Cysteinyl-leukotriene$_1$ receptor is a potent target for the prevention and treatment of human urological cancer

MASAHIDE MATSUYAMA and RIKIO YOSHIMURA

Department of Urology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan

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Abstract. Leukotrienes (LTs) are biologically active fatty acids derived from the oxidative metabolism of arachidonic acid through the 5-lipoxygenase pathway. LTs work to contract airway smooth muscle, increase vascular permeability and mucus secretions, and attract and activate inflammatory cells in the airways of patients with asthma. Recently, it was reported that the LTD_4 receptor (cysteinylLT_1 receptor; CysLT_1R) plays an important role in carcinogenesis. In this study, CysLT_1R expression was examined in human urological cancer cell lines (renal cell carcinoma, bladder cancer, prostate cancer and testicular cancer) using immunohistochemistry and RT-PCR. The effect of CysLT_1R antagonist on these cells was also examined using the MTT assay, Hoechst staining and flow cytometry. CysLT_1R expression was significantly more extensive and intense in the malignant tissues than in normal tissues. Furthermore, CysLT_1R antagonist induced a reduction in malignant cell viability through early apoptosis. These results demonstrate that CysLT_1R expressed in urological cancer may play a crucial role in carcinogenesis. CysLT_1R may therefore be a novel target in the treatment of urological cancer.

Introduction

Urological cancer includes renal cell carcinoma (RCC), bladder cancer (BC), prostate cancer (PC) and testicular cancer (TC).

Recently, with more frequent routine medical check-ups and progress in diagnostic imaging techniques, there has been an increase in the early diagnosis of RCC. However, the cause of RCC remains unknown, and it generally does not respond well to radiotherapy and chemotherapy compared to many other types of cancer. Moreover, anti-cancer drugs such as interleukin-2 are used with only relative success, leaving surgery the only current therapeutic option. New molecular targets are therefore needed for the prevention and treatment of RCC.

The natural history of BC is not well understood, but exposure to carcinogens, including aromatic amines, is considered a major risk factor for its development. Workers exposed to aromatic amines frequently have mutated p53, a tumor-suppressor gene involved in the carcinogenesis of numerous types of cancer.

PC constitutes 32% of all cancer cases in American men, and is on the increase worldwide. Due to increased screening, PC is frequently diagnosed at a clinically localized stage, making it amenable to therapy. Nevertheless, it remains the second most common cause of cancer-related death in men. Patients generally respond to androgen-deprivation therapy, but the vast majority eventually experience disease progression and become refractory to sustained hormonal manipulation. Typically in such patients, progress is associated with a rise in serum prostate-specific antigen levels. Unfortunately, standard therapeutic options at thiws stage of disease progression are limited, and although chemotherapy for hormone-refractory PC patients has exhibited some success, the response is generally short-lived (1).

TC is very rare, with over 90% of cases being germ cell tumors (seminoma and non-seminoma), and the remaining percentage non-germinal tumors. The survival rate of TC patients has improved in recent years, reflecting the development and refinement of effective combination chemotherapy. However, improvements in the treatment of TC are still necessary.

Angiogenetic factors play an important role in urological and other types of cancer. In recent years, the expression of angiogenic factors in solid human cancer has been widely reported (2). Growth factors secreted by tumor cells such as fibroblast growth factor and transforming growth factor have been found to increase neovascularization *in vivo* and *in vitro* (3).

The metabolism of arachidonic acid (AA) by either the cyclooxygenase (COX) or lipoxygenase (LOX) pathway generates eicosanoids. These have been implicated in the pathogenesis of a variety of human diseases, including cancer, and are significantly involved in cancer promotion, progression and metastasis. Studying these pathways in specimens from patients with urological cancer, we demonstrated that COX-2 and 5-LOX were overexpressed in human urological cancer tissue (4-11).

Correspondence to: Dr Rikio Yoshimura, Department of Urology, Osaka City University Hospital, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
E-mail: jasmin@med.osaka-cu.ac.jp

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Leukotriene (LT) is a member of 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 LTA_4, which can be converted into either LTB_4 or cysteinyl(Cys)LTs (LTC_4, LTD_4 and LTE_4). LTD_4 is the most important component of CysLTs ($CysLT_1$ and $CysLT_2$), and the $CysLT_1$ receptor ($CysLT_1R$) is specific for LTD_4 (12). Generally, CysLTs are important mediators of human bronchial asthma, and many CysLT receptor antagonists are clinically used for the treatment of human bronchial asthma. A few reports have addressed the relationship between $CysLT_1R$ and colon cancer (13,14). To date, only reports published by our team have examined the relationship between $CysLT_1R$ and urological cancer (15-18).

Our research focuses on the relationship between $CysLT_1R$ and urological cancer, and on the anti-cancer effects of the $CysLT_1R$ antagonist.

