Cysteinyl-leukotriene$_1$ receptor antagonist prevents urological cancer cell growth through early apoptosis

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Abstract. Recent studies have demonstrated that the cysteinyl-leukotriene$_1$ receptor ($CysLT_1R$) antagonist induces the growth arrest of cancer cells through apoptosis. In this study, we examined the effects of the $CysLT_1R$ antagonist on cell proliferation in urological cancer cell lines, including renal cell carcinoma, bladder cancer, prostate cancer and testicular cancer cells. The inhibitory effect of the $CysLT_1R$ antagonist on the urological cancer cells was investigated using the MTT assay and flow cytometry. The $CysLT_1R$ antagonist induced a reduction in cell viability with a half-maximal concentration of growth inhibition in all the urological cancer cell lines, and arrested the growth of the cells through early apoptosis. In conclusion, the $CysLT_1R$ antagonist may mediate potent anti-proliferative effects against urological cancer cells through early apoptosis, and may therefore serve as a novel therapeutic target in the treatment of urological cancer.

Introduction

Angiogenetic factors play important roles in urological as well as in other types of cancer. In recent years, the expression of angiogenic factors in solid human tumors has been widely reported (1). 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* (2).

The metabolism of arachidonic acid (AA) by 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, where they are significantly involved in tumor promotion, progression and metastasis.

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 LTA_4, which can be converted into 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 (3). Generally, CysLTs are important mediators of human bronchial asthma, and many CysLT receptor antagonists are clinically used in its treatment.

We previously demonstrated that the 5-LOX inhibitor prevents urological cancer cell growth through apoptosis (4). However, few reports have addressed the relationship between $CysLT_1R$ and carcinogenesis, and fewer still have provided evidence suggesting that the $CysLT_1R$ antagonist induces the growth arrest of cancer cells through apoptosis.

With this background, the present study aimed to examine the inhibitory effect of the $CysLT_1R$ antagonist in renal cell carcinoma (RCC), bladder cancer (BC), prostate cancer (PC) and testicular cancer (TC) cell lines.

Materials and methods

Reagents and materials. RPMI-1640 was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) and a penicillin-streptomycin mixture were from BioWhittaker (Walkersville, MD, USA). Trypsin/EDTA was from Gibco BRL (Rockville, MD, USA). Montelukast is a selective and orally active $CysLT_1R$ antagonist (LKT Laboratories, Inc., MN, USA) with demonstrated effectiveness for treating allergic asthma and allergic rhinitis in adults as well as in children as young as 12 months of age for allergic asthma and 6 months of age for allergic rhinitis (5).

Cell cultures. The human RCC cell line (Caki-1), human BC cell line (T24), human PC cell lines (LNCaP, PC3 and

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Table I. Effect of the $CysLT_1R$ antagonist on the viability of human urological cancer cells as determined by the MTT assay.

$CysLT_1R$ antagonist	12.5 μM (%)	25 μM (%)	50 μM (%)	100 μM (%)
RCC cell line				
Caki-1	107.2	116.8	122.4	15.6
BC cell line				
T24	101.3	102.6	81.2	18.0
PC cell lines				
PC3	103.2	98.9	102.6	18.9
DU-145	116.5	43.6	19.8	12.4
LNCaP	101.0	101.8	23.4	11.8
TC cell line				
NEC-8	108.4	115.4	121.2	21.8

Concentration-response analysis of the viability of human urological cancer cells treated with the $CysLT_1R$ antagonist (12.5-100 μM), carried out using the MTT assay. Data are expressed as the percentage of control culture conditions.

DU-145) and human TC cell line (NEC-8) were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were grown in a culture flask (Nunc, Roskilde, Denmark) in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37˚C. The media were changed every 3 days, and cells were separated via trypsinization using trypsin/EDTA upon reaching subconfluence.

