

Overexpression of cysteinyl LT₁ receptor in prostate cancer and CysLT₁R antagonist inhibits prostate cancer cell growth through apoptosis

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Abstract. The metabolism of arachidonic acid by either cyclooxygenase or lipoxygenase is believed to play an important role in carcinogenesis. Leukotriene (LT) D₄ is a proinflammatory mediator derived from arachidonic acid through various enzymatic steps, and 5-lipoxygenase is an important factor in generating LTD₄. We investigated LTD₄ receptor (cysteinyl LT₁ receptor: CysLT₁R) expression in prostate cancer (PC), as well as the effects of CysLT₁R antagonist on cell proliferation in PC cell lines. CysLT₁R expression in PC patients, prostatic intraepithelial neoplasia (PIN), benign prostatic hyperplasia (BPH), and normal prostate (NP) tissues were examined. CysLT₁R expression was detected by immunohistochemistry. Effects of CysLT₁R antagonist on PC cell growth were examined by MTT assay. Flow cytometry and Hoechst staining were used to determine whether or not the CysLT₁R antagonist induces apoptosis. Initially, only slight CysLT₁R expression was detected in BPH and NP tissues and marked CysLT₁R expression was detected in PIN and PC tissues. CysLT₁R expression was higher in high-grade cancer than in low-grade cancer. Furthermore, CysLT₁R antagonist caused marked inhibition of PC cells in a concentration- and time-dependent manner through early apoptosis. In conclusion, CysLT₁R is induced in PC, and the results suggest that CysLT₁R antagonist may mediate potent anti-proliferative effects of PC cells. Thus, the target of CysLT₁R may become a new therapy in the treatment of PC.

Introduction

Prostate cancer (PC) in American men statistically comprises 32% of all cancers, including lung and colon cancers, and is increasing world-wide. Prostate cancer differs from other urinary tract tumors due to hormone dependency. With respect to the metastasis of cancer cells, angiogenetic factors play an important role in this organ as in others (1). Although various potential angiogenetic factors have been identified in PC, it is still unclear by which process PC cells become angiogenic.

The metabolism of arachidonic acid (AA) by either the cyclooxygenase (COX) pathway 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 PC, we demonstrated that COX-2 and 5-LOX were overexpressed in PC tissues (2,3). Leukotriene (LT) belongs to an important group of proinflammatory mediators, and LT 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 CysLTs (CysLT₁, CysLT₂) and CysLT₁ receptor (CysLT₁R) is specific for LTD₄ (4). Generally, CysLTs are important mediators of human bronchial asthma and many CysLTs receptor antagonists are clinically used in treating human bronchial asthma. A few studies have addressed the relationship between CysLT₁R and colon cancer (5,6). To date, no study has addressed the relationship between CysLT₁R and human PC. Our team has already demonstrated that 5-LOX is overexpressed in human urological cancer (3,7-9).

With this background, the purpose of our study was to examine the expression of CysLT₁R in human PC tissues and PC cell lines, to evaluate the inhibitory effect of CysLT₁R antagonist on human PC cell lines, and to determine whether

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or not the CysLT₁R antagonist induces apoptosis of such PC cells.

Materials and methods

Tumor specimens. Tumor specimens were obtained from 151 patients with PC, 20 with prostatic intraepithelial neoplasia (PIN), 20 with benign prostatic hyperplasia (BPH), and 20 patients with normal prostate tissues (NP) who underwent total cystectomy due to bladder cancer. The 151 patients were aged 53-86 years (mean age 69±7.5 years). The tumor histopathologies were classified using Gleason score as follows: low group, ≤5; middle group, 6 and 7; high group, ≥8. There were 49 patients in the low group, 57 in the middle group, and 45 in the high group. The age of the 20 patients with PIN averaged 66±6.1 (52-73) years, and that of the 20 patients with BPH averaged 67±5.6 (59-76) years, and all had nodular hyperplasia. The age of the 20 patients with NP averaged 67±1.1 (54-79) years (Table I). Their prostate volumes were normal size (volume: <25 cm³). Tumor tissues, non-tumor tissues, vascular endothelium, and interstitial tissues from the subjects were immunostained with antibodies for CysLT₁R.