Materials and methods

Tumor specimens. Tissue specimens were obtained from the Osaka City University Hospital. Cancer tissue, non-cancer tissue, vascular endothelium and interstitial tissue from the subjects were preserved in 10% formalin, embedded in paraffin and serially sectioned onto microscope slides at a thickness of 4 μm.

Renal cell carcinoma. RCC specimens were obtained from 58 patients with RCC, and were paired with normal kidney (NK) tissue from 20 patients who underwent total nephroureterectomy due to ureteral cancer.

Bladder cancer. BC specimens were obtained from 90 patients with BC (including 32 who underwent total cystectomy and 58 who underwent transureteral resection of bladder tumors), and were paired with normal bladder (NB) tissue from 30 patients who underwent total prostatectomy due to prostate cancer.

Prostate cancer. PC specimens were obtained from 151 patients with PC, 20 patients with benign prostatic hyperplasia (BPH) and 20 patients with prostatic intraepithelial neoplasia (PIN) who underwent total prostatectomy or subcapsular prostatectomy, and were paired with normal prostate (NP) tissue from 20 patients who underwent total cystectomy due to bladder cancer.

Testicular cancer. TC specimens were obtained from 30 patients with TC, and were paired with normal testis (NT) tissue from 10 patients who underwent orchiectomy for prostate cancer.

Immunohistochemical staining. Immunohistochemical staining was performed with the Vectastain avidin-biotin peroxidase complex kit (Vector Laboratories, CA, USA) as previously described (19). Primary antibodies against goat $CysLT_1R$ (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. Stained specimens were classified into 5 grades (0-4) according to staining intensity and the number of positive cells, assessed by two observers in a blinded manner on two separate occasions using coded slides. An average score was calculated. A grade of 4 indicated that all staining was maximally intense throughout the specimen, while 0 indicated that staining was absent throughout the specimen. Micro-anatomical staining sites were also recorded. This method was perfomed as previously described (19). Results are presented as the mean ± SD. Data analysis was performed using ANOVA (20).

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 (LKT Laboratories, MN, USA) is a selective and orally active $CysLT_1R$ antagonist with demonstrated effectiveness in treating allergic asthma and allergic rhinitis in adults and children as young as 12 months of age (allergic asthma) and 6 months of age (allergic rhinitis) (21).

Cell cultures. The human RCC cell line Caki-1 and normal prostate stromal cells (NPCs) were provided by Dr Shinichi Ikemoto (Department of Urology, Osaka City University School of Medicine, Osaka, Japan). The human BC cell line T24, PC cell lines LNCaP, PC3 and DU-145, TC cell line NEC-8 and normal proximal tubular endothelial cells (PRTECs) were obtained from the Health Science Research Resources Bank (Osaka, Japan).

Cells were grown in culture flasks (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% CO_2 atmosphere at 37˚C. The media were changed every 3 days, and the cells were separated via trypsinization using trypsin/EDTA upon reaching subconfluence.

Cell proliferative studies. Approximately $1.0x10^4$ cells placed on 8x8-mm diameter multichamber slides (Nunc, Copenhagen, Denmark) were treated with $CysLT_1R$ antagonist (12.5-100 μM) dissolved in ethanol. The final concentration of ethanol was <0.05%. Cell viability was measured on day 1 using a microplate reader with a modified 3-[4,5-dimethylthiazol-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) assay (WST-1 assay; Dojindo, Kumamoto, Japan). Results are presented as the percentage of control-culture conditions.

RT-PCR of $CysLT_1R$. Total RNA was isolated from the human urological cancer cell lines using the RNAqueous kit (Ambion, Japan) and examined for the presence of the $CysLT_1R$ gene using sense primer 5'-CTGCTCGCTTCGCTACTTGGA-3', and antisense primer 5'-CGGCACCTGTCCTACGAGTTG-3' (Takara RNA PCR™ kit; Takara Bio, Japan). The first step of PCR was carried out for 2 min at 94˚C. Subsequent steps involved 35 cycles as follows: 30 sec at 94˚C, 45 sec at 60˚C and 60 sec at 72˚C. The presence of the $CysLT_1R$ gene (650 bp) was visualized on a 0.7% agarose gel.

Flow cytometry (Annexin V and propidium iodide staining). The effect of $CysLT_1R$ antagonist on the human urological cancer cell lines was determined by dual staining with Annexin V-FITC

