

Expression of cysteinylLT₁ receptor in human testicular cancer and growth reduction by its antagonist 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 pro-inflammatory mediator derived from arachidonic acid through various enzymatic steps, and 5-lipoxygenase is an important factor in generating LTD₄. We investigated LTD₄ receptor (cysteinylLT₁ receptor; CysLT₁R) expression in testicular cancer (TC), as well as the effects of the CysLT₁R antagonist on cell proliferation in a TC cell line. CysLT₁R expression in tissue from TC patients and normal testes (NTs) was detected using immunohistochemistry and RT-PCR. The effects of the CysLT₁R antagonist on TC cell growth were examined using the MTT assay. Flow cytometry was used to determine whether or not the CysLT₁R antagonist induces apoptosis. Immunohistochemistry indicated that CysLT₁R expression was strong in all types of TC tissues, but very weak in NT tissues. The TC cell line expressed CysLT₁R mRNA as detected by RT-PCR. MTT and flow cytometry revealed that the CysLT₁R antagonist caused marked inhibition of TC cells through early apoptosis. In conclusion, CysLT₁R was induced in TC. The results suggest that the CysLT₁R antagonist may mediate potent anti-proliferative effects against TC cells. Thus, CysLT₁R may become a new therapeutic target for the treatment of TC.

Introduction

Testicular cancer (TC) is relatively rare, but angiogenic factors are considered to play an important role in invasion in both TC cells and other organs. 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 demonstrated to increase neovascularization *in vivo* and *in vitro* (2).

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 tumor promotion, progression and metastasis. Studying these pathways in specimens from patients with TC, we demonstrated that COX-2 and 5-LOX were overexpressed in TC tissues (3,4).

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 CysLTs (CysLT₁, CysLT₂), and CysLT₁ receptor (CysLT₁R) is specific for LTD₄ (5). CysLTs are important mediators of human bronchial asthma, and many CysLT receptor antagonists are clinically used in its treatment. A few reports have addressed the relationship between CysLT₁R and colon cancer (6,7). To date, no report has addressed the relationship between CysLT₁R and human TC. Our team has already demonstrated that 5-LOX is overexpressed in human urological cancer (4,8-10).

Based on these findings, the present study aimed to examine the expression of CysLT₁R in human TC tissues in order to evaluate the inhibitory effect of the CysLT₁R antagonist on human TC cells, and to determine whether the CysLT₁R antagonist induces apoptosis in these cells.

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Materials and methods

Tumor specimens. Tissue specimens were obtained from 30 patients with TC and 10 patients with normal testes (NT) who underwent total orchiectomy for prostate cancer. Tumor histopathology was determined by pathologists. 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 4 μ m.

Immunohistochemistry and patient samples. TC and NT slides were deparaffinized, then immunohistochemical staining was performed with the VectaStain Avidin-Biotin Peroxidase Complex Kit (Vector Laboratories, Burlingame, CA, USA) as previously described (11). 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.

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 CysLT₁R antagonist (LKT Laboratories Inc., MN, USA) with demonstrated effectiveness for treating allergic asthma and allergic rhinitis in adults and in children as young as 12 months of age (allergic asthma) and 6 months of age (allergic rhinitis) (12).

Cell cultures. The human TC cell line NEC-8 was 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 of penicillin and 100 μ g/ml of streptomycin in a humidified 5% CO₂ atmosphere at 37°C. Media were changed every 3 days and, upon reaching subconfluence, cells were separated via trypsinization using trypsin/EDTA.

Cell proliferation 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 at a final concentration of <0.05%. Cell viability was measured on 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.

RT-PCR of CysLT₁R. Total RNA was isolated from NEC-8 cells using the RNeasy[®] Kit (Ambion, Japan) and checked for the presence of the CysLT₁R gene using primers (sense 5'-CTGCTCGCTTCGCTACTTGG-3', antisense 5'-CGG CACCTGTCCTACGAGTTG-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 CysLT₁R gene (650 bp) was visualized on a 0.7% agarose gel.