Immunohistochemistry and patient samples. PC, PIN, BPH and NP tissue specimens were preserved in 10% formalin and embedded in paraffin, serially sectioned onto microscope slides at a thickness of 4 μm, and then deparaffinized. Immunohistochemical staining was performed with the VectaStain avidin-biotin peroxidase complex kit (Vector Laboratories; Burlingame, CA, USA) as previously described (10). Primary antibodies against goat CysLT₁R (Abcam, Cambridge, UK) were diluted 1:50 with 1% bovine serum albumin in phosphate-buffered saline (PBS), and left 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 was 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 0-4 according to the intensity of staining and the number of positive cells (10). Observers assessed all tissues to assign the score. Scale 4 implied all staining was maximally intense throughout the specimen, while scale 0 implied staining was absent throughout the specimen. The micro-anatomical sites of staining were also recorded. In order to quantify the expressions 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 contributes to excluding subjective variability.

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

Table I. Summary of the various tumor specimens.

Histopathology	No. pts.	Median age (range)
Prostate cancer	151	69±7.5 (53-86)
Low group (Gleason score, ≤5)	49	68±7.5 (55-84)
Middle group (Gleason score, 6)	57	69±8.2 (53-86)
High group (Gleason score, ≥8)	45	67±5.7 (55-76)
Prostatic intraepithelial neoplasia	20	66±6.1 (52-73)
Benign prostatic hyperplasia	20	67±5.6 (59-76)
Normal prostate tissue	20	67±1.1 (54-71)

The tumor histopathologies were diagnosed by pathologists at the Department of Pathology.

Cell cultures. The human PC cell lines LNCaP, PC-3, DU-145 and normal stromal prostate cell lines (NPCs) were obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were grown in culture flask (Nunc, Roskilde, Denmark) in RPMI-1640 supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in a humidified 5% CO₂ atmosphere at 37°C. The media were changed every 3 days, and the cells were separated via trypsinization using trypsin/EDTA when they reached subconfluence.

Cell-proliferative studies. Cells (~1.0x10⁴) placed onto 8x8 mm diameter multichamber slides (Nunc, Copenhagen, Denmark) were treated with 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 effects of CysLT₁R antagonist on PC cells were determined by dual staining with Annexin V-FITC and propidium iodide using Annexin V-FITC apoptosis detection kit I (Biosciences Pharmingen). Annexin V-FITC and propidium iodide (PI) were added to the cellular suspension as in the manufacturer's instructions, and sample fluorescence of 10000 cells was analyzed by flow cytometry, FACScan (Becton Dickinson, Germany). Cells which were Annexin V-FITC-positive and PI-negative were identified as early apoptosis. Cells which were Annexin V-FITC-positive and PI-positive were identified as late apoptosis or necrosis.

Identification of DNA fragmentation. The assay was performed by the TdT-mediated dUTP nick-end labelling (TUNEL) method using APO-Direct™ kit (Becton Dickinson).