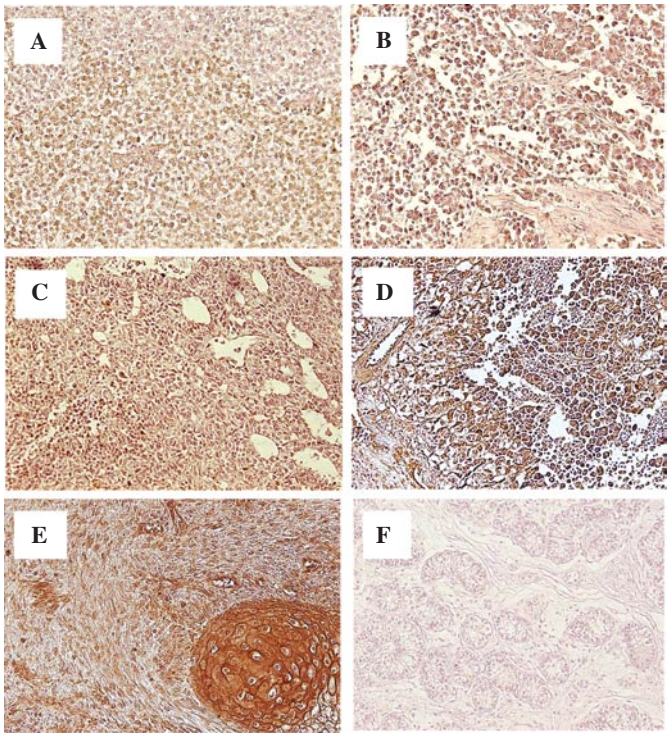

Figure 1. Representative $CysLT_1R$ expression in testis tissues. Very weak $CysLT_1R$ expression was observed in normal testis tissue (F). In contrast, significantly strong $CysLT_1R$ expression was observed in cancer tissue, including the nuclei and cytoplasm of all testicular cancer samples (A, seminoma; B, embryonal carcinoma; C, yolk sac tumors; D, choriocarcinoma; E, teratoma).

and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection kit I (Biosciences Pharmingen, USA). Annexin V-FITC and PI were added to the cellular suspension according to the manufacturer's instructions, and the sample fluorescence of $1.0x10^4$ cells was analyzed by flow cytometry, conducted with FACScan (Becton Dickinson, Germany). Annexin V-FITC-positive and PI-negative cells were identified as early apoptotic. Annexin V-FITC-positive and PI-positive cells were identified as late apoptotic or necrotic.

Flow cytometry (identification of DNA fragmentation). Flow cytometry was performed according to the TdT-mediated dUTP nick end-labeling (TUNEL) method using the Apo-Direct™ kit (Becton Dickinson). Following the experiments, the human urological cancer cell lines in suspension ($1x10^6$/ml) were fixed with 1% PBS, washed in PBS and suspended in 70% (v/v) ice-cold ethanol, then stored at -20˚C until use. The positive and negative controls and the samples were stained with FITC-dUTP by incubation in terminal deoxynucleotidyl transferase buffer according to the manufacturer's instructions, and the sample fluorescence of $1x10^4$ cells was analyzed by flow cytometry (Becton Dickinson). Results are expressed as the percentage of TUNEL-positive cells.

Detection of apoptosis by Hoechst staining. DNA chromatin morphology was assessed using Hoechst staining. The human urological cancer cell lines ($5x10^5$ cells) were incubated with 100 μM $CysLT_1R$ antagonist for 24 h. Cells were washed with RPMI-1640 and labeled with 8 mg/ml Hoechst 33342 (Sigma-Aldrich Japan K.K. Tokyo, Japan) for 10 min; PI (Sigma-Aldrich Japan K.K.) was then added (10 mg/ml final concentration), and the cells were examined by fluorescence microscopy.

Results

Immunohistochemistry. Immunohistochemistry revealed strong $CysLT_1R$ expression in all the tissue samples.

Renal cell carcinoma. $CysLT_1R$ was strongly expressed in RCC tissue samples of all grades, while very weak expression of $CysLT_1R$ was detected in NK tissue.

Bladder cancer. $CysLT_1R$ was strongly expressed in BC tissue samples of all grades, while very weak expression of $CysLT_1R$ was detected in NB tissue.

Prostate cancer. $CysLT_1R$ was strongly expressed in PC and PIN tissue samples of all grades, while very weak expression of $CysLT_1R$ was detected in BPH and NP tissue.

Testicular cancer. $CysLT_1R$ was strongly expressed in all TC tissue samples, while very weak expression of $CysLT_1R$ was detected in NT tissue (Fig. 1).

Statistical analysis of immunohistochemistry. Tissue samples were classified into categories and examined for the intensity of $CysLT_1R$ immunostaining.

Renal cell carcinoma. RCC tissues were classified as epithelium or blood vessels. $CysLT_1R$ expression was significantly more extensive and intense in all the RCC groups (G1, 1.9±0.7; G2, 2.4±1.0; G3, 2.6±0.7) compared to its expression in the NP tissues (1.3±0.6) (epithelium only). Furthermore, $CysLT_1R$ expression was higher in high-grade compared to low-grade cancer. However, $CysLT_1R$ expression presented no significant differences in blood vessel RCC and NK tissues.