Flow cytometry

Annexin V and propidium iodide staining. The effect of the CysLT₁R antagonist on NEC-8 cells was determined by dual staining with Annexin V-FITC and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit I (Biosciences Pharmingen). Annexin V-FITC and PI were added to the cellular suspension as per the manufacturer's instructions, and sample fluorescence of 1.0×10^4 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 by the TdT-mediated dUTP Nick End Labelling (TUNEL) method using the Apo-Direct[™] kit (Becton Dickinson). Following the experiments, NEC-8 cells in suspension (1×10^6 /ml) were fixed with 1% PBS, washed in PBS, and suspended in 70% (v/v) ice-cold ethanol, then 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 according to the manufacturer's instructions, and the sample fluorescence of 1.0×10^4 cells was analyzed by flow cytometry (Becton Dickinson). Results are presented as the percentage of TUNEL-positive cells.

Results

CysLT₁R expression in testis tissue. Very weak CysLT₁R expression was observed in the NT case (Fig. 1F). In contrast, significantly strong CysLT₁R expression was observed in cancer tissues, including the nuclei and cytoplasm, in all TC types (Fig. 1A, seminoma; B, embryonal carcinoma; C, yolk sac tumors; D, choriocarcinoma; and E, teratoma). Immunostaining with PBS was negative in all subjects (data not shown).

CysLT₁R antagonist-induced growth inhibition in TC cells as determined by the MTT assay. Treatment with the CysLT₁R antagonist induced a reduction in cell viability with the half-maximal concentration of growth inhibition of NEC-8 cells in the range of 12.5-100 μ M (Fig. 2). The CysLT₁R antagonist halted the growth of NEC-8 cells.

CysLT₁R expression in TC cells. Using specific primers for CysLT₁R and GAPDH amplification predicted fragments of 650 and 400 bp. NEC-8 cells expressed CysLT₁R mRNA bands (Fig. 3, lane 2). CysLT₁R mRNA bands with 100 μ M CysLT₁R antagonist in NEC-8 cells were down-regulated (Fig. 3, lane 4).

CysLT₁R antagonist-induced apoptosis indicated by flow cytometry. NEC-8 without CysLT₁R antagonist treatment is shown in Fig. 4. Ninety percent of NEC-8 cells treated with