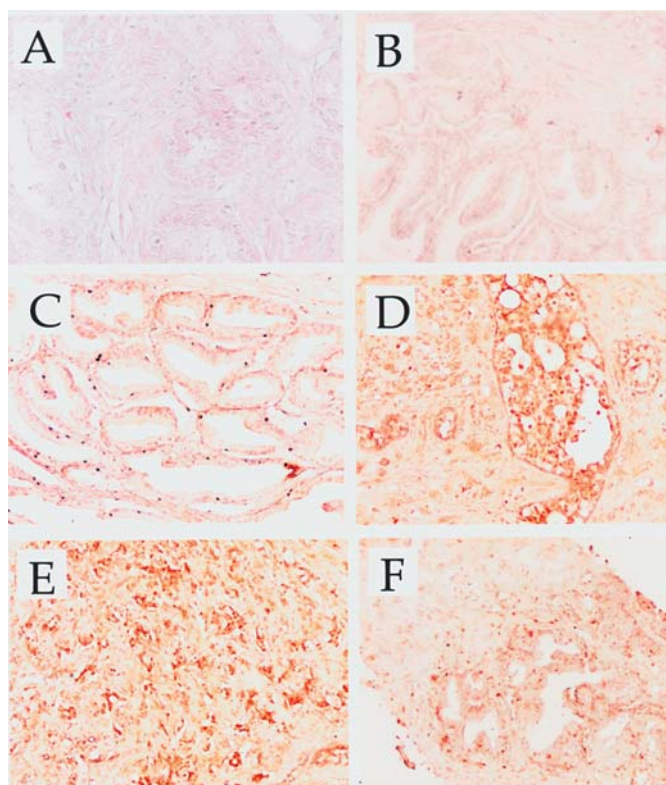


Figure 1. Representative CysLT₁R expression in prostate tissues. Very weak CysLT₁R expression was found in BPH (B) and NP cases (A). In contrast, significantly strong CysLT₁R expression was observed in cancer tissues including nuclei and cytoplasm in all PC groups (C, Low group; D, Middle group; E, High group) and PIN group (F).

Following the experiments, PC cells in suspension ($1 \times 10^6/\text{ml}$) 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 in the manufacturer's instructions, and sample fluorescence of 10000 cells was analyzed by flow cytometry (Becton Dickinson). Results are given as % of TUNEL-positive cells.

Detection of apoptosis. DNA chromatin morphology was assessed using Hoechst staining. PC cells were incubated with $100 \mu\text{M}$ CysLT₁R antagonist for 24 h. Cells were washed by RPMI-1640 and labeled with 8 mg/ml of Hoechst 33342 (Sigma-Aldrich Japan K.K., Tokyo, Japan) for 10 min; PI (Sigma-Aldrich Japan K.K.) was added (10 mg/ml final concentration), and the cells were examined by fluorescence microscopy.

Statistical analysis. All results are presented as the mean \pm SD. Analysis of data was performed using the analysis of variance (ANOVA) (11).

Results

CysLT₁R expression of prostate tissues. To examine the expression and localization of CysLT₁R in NP, BPH, PIN and PC tissues (Fig. 1B) immunohistochemistry was performed.

Table II. Statistical analysis of CysLT₁R expression.

	Epithelium	Blood vessel	Stromal tissue
PC	2.6 ± 1.0^a	1.3 ± 0.5	1.2 ± 0.5
Low group	2.1 ± 0.8^a	1.2 ± 0.3	1.0 ± 0.2
Middle group	2.7 ± 0.9^a	1.2 ± 0.5	1.1 ± 0.5
High group	3.2 ± 0.8^a	1.4 ± 0.6	1.4 ± 0.6
PIN	1.8 ± 0.9^a	1.2 ± 0.3	1.1 ± 0.3
BPH	1.3 ± 0.5	1.3 ± 0.4	1.2 ± 0.4
NP	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.1

Scaled 0-4 on the coded sections by two observers in a blinded manner. 0, no staining; 4, maximum intensity. Statistical analysis was performed using the ANOVA (p-value). Only epithelium, CysLT₁R expression was significantly more extensive and intense in PC and PIN tissue than BPH and NP tissues. However, CysLT₁R expression in blood vessels and stromal tissues were at the basic level in PC, PIN, BPH and NP tissues ($^aP < 0.01$).

Table III. Comparison of CysLT₁R expression in each grade of PC.