Bladder cancer. BC tissues were classified as epithelium, blood vessels or stromal tissue. $CysLT_1R$ expression was significantly more extensive and intense in all the BC groups (all groups, 1.6±0.8; G1, 1.3±0.5; G2, 1.7±0.9; G3, 1.7±0.9) compared to its expression in NB tissues (0.4±0.3) (epithelium only). Furthermore, $CysLT_1R$ expression was higher in high-grade (G2 and G3) compared to low-grade (G1) cancer and in advanced-stage (pT2 or higher) (2.4±0.7) compared to early-stage (pT1 or lower) (1.1±0.5) cancer. However, $CysLT_1R$ expression in blood vessels and stromal tissue was at base levels in the BC and NB tissues (Table I).

Prostate cancer. PC tissues were classified as epithelium, blood vessels or stromal tissue. $CysLT_1R$ expression was significantly more extensive and intense in all the PC groups (all groups, 2.6±1.0; G1, 2.1±0.8; G2, 2.7±0.9; G3, 3.2±0.8) and in PIN tissues (1.8±0.9) compared to its expression in BPH (1.3±0.5) and NP (1.2±0.1) tissues (epithelium only). Furthermore, $CysLT_1R$ expression was higher in high-grade than in low-grade cancer. However, $CysLT_1R$ expression in blood vessels and stromal tissues was at base levels in the PC, PIN, BPH and NP tissues.

Reverse transcription-polymerase chain reaction. Using specific primers for $CysLT_1R$ and GAPDH, amplification predicted fragments of 650 and 400 bp.

Renal cell carcinoma. The RCC cell line expressed $CysLT_1R$ mRNA bands. These were down-regulated following treatment with 100 μM $CysLT_1R$ antagonist.

Table I. Statistical analysis of $CysLT_1R$ expression.

	Epithelium	Blood vessel	Stromal tissue	Mean age
Bladder cancer (n=90)	1.6±0.8[b]	0.4±0.3	0.3±0.2	68.1±7.5
Grade 1 (n=30)	1.3±0.5[b]	0.4±0.3	0.3±0.2	68.8±8.5
Grade 2 (n=30)	1.7±0.9[a,b]	0.3±0.3	0.3±0.3	67.6±6.7
Grade 3 (n=30)	1.7±0.9[a,b]	0.4±0.3	0.3±0.2	67.9±7.4
Early-stage (n=55)	1.1±0.5	0.3±0.2	0.3±0.2	68.0±7.3
Advanced-stage (n=35)	2.4±0.7[b]	0.4±0.4	0.3±0.2	68.3±7.8
Normal bladder (n=30)	0.4±0.3	0.3±0.2	0.3±0.3	65.8±7.2

Classification of the coded sections by two observers in a blinded manner using a scale of 0-4. 0, no staining; 4, maximum intensity. Statistical analysis was performed using ANOVA (p-value). In the epithelium alone, $CysLT_1R$ expression was significantly more extensive and intense in bladder cancer (BC) tissue compared to normal bladder (NB) tissue. Furthermore, $CysLT_1R$ expression was higher in high-grade (grades 2 and 3) compared to low-grade (grade 1) cancer ([a]$p<0.05$), and was higher in advanced-stage (pT2 or higher) compared to early-stage (pT1 or lower) cancer. However, $CysLT_1R$ expression in blood vessels and stromal tissue was at base levels in the BC and NB tissues ([b]$p<0.01$).

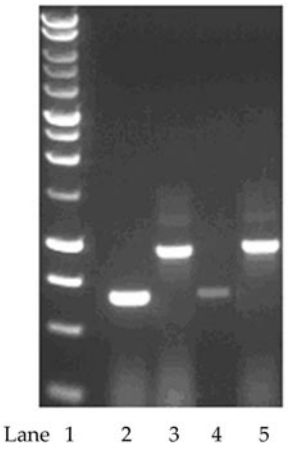

Figure 2. RT-PCR analysis of $CysLT_1R$ in bladder cancer cells. Using specific primers for $CysLT_1R$ and GAPDH, amplification predicted fragments of 650 and 400 bp. BC cells expressed $CysLT_1R$ mRNA bands (lane 1, marker; lane 2, T24; lane 3, GAPDH; lane 4, T24 with 100 μM $CysLT_1R$ antagonist; lane 5, GAPDH with 100 μM $CysLT_1R$ antagonist).

Bladder cancer. The BC cell line expressed $CysLT_1R$ mRNA bands. These were down-regulated following treatment with 100 μM $CysLT_1R$ antagonist (Fig. 2).

Prostate cancer. The PC cell lines expressed $CysLT_1R$ mRNA bands. These were down-regulated following treatment with 100 μM $CysLT_1R$ antagonist.

Testicular cancer. The TC cell line expressed $CysLT_1R$ mRNA bands.These were down-regulated following treatment with 100 μM $CysLT_1R$ antagonist.

MTT assay. Using the MTT assay, similar results were obtained for all the cells lines.

Renal cell carcinoma. In the RCC cell line, $CysLT_1R$ antagonist induced a reduction in cell viability with a half-maximal concentration of growth inhibition in the range of 12.5-100 μM, while having no effect on PRTEC proliferation (Table II).