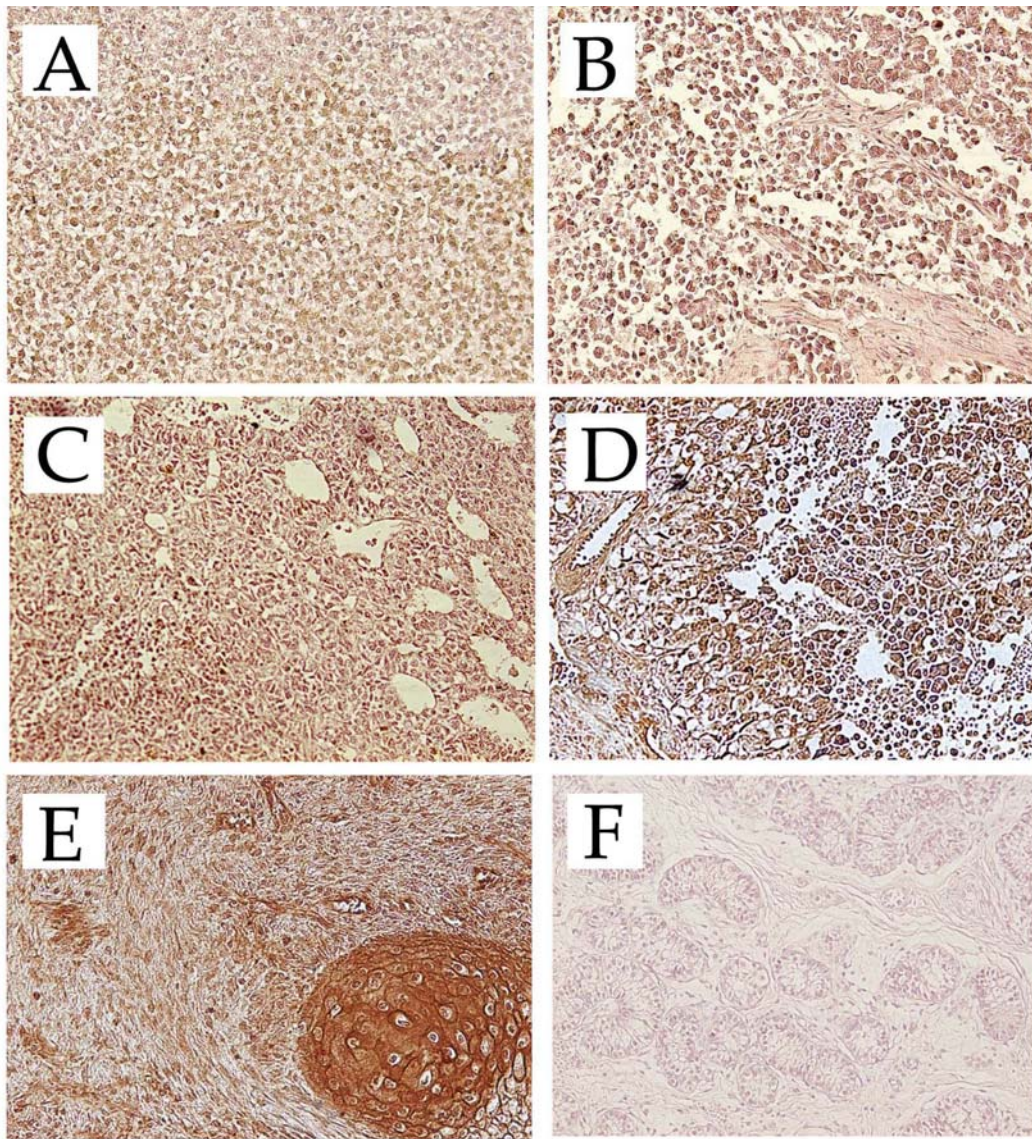


Figure 1. Representative CysLT₁R expression in testis tissues. Very weak CysLT₁R expression was noted in normal testis tissue (F). In contrast, significantly strong CysLT₁R expression was observed in cancer tissues, including the nuclei and cytoplasm, in all testicular cancer types (A, seminoma; B, embryonal carcinoma; C, yolk sac tumors; D, choriocarcinoma; E, teratoma).

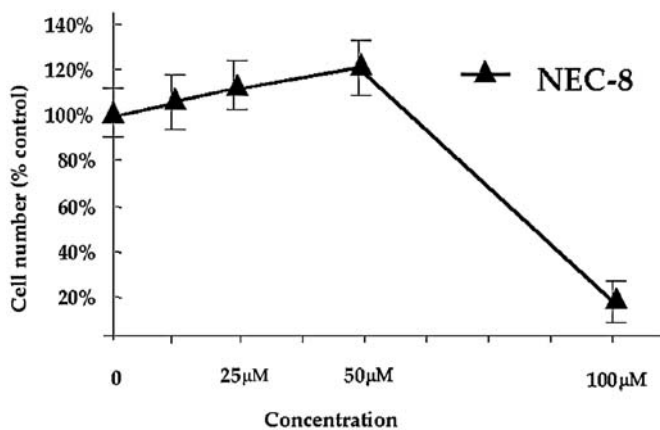


Figure 2. Concentration-dependent effects of the CysLT₁R antagonist on human TC cells. The CysLT₁R antagonist induced a reduction in cell viability with half-maximal concentration of growth inhibition of NEC-8 cells in the range of 12.5-100 μM. The CysLT₁R antagonist halted the growth of NEC-8 cells.

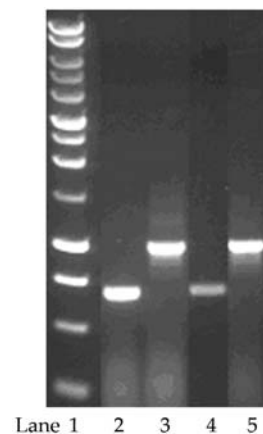


Figure 3. RT-PCR analysis of CysLT₁R in human TC cells. Using specific primers for CysLT₁R and GAPDH amplification predicted fragments of 650 and 400 bp. NEC-8 cells expressed CysLT₁R mRNA bands. CysLT₁R mRNA bands with 100 μM CysLT₁R antagonist in NEC-8 were down-regulated. Lane 1, marker; 2, NEC-8; 3, GAPDH; 4, NEC-8 with 100 μM CysLT₁R antagonist; and 5, GAPDH with 100 μM CysLT₁R antagonist.