PC Epithelium	P-value (ANOVA)		
	Low group	Middle group	High group
Low group		0.003	<0.001
Middle group	0.003		0.004
High group	<0.001	0.004	

Very weak CysLT₁R expression was found in BPH and NP cases (Fig. 1A). In contrast, significantly strong CysLT₁R expression was observed in cancer tissues including nuclei and cytoplasm in all PC groups (Fig. 1C; Low group, 1D; Middle group, 1E; High group) and PIN group (Fig. 1F). Immunostaining with PBS was negative in all subjects (data not shown).

Statistical analysis of CysLT₁R expression. The extent and intensity of staining of PC tissues using CysLT₁R antibody were scaled 0-4 by two observers in a blinded manner. CysLT₁R expression was significantly more extensive and intense in all PC groups (All groups, 2.6 ± 1.0 ; Low group, 2.1 ± 0.8 ; Middle group, 2.7 ± 0.9 ; High group, 3.2 ± 0.8) and PIN tissues (1.8 ± 0.9) than in tissues of BPH (1.3 ± 0.5) and NP tissues (1.2 ± 0.1) only the epithelium (Table II). CysLT₁R expression was higher in High group cancer than Low group cancer (Table III). However, CysLT₁R expression in blood vessels and stromal tissues were at basic levels in PC, PIN, BPH and NP tissues (Table II).

CysLT₁R antagonist-induced growth inhibition in PC cells by MTT assay. To investigate the effects of CysLT₁R antagonist

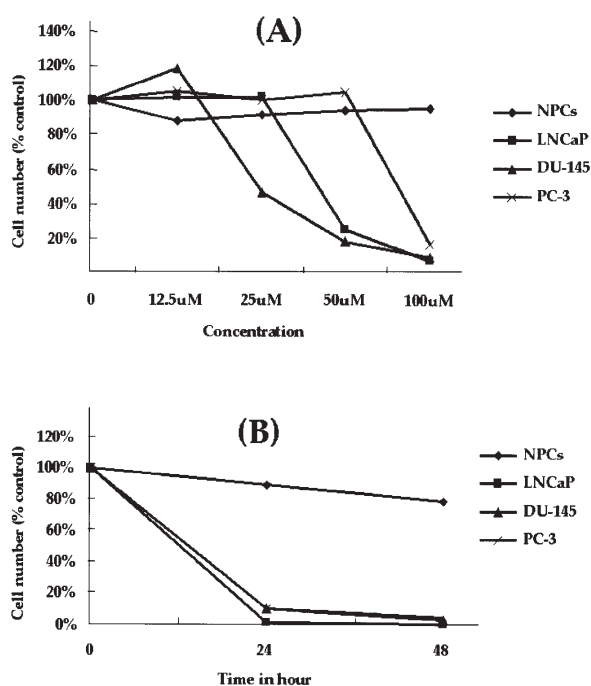


Figure 2. Effects of CysLT₁R antagonist in a concentration-dependent manner. CysLT₁R antagonist induced the reduction of cell viability with half-maximal concentration of growth inhibition of PC cell lines in the range of 12.5-100 μM (A). Cell count at days 1 and 2 clearly showed marked inhibition of cell proliferation using 100 μM of CysLT₁R antagonist. CysLT₁R antagonist stopped the growth of PC cells (B).

on PC cell proliferation, we analyzed cell viability *in vitro* by modified MTT assay. As shown in Fig. 2, although CysLT₁R antagonist had no effect on NPC proliferation, CysLT₁R antagonist induced the reduction of cell viability with the half-maximal concentration of growth inhibition of all PC cell lines (LNCaP, PC-3, DU-145) (Fig. 2A) in the range of 12.5-100 μM. Furthermore, counting cells at days 1 and 2 clearly showed marked inhibition of cell proliferation using 100 μM of CysLT₁R antagonist (Fig. 2B). CysLT₁R antagonist stopped the growth of all PC cells.

CysLT₁R antagonist-induced apoptosis by flow cytometry. To evaluate whether or not cell death induced by CysLT₁R antagonist was through apoptosis, we used flow cytometry. All PC cell lines and NPCs without CysLT₁R antagonist are shown in Fig. 3. Almost all PC cells (LNCaP, PC-3, DU-145) with treatment of 100 μM CysLT₁R antagonist induced early apoptosis. However, NPCs with treatment of 100 μM CysLT₁R antagonist could not induce apoptosis (Fig. 3).

Furthermore, 100 μM CysLT₁R antagonist induced DNA fragmentation in all PC cells. However, 100 μM of CysLT₁R antagonist could not induce DNA fragmentation in NPCs (Fig. 4).

Effect of CysLT₁R antagonist in induction of apoptosis on human PC cells. To evaluate whether or not cell death induced by CysLT₁R antagonist was through apoptosis, we evaluated the chromatin morphology of PC cells using Hoechst 33342 staining. Cells treated with CysLT₁R antagonist showed significant chromatin condensation, cellular shrinkage, small

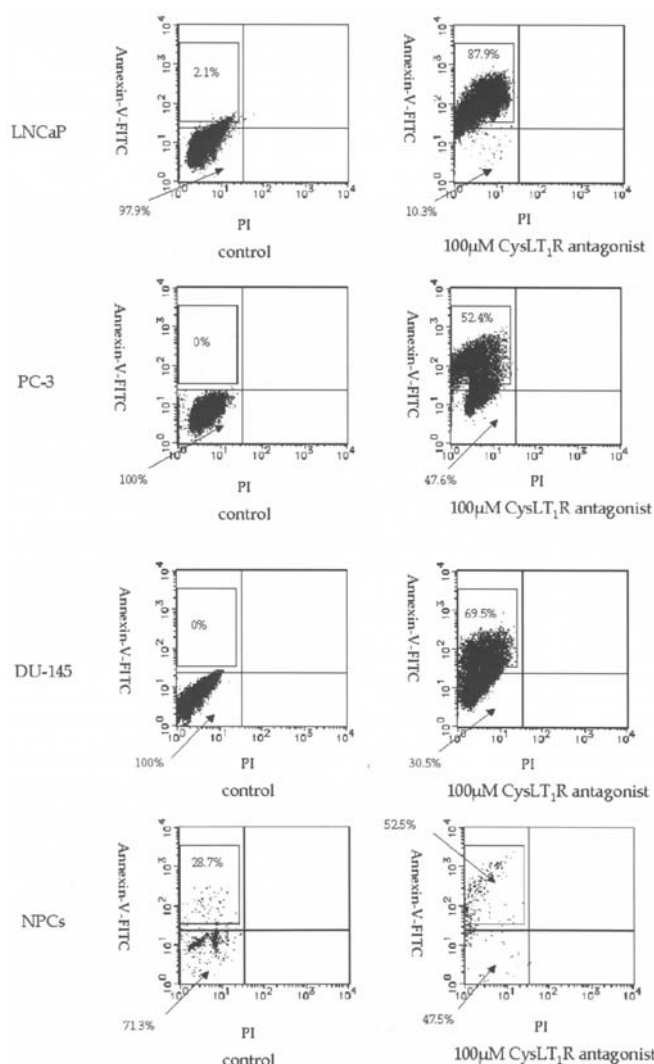


Figure 3. Effects of CysLT₁R antagonist on early and late apoptosis by flow cytometry. Almost all PC cells (LNCaP, PC-3, DU-145) with treatment of 100 μM CysLT₁R antagonist induced early apoptosis. However, NPCs with treatment of 100 μM CysLT₁R antagonist could not induce apoptosis. The higher left quadrants represent early apoptosis (Annexin V-FITC-positive cells and PI-negative cells). The higher 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.

membrane-bound bodies (apoptotic bodies), and cytoplasmic condensation. These cellular changes are typical characteristics of apoptosis (Fig. 5B, LNCaP; 5D, PC-3; 5F, DU-145). All PC cell lines without CysLT₁R antagonist maintained normal chromatin patterns and cell size (Fig. 5A, LNCaP; 5C, PC-3; 5E, DU-145).

Discussion

LTs are biologically active fatty acids derived from the oxidative metabolism of AA (12,13), and they are derived from AA via 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.

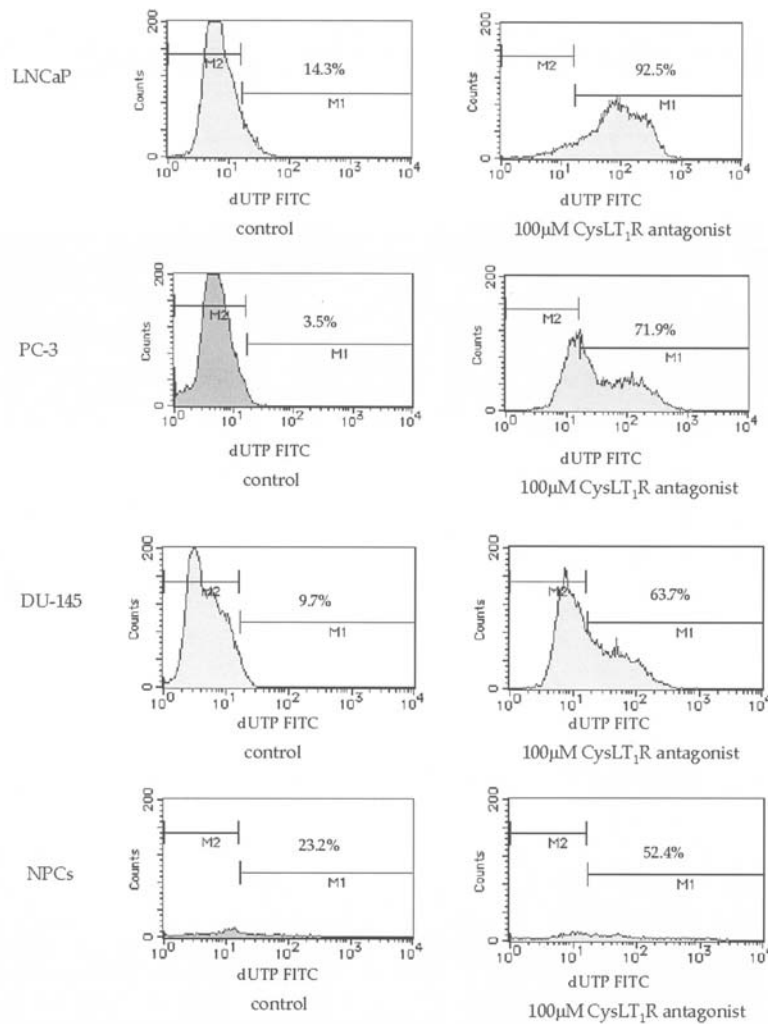
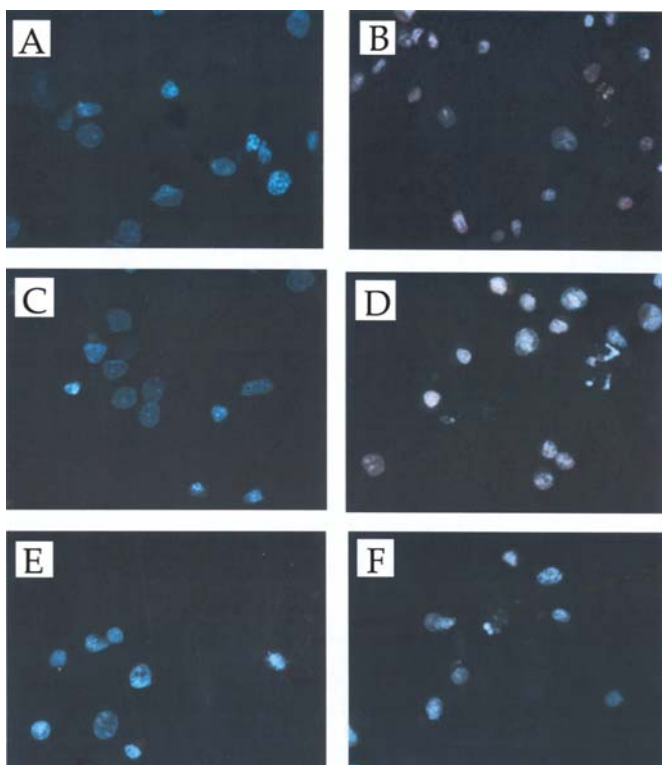