Bladder cancer. In the BC cell line, $CysLT_1R$ antagonist induced a reduction in cell viability with a half-maximal

Table II. Effects of $CysLT_1R$ antagonist on the viability of human urological cancer cells.

$CysLT_1R$ antagonist	12.5 μM	25 μM	50 μM	100 μM
RCC cell line				
Caki-1	103.8	112.6	118.4	15.1
BT cell line				
T24	101.3	102.6	81.2	18.0
PC cell lines				
LNCaP	102.4	101.8	26.9	6.8
PC3	103.3	100.2	102.6	19.5
DU-145	117.4	47.2	20.7	7.5
TC cell line				
NEC-8	105.4	112.8	120.4	21.1
Normal proximal tubular endothelial cells	98.2	93.6	101.7	105.3
Normal prostate stromal cells	87.5	92.7	92.9	97.1

Dose-response analysis of viability in human urological cancer cells treated with $CysLT_1R$ antagonist (12.5-100 μM), measured using the MTT assay and expressed as the percentage of control culture conditions.

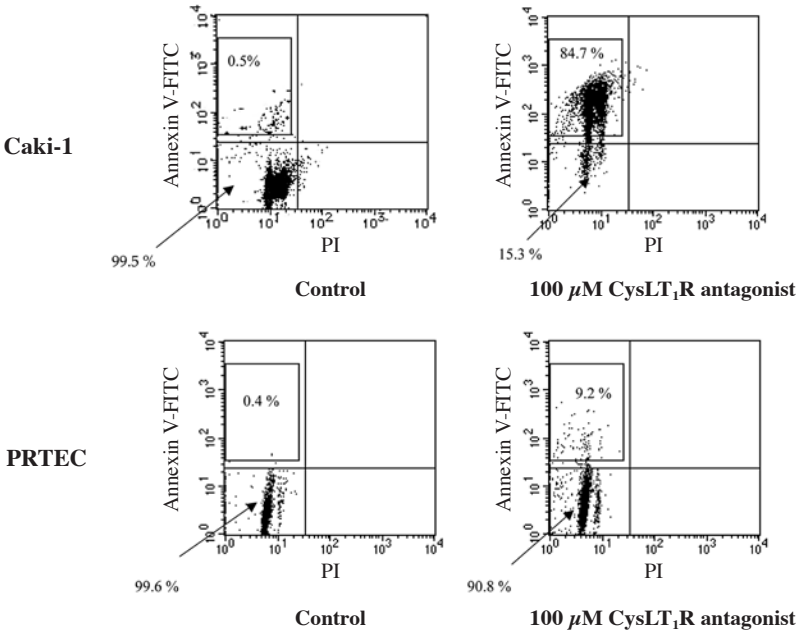

Figure 3. Flow cytometric analysis of the effects of $CysLT_1R$ antagonist on early and late apoptosis in renal cell carcinoma (RCC) cells. Almost 100% of the RCC cells treated with 100 μM $CysLT_1R$ antagonist underwent early apoptosis. However, normal proximal tubular endothelial cells treated with 100 μM $CysLT_1R$ antagonist did not undergo apoptosis. The upper left quadrants represent early apoptosis (Annexin V-FITC-positive and PI-negative cells). The upper right quadrants represent late necrosis and necrosis (Annexin V-FITC-positive and PI-positive cells). FITC-Annexin V/PI flow cytometry diagrams of a representative experiment are presented.

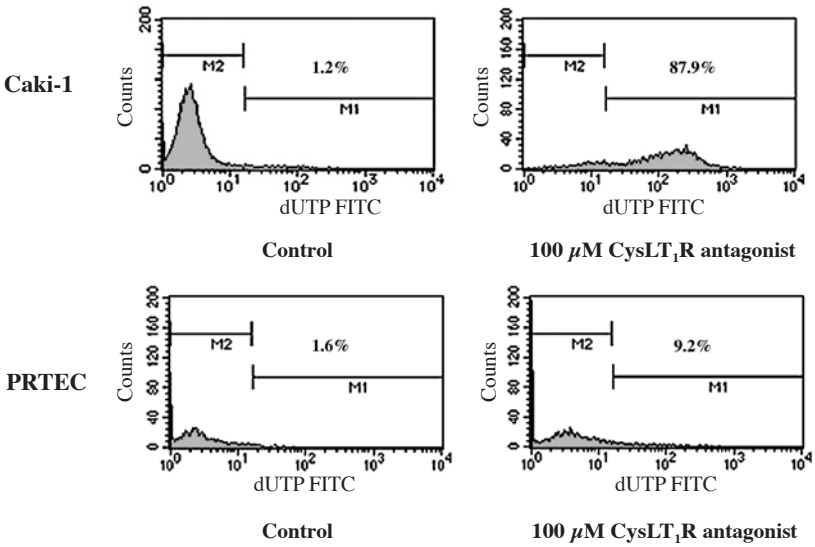

Figure 4. Effects of $CysLT_1R$ antagonist on the induction of DNA fragmentation in renal cell carcinoma (RCC) cells. $CysLT_1R$ antagonist (100 μM) induced DNA fragmentation in RCC cells. However, 100 μM $CysLT_1R$ antagonist did not induce DNA fragmentation in proximal tubular endothelial cells. Typical flow cytometry analysis histograms of a representative experiment are presented.

concentration of growth inhibition in the range of 12.5-100 μM (Table II).