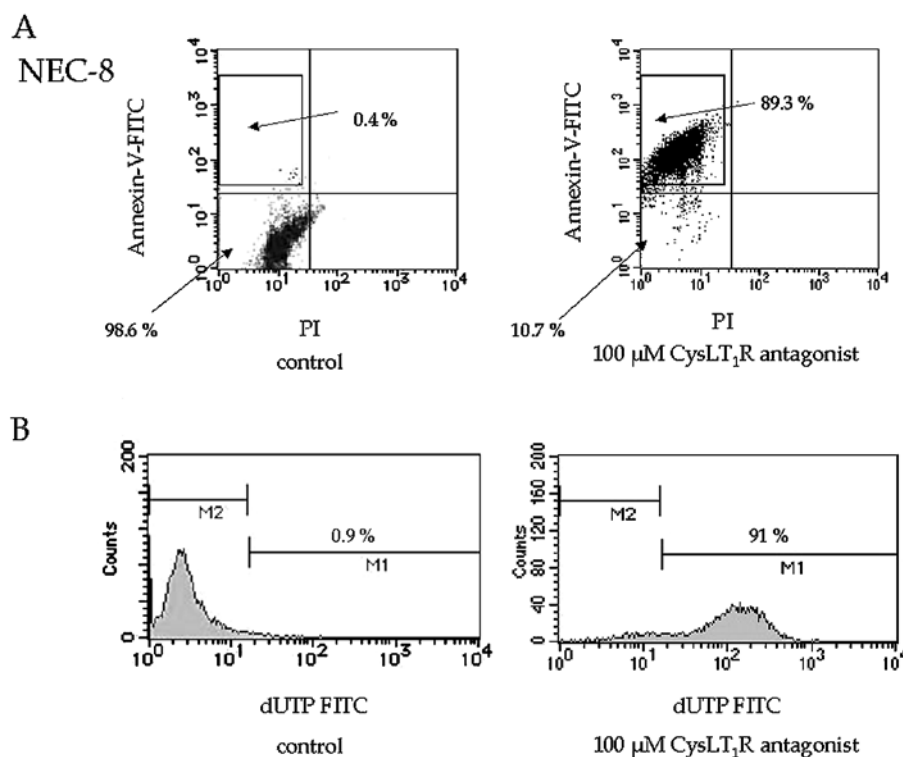


Figure 4. Effects of CysLT₁R antagonist on apoptosis by flow cytometry, and CysLT₁R antagonist-induced DNA fragmentation in human TC cells. A total of 90% of NEC-8 cells with 100 μM CysLT₁R antagonist exhibited early apoptosis (A). Diagrams of FITC-Annexin V/PI flow cytometry in a representative experiment are presented. CysLT₁R antagonist (100 μM) induced DNA fragmentation in NEC-8 cells (B). Histograms of typical flow cytometry analysis in a representative experiment are presented.

100 μM CysLT₁R antagonist exhibited early apoptosis, but not late apoptosis (Fig. 4A). The CysLT₁R antagonist (100 μM) induced DNA fragmentation in NEC-8 cells (Fig. 4B).

Discussion

Leukotrienes (LTs) are biologically active fatty acids derived from the oxidative metabolism of arachidonic acid (AA) (13,14) 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 CysLT₄ is specific for LTD₄. LTs are potent biochemical mediators, released from mast cells, eosinophils and basophils, that 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 (15). 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 (particularly LTB₄, LTC₄, LTD₄ and LTE₄). Our previous studies reported that 5-LOX was overexpressed in urological cancers and that the 5-LOX inhibitor may attenuate the growth of human urological cancers and induce apoptosis through the AA pathway (4,8-10,16). Based on these findings, we examined whether or not CysLT₁R is expressed in human

testicular cancer (TC) tissues, and whