinophils and basophils. LTs work to contract airway smooth muscle, increase vascular permeability, increase mucus secretions and attract and activate inflammatory cells in the airways of patients with asthma (13). The action of LTs can be blocked through either of two specific mechanisms, i) the inhibition of LT production, and ii) the antagonism of LT binding to cellular receptors.

On the other hand, the 5-LOX inhibitor inhibits LT formation, especially LTB₄, LTC₄, LTD₄ and LTE₄. Our previous studies demonstrated that 5-LOX is overexpressed in urological cancers, and the 5-LOX inhibitor may attenuate the growth of human urological cancers and induce apoptosis through the AA pathway (2,6-8,14). With this as our basis, we examined whether or not CysLT₁R would be expressed in human RCC tissues, and whether or not it would prevent RCC cell growth as CysLT₁R antagonist.

In our study, we found CysLT₁R to be expressed strongly in RCC tissues. The extent and intensity of CysLT₁R expression in RCC tissues were greater than in NK tissues. Furthermore, CysLT₁R expression was higher in high-grade than in low-grade cancer. Next, we demonstrated by MTT assay that the co-incubation of RCC cells with the CysLT₁R antagonist halted the growth of RCC cells and potently inhibited cell growth in a dose-dependent manner. These results indicate that CysLT₁R is essential for the cell growth of RCC cells.

The mechanism by which the CysLT₁R antagonist induces growth prevention in RCC cells needed clarification. To address this issue, we examined whether or not apoptosis was involved in growth suppression in such cancer cells. The CysLT₁R antagonist (100 μ M) strongly induced early apoptosis in RCC cells, as shown by flow cytometry. Our results demonstrated that apoptosis may be involved by the CysLT₁R antagonist in mechanisms to prevent cell growth in RCC cells. Thus, our research provides the first confirmation that, through apoptosis, the CysLT₁R antagonist inhibits RCC cell growth.

Regarding CysLT₁R and colon cancer, Ohd *et al* reported that CysLT₁R was overexpressed in human colorectal cancer and was significantly correlated to COX-2 and 5-LOX (4). The expression of CysLT₁R was higher in high-grade and early-stage carcinoma, suggesting typical differences in every cancer. Furthermore, survival time was slightly shorter in patients with high-intensity CysLT₁R staining than in those with low-intensity staining. We also demonstrated that CysLT₁R was overexpressed in human prostate cancer, and that the expression of CysLT₁R was higher in high-grade than in low-grade cancer. Furthermore, the CysLT₁R antagonist inhibited prostate cancer cell growth through apoptosis (15).

This result indicates a relationship between patient survival and the expression of CysLT₁R. In addition, these reports support findings which suggest that the CysLT₁R antagonist can prevent cell growth in other types of cancer.

In summary, our study provides evidence that the cell growth and apoptosis of human RCC cells are regulated by CysLT₁R. The growth inhibition of RCC cells by blocking CysLT₁R was associated with induction of apoptosis. Thus, the CysLT₁R antagonist provides a novel approach to anticancer therapies.

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