Prostate cancer. In the PC cell lines, $CysLT_1R$ antagonist induced a reduction in cell viability with a half-maximal concentration of growth inhibition in the range of 12.5-100 μM, while having no effect on NPC proliferation (Table II).

Testicular cancer. In the TC cell line, $CysLT_1R$ antagonist induced a reduction in cell viability with a half-maximal concentration of growth inhibition in the range of 12.5-100 μM (Table II).

Flow cytometry. Using flow cytometry, similar results were obtained for all the cell lines.

Renal cell carcinoma. In the RCC cell line, 100 μM $CysLT_1R$ antagonist induced early apoptosis, not late apoptosis or necrosis and DNA fragmentation. FITC-Annexin V/PI flow cytometry diagrams and typical flow cytometry analysis histograms are presented in Figs. 3 and 4.

Bladder cancer. In the BC cell line, 100 μM $CysLT_1R$ antagonist induced early apoptosis, not late apoptosis or necrosis and DNA fragmentation.

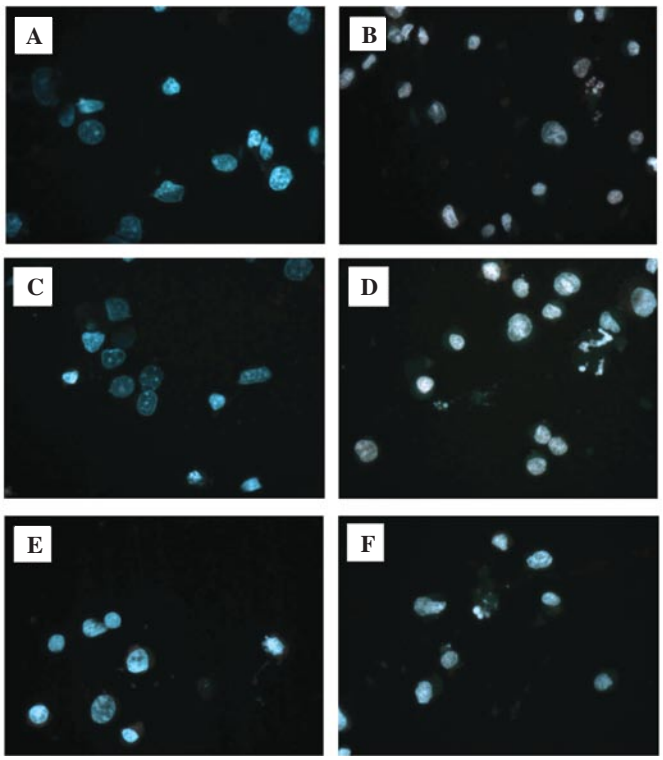

Figure 5. Effects of CysLT$_1$R antagonist on the induction of apoptosis in prostate cancer cells. Cells treated with CysLT$_1$R antagonist showed significant chromatin condensation, cellular shrinkage, small membrane-bound bodies (apoptotic bodies) and cytoplasmic condensation. These cellular changes are typical characteristics of apoptosis (B, LNCaP; D, PC3; F, DU-145). All prostate cancer cell lines without CysLT$_1$R antagonist maintained normal chromatin patterns and cell size (A, LNCaP; C, PC3; E, DU-145).

Prostate cancer. In the PC cell lines, 100 μM CysLT$_1$R antagonist induced early apoptosis, not late apoptosis or necrosis and DNA fragmentation.

Testicular cancer. In the TC cell line, 100 μM CysLT$_1$R antagonist induced early apoptosis, not late apoptosis or necrosis and DNA fragmentation.

Hoechst staining. Using Hoechst staining, similar cellular changes typical of apoptosis were observed in all the cell lines. Without CysLT$_1$R antagonist treatment, the cell lines maintained normal chromatin patterns and cell size.

Renal cell carcinoma. The RCC cell line treated with 100 μM CysLT$_1$R antagonist showed significant chromatin condensation, cellular shrinkage, small membrane-bound bodies (apoptotic bodies) and cytoplasmic condensation.

Bladder cancer. The BC cell line treated with 100 μM CysLT$_1$R antagonist showed significant chromatin condensation, cellular shrinkage, apoptotic bodies and cytoplasmic condensation.

Prostate cancer. The PC cell lines treated with 100 μM CysLT$_1$R antagonist showed significant chromatin condensation, cellular shrinkage, apoptotic bodies and cytoplasmic condensation (Fig. 5).