Figure 4. CysLT₁R antagonist induces DNA fragmentation in PC cells. CysLT₁R antagonist (100 μM) induced DNA fragmentation in all PC cells. However, 100 μM of CysLT₁R antagonist could not induce DNA fragmentation in NPCs. Typical flow cytometry analysis histograms in a representative experiment are presented.



LTD₄ plays the most important role of 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 (14). The action of LTs can be blocked through either of two specific mechanisms, 1) inhibition of LT production and 2) antagonism of LT binding to cellular receptors.

On the other hand, 5-LOX inhibitor inhibits LT formation (especially LTB₄, LTC₄, LTD₄ and LTE₄). Our previous studies demonstrated that 5-LOX is overexpressed in urological

Figure 5. Effects of CysLT₁R antagonist in induction of apoptosis on human PC cells. Cells treated with CysLT₁R antagonist showed significant chromatin condensation, cellular shrinkage, small membrane-bound bodies (apoptotic bodies), and cytoplasmic condensation. These cellular changes are typical characteristics of apoptosis (B, LNCaP; D, PC-3; F, DU-145). All PC cell lines without CysLT₁R antagonist maintained normal chromatin patterns and cell size (A, LNCaP; C, PC-3; E, DU-145).

cancers and 5-LOX inhibitor may attenuate growth of human urological cancers and induced apoptosis through AA pathway (3,7-9,15).

With this background, we examined whether or not CysLT₁R would be expressed in human PC tissues, and whether or not it would prevent PC cell growth as CysLT₁R antagonist.

We found CysLT₁R strongly expressed in PC tissues. The extent and intensity of CysLT₁R expression in PC tissues were greater than in NP tissues. Furthermore, CysLT₁R expression was higher in high group cancer than low group cancer. Next, we demonstrated that co-incubation of PC cells with CysLT₁R antagonist stopped the growth of PC cells and potently inhibited cell growth in a dose- and time-dependent manner by MTT assay. These results indicate that CysLT₁R is essential for cell growth of PC cells. The mechanism by which CysLT₁R antagonist induces growth prevention in PC cells needed clarification. To address this problem, we examined whether or not apoptosis was involved in growth suppression in such cancer cells. CysLT₁R antagonist (100 μ M) strongly induced early apoptosis in all PC cells by flow cytometry and Hoechst staining. Our results demonstrated that apoptosis may be involved in the mechanisms by CysLT₁R antagonist to prevent cell growth in PC cells. Thus, our research provides the first confirmation that through apoptosis CysLT₁R antagonist inhibits prostate cancer cell growth.

Regarding CysLT₁R and colon cancer, Ohd *et al* have reported that CysLT₁R is overexpressed in human colorectal cancer and CysLT₁R is significantly correlated to COX-2, 5-LOX (5). The expression of CysLT₁R is higher in high-grade and early-stage carcinoma, suggesting typical differences in each cancer. Survival time is slightly shorter in patients with high-intensity CysLT₁R staining than in those with low-intensity staining. This result indicates a relationship between patient survival and the expression of CysLT₁R. These studies support the data that CysLT₁R antagonist can prevent cancer cell growth.

In summary, our study provides evidence that cell growth and apoptosis of human PC cells are regulated by CysLT₁R. Growth inhibition of PC cells by blocking CysLT₁R was associated with induction of apoptosis. Thus, CysLT₁R antagonist provides a novel approach to anti-cancer therapies.

Acknowledgements

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