Cell-proliferation studies. Approximately $1.0x10^4$ cells placed on 8x8-mm diameter multichamber slides (Nunc, Copenhagen, Denmark) were treated with the $CysLT_1R$ antagonist dissolved in ethanol. The final concentration of ethanol was <0.05%. Cell viability was measured at 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) and presented as the percentage of control culture conditions.

Flow cytometry

Annexin V and propidium iodide staining. The effect of the $CysLT_1R$ antagonist on urological cancer cells was determined by dual staining with Annexin V-FITC and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection kit I (Biosiences Pharmingen). Annexin V-FITC and PI were added to the cellular suspension as specified in the manufacturer's instructions, and a sample fluorescence of $1.0x10^4$ cells was analyzed by flow cytometry, carried out with FACScan (Becton Dickinson, Germany). Cells that were Annexin V-FITC-positive and PI-negative were identified as early apoptotic. Cells that 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, urological cancer cells 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 sample were stained with FITC-dUTP by incubation in terminal deoxynucleotidyl transferase buffer as specified in the manufacturer's instructions, and the sample fluorescence of $1.0x10^4$ cells was analyzed using flow cytometry (Becton Dickinson). Results are expressed as the percentage of TUNEL-positive cells.

Results

$CysLT_1R$ antagonist-induced growth inhibition in urological cancer cells. To investigate the effects of the $CysLT_1R$ antagonist on urological cancer cell proliferation, cell viability was analysed *in vitro* using a modified MTT assay. The $CysLT_1R$ antagonist induced a reduction in cell viability at the half-maximal concentration of growth inhibition of urological cancer cells in the range of 12.5-100 μM, and arrested the growth of all the urological cancer cell lines (Table I).

$CysLT_1R$ antagonist-induced apoptosis as shown by flow cytometry. The effects of the $CysLT_1R$ antagonist on the apoptosis of the urological cancer cells compared with the controls are shown in Fig. 1. Upon treatment with 100 μM $CysLT_1R$ antagonist, nearly all the urological cancer cells underwent early but not late apoptosis (Fig. 1). $CysLT_1R$ antagonist (100 μM) induced DNA fragmentation in the urological cancer cells (Fig. 2).

Discussion

Leukotrienes (LTs) are biologically active fatty acids derived from the oxidative metabolism of arachidonic acid (AA) (6,7) via the 5-LOX pathway. The activity of 5-LOX leads to the formation of unstable LTA_4, which is 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 released from mast cells, eosinophils and basophils