Testicular cancer. The TC cell line treated with 100 μM CysLT$_1$R antagonist showed significant chromatin condensation, cellular shrinkage, apoptotic bodies and cytoplasmic condensation.

Discussion

Leukotrienes (LTs) are biologically active fatty acids derived from the oxidative metabolism of arachidonic acid (AA) (22,23) through the 5-LOX pathway. The activity of 5-LOX leads to the formation of unstable LTA_4, which can be converted into either LTB_4 or CysLTs (LTC_4, LTD_4 and LTE_4). CysLTs are components of a slow-reacting substance of anaphylaxis. LTD_4 plays the most important role in CysLTs (CysLT$_1$ and CysLT$_2$), and CysLT$_1$ is specific for LTD_4. LTs are potent biochemical mediators that are released from mast cells, eosinophils and basophils. They work to contract airway smooth muscle, increase vascular permeability and mucus secretions, and attract and activate inflammatory cells in the airways of patients with asthma (24). The action of LTs can be blocked through either one of two specific mechanisms: i) the inhibition of LT production, or ii) the antagonism of LT binding to cellular receptors.

By contrast, the 5-LOX inhibitor inhibits LT formation (in particular LTB_4, LTC_4, LTD_4 and LTE_4). Our previous studies found that 5-LOX was overexpressed in urological cancer, and that the 5-LOX inhibitor may attenuate the growth of human urological cancer and induce apoptosis through the AA pathway (8-11,25). On the basis of these findings, we examined whether or not CysLT$_1$R is expressed in human urological cancer tissue, and whether or not it is possible to prevent urological cancer cell growth by means of CysLT$_1$R antagonist.

In this study, immunohistochemistry revealed strong expression of CysLT$_1$R in urological cancer tissue. The extent and intensity of CysLT$_1$R expression were greater in the urological cancer tissue than in normal tissue. In RCC and PC, CysLT$_1$R expression was higher in high-grade compared to low-grade cancer. In the BC cell line, CysLT$_1$R expression was higher in high-grade and advanced-stage cancer compared to low-grade and early-stage cancer.

Limited data regarding CysLT$_1$R and urological cancer have been reported in previous studies. We expect that further research will be undertaken. Theories concerning the correlation between cancer grade or stage and CysLT$_1$R expression are still controversial. In the near future, this may be elucidated through competitive PCR.

Using RT-PCR, we found that CysLT$_1$R was expressed in urological cancer cell lines, and that this expression was down-regulated by 100 μM CysLT$_1$R antagonist.

Next, using the MTT assay, we demonstrated that the co-incubation of urological cancer cells with CysLT$_1$R antagonist arrested the growth of urological cancer cells and potently inhibited cell growth in a dose-dependent manner. These results indicate that CysLT$_1$R is essential for the cell growth of urological cancer cells.

The mechanism by which CysLT$_1$R antagonist suppresses growth in urological cancer cells requires clarification. To address this issue, we examined whether or not apoptosis was involved in growth suppression in the urological cancer cells. CysLT$_1$R antagonist (100 μM) strongly induced early apoptosis in urological cancer according to the results of flow cytometry and Hoechst staining. Thus, apoptosis may be involved in the mechanisms by which CysLT$_1$R antagonist prevents cell growth in urological cancer cells. These results

provide the first confirmation that $CysLT_1R$ antagonist inhibits urological cancer cell growth through apoptosis.

In a study on $CysLT_1R$ and colon cancer, Ohd *et al* reported that $CysLT_1R$ was overexpressed in human colorectal cancer and was significantly correlated to COX-2 and 5-LOX (13). The expression of $CysLT_1R$ was higher in high-grade and early-stage cancer, suggesting typical differences in colon cancer (13). Furthermore, survival time was slightly shorter in patients with high-intensity $CysLT_1R$ staining than in those with low-intensity staining (14). These reports suggest that there are various relationships between $CysLT_1R$ and other types of cancer, and that $CysLT_1R$ antagonist can prevent cell growth in other types of cancer besides urological cancer.

These findings suggest that $CysLT_1R$ expression is strong in urological cancer, though the anti-cancer effect of $CysLT_1R$ antagonist is weak in urological cancer patients in a single administration at a clinical dose. $CysLT_1R$ antagonist is therefore suitable for chemopreventive therapy.

In conclusion, there is no question that $CysLT_1R$ is involved in the initiation and promotion of urological cancer. It may be possible to apply $CysLT_1R$ antagonist as an anti-cancer drug in the area of cancer prevention. However, $CysLT_1R$ antagonist at a clinical dose is not expected to have a suppressive effect on the cancer. Though the clinical application of $CysLT_1R$ antagonist requires further research and consideration, targeting $CysLT_1R$ may provide a novel strategy for the prevention and treatment of human urological cancer.

References

1. Oh WK and Kantoff PW: Management of hormone refractory prostate cancer: current standards and future prospects. J Urol 160: 1220-1229, 1998.
2. Weidner N, Folkman J, Pozza F, Bevilaqua P, Allred EN and Moore DH: Tumor angiogenesis: a new significant and independent prognostic indicator in early stage brest carcinoma. J Natl Cancer Inst 84: 1875-1887, 1992.
3. Lafyatis R, Thompson NL, Remmers EF, Flanders KC, Roche NS and Kim SJ: Transforming growth factor-beta production by synovial tissues from rheumatoid patients and streptococcal cell wall arthritic rats. Studies on secretion by synovial fibroblast-like cells and immunohistologic localization. J Immunol 143: 1142-1148, 1989.
4. Yoshimura R, Matsuyama M, Kawahito Y, *et al*: Study of cyclooxygenase-2 in renal cell carcinoma. Int J Mol Med 13: 229-233, 2004.
5. Yoshimura R, Sano H, Mitsuhashi M, Kohno M, Chargui J and Wada S: Expression of cyclooxygenase-2 in patients with bladder carcinoma. J Urol 165: 1468-1472, 2001.
6. Yoshimura R, Sano H, Masuda C, *et al*: Expression of cyclooxygenase-2 in prostate carcinoma. Cancer 89: 589-596, 2000.
7. Hase T, Yoshimura R, Matsuyama M, *et al*: Cyclooxygenase-1 and -2 in human testicular tumours. Eur J Cancer 39: 2043-2049, 2003.
8. Matsuyama M, Yoshimura R, Mitsuhashi M, *et al*: 5-lipoxygenase inhibitors attenuate growth of human renal cell carcinoma and induce apoptosis through arachidonic acid pathway. Oncol Rep 14: 73-79, 2005.
9. Yoshimura R, Matsuyama M, Tsuchida K, Kawahito Y, Sano H and Nakatani T: Expression of lipoxygenase in human bladder carcinoma and growth inhibition by its inhibitors. J Urol 170: 1994-1999, 2003.
10. Matsuyama M, Yoshimura R, Mitsuhashi M, *et al*: Expression of lipoxygenase in human prostate cancer and growth reduction by its inhibitors. Int J Oncol 24: 821-827, 2004.
11. Yoshimura R, Matsuyama M, Mitsuhashi M, *et al*: Relationship between lipoxygenase and human testicular cancer. Int J Mol Med 13: 389-393, 2004.
12. Lynch KR, O'Neill GP, Liu G, *et al*: Characterization of human cysteinyl leukotriene $CysLT_1$ receptor. Nature 399: 789-793, 1999.
13. Ohd JF, Wikstrom K and Sjolander A: Leukotrienes induce cell-survival signaling in intestinal epithelial cells. Gastroenterology 119: 1007-1018, 2000.
14. Nielsen CK, Campbell JI, Ohd JF, *et al*: A novel localization of the G-protein-coupled $CysLT_1$ receptor in the nucleus of colorectal adenocarcinoma cells. Cancer Res 65: 732-742, 2005.
15. Funao K, Matsuyama M, Naganuma T, *et al*: The cysteinylLT_1 receptor in human renal cell carcinima. Mol Med Rep 1: 185-189, 2008.
16. Matsuyama M, Funao K, Hayama T, *et al*: Relationship between cysteinylLT_1 receptor and human transitional cell carcinoma in bladder. Urology 73: 916-921, 2009.
17. Matsuyama M, Hayama T, Funao K, *et al*: Overexpression of cysteinylLT_1 receptor in prostate cancer and $CysLT_1R$ antagonist inhibits prostate cancer cell growth through apoptosis. Oncol Rep 18: 99-104, 2007.
18. Matsuyama M, Funao K, Kawahito Y, *et al*: The expression of cysteinylLT_1 receptor in human testicular cancer and growth reduction by its antagonist through apoptosis. Mol Med Rep 2: 163-167, 2009.
19. Sano H, Hla T, Maier JAM, *et al*: In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. J Clin Invest 89: 97-108, 1992.
20. Fitzgerald SM and Flinn S: Evaluating research studies using the analysis of variance (ANOVA): issues and interpretations. J Hand Ther 13: 56-60, 2000.
21. Storms W: Update on montelukast and its role in the treatment of asthma, allergic rhinitis and exercise-induced bronchoconstriction. Expert Opin Pharmacother 8: 2173-2187, 2007.
22. Horwitz RJ, McGill KA and Busse WW: The role of leukotriene modifiers in the treatment of asthma. Am J Respir Crit Care Med 157: 1363-1371, 1998.
23. Wenzel SE: New approaches to anti-inflammatory therapy for asthma. Am J Med 104: 287-300, 1998.
24. Drazen J: Clinical pharmacology of leukotriene receptor antagonists and 5-lipoxygenase inhibitors. Am J Respir Crit Care Med 157: 233-237, 1998.
25. Matsuyama M, Yoshimura R, Tsuchida K, *et al*: Lipoxygenase inhibitors prevent urological cancer cell growth. Int J Mol Med 13: 665-668, 2004.