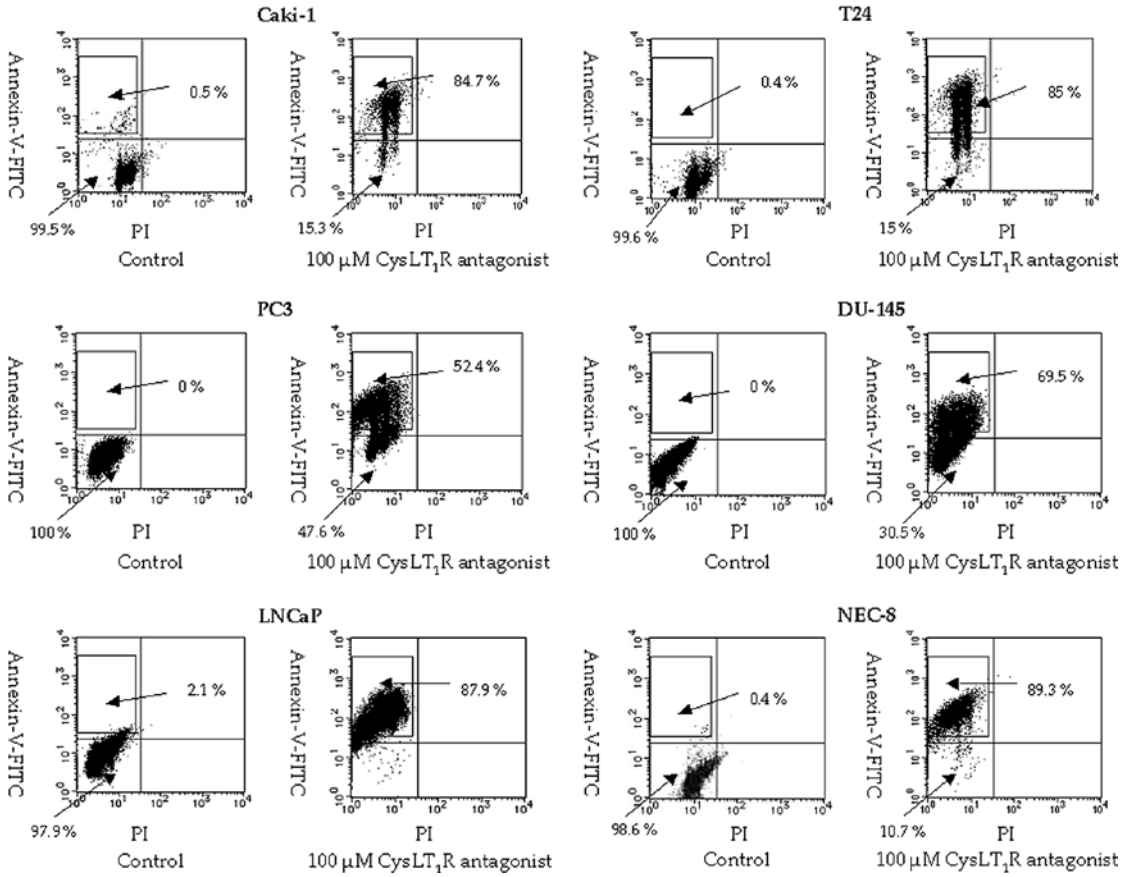

Figure 1. Effects of the $CysLT_1R$ antagonist on apoptosis by flow cytometry. Nearly all the urological cancer cells upon treatment with 100 μM $CysLT_1R$ antagonist underwent early apoptosis. FITC-Annexin V/PI flow cytometry diagrams of a representative experiment are shown.

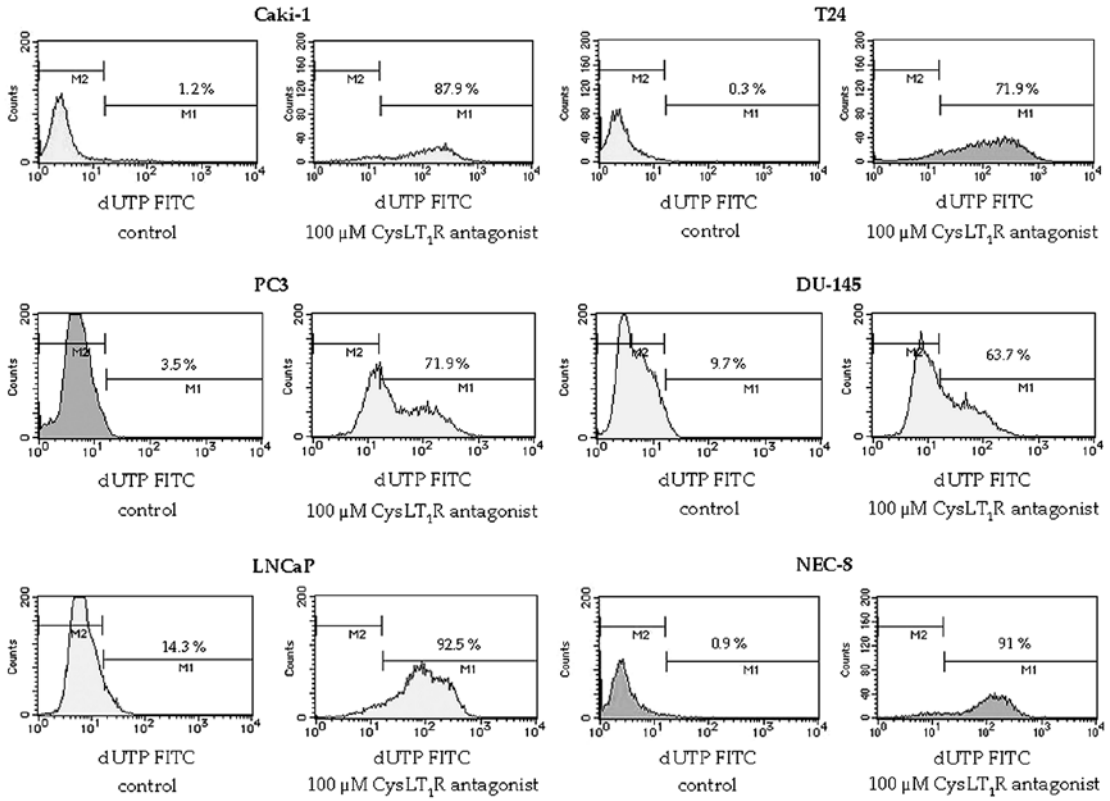

Figure 2. Effects of the $CysLT_1R$ antagonist on DNA fragmentation by flow cytometry. The $CysLT_1R$ antagonist (100 μM) induced DNA fragmentation in the urological cancer cell lines. Typical flow cytometry histograms of a representative experiment are shown.

that work to contract airway smooth muscle, increase vascular permeability, increase mucus secretions and to attract and activate inflammatory cells in the airways of patients with asthma (8). The action of LTs can be blocked through one of two specific mechanisms: i) the inhibition of LT production, or ii) the antagonism of LT binding to cellular receptors.

The 5-LOX inhibitor inhibits LT formation (especially LTB_4, LTC_4, LTD_4 and LTE_4). In our previous study, the 5-LOX inhibitor attenuated the growth of human urological cancer and induced apoptosis through the AA pathway (4). With this background, we examined whether or not the $CysLT_1R$ antagonist prevents urological cancer cell growth.

In the present study, we demonstrated using an MTT assay that co-incubation of urological cancer cells with the $CysLT_1R$ antagonist halted the growth of urological cancer cells, and potently inhibited cell growth in a dose-dependent manner. The results indicated that $CysLT_1R$ is essential for the cell growth of urological cancer cells. However, the mechanism by which the $CysLT_1R$ antagonist induced growth inhibition in urological cancer cells required further clarification. To address this problem, we examined whether or not apoptosis was involved in growth suppression in these cancer cell lines. The $CysLT_1R$ antagonist (100 μM) strongly induced early apoptosis in urological cancer cells as determined by flow cytometry, suggesting that apoptosis may be one of the mechanisms through which the $CysLT_1R$ antagonist prevents urological cancer cell growth. This provides the first indication that, through early apoptosis, the $CysLT_1R$ antagonist inhibits urological cancer cell growth.

Regarding $CysLT_1R$ and colon cancer, Ohd *et al* found that $CysLT_1R$ was overexpressed in human colorectal cancer, and was significantly correlated with COX-2 and 5-LOX. They demonstrated that the expression of $CysLT_1R$ was higher in high-grade and early-stage carcinoma, suggesting typical differences in colon cancer (9). Furthermore, survival time was found to be slightly shorter in patients with high-intensity $CysLT_1R$ staining than in those with low-intensity staining (10). In addition, we previously found that $CysLT_1R$ was overexpressed in human PC and RCC, and that the expression of $CysLT_1R$ was higher in high- than in low-grade cancer (11,12). Together, these reports suggest that there is a relationship between $CysLT_1R$ and various cancers, and that the $CysLT_1R$ antagonist can prevent cell growth in other types of cancer besides urological cancer.

In conclusion, our study provides evidence that the mechanisms of the cell growth and apoptosis of urological cancer cells involve $CysLT_1R$. Growth inhibition of urological cancer cells by blocking $CysLT_1R$ is associated with the induction of apoptosis. Thus, the $CysLT_1R$ antagonist may serve as a novel target for anti-cancer therapies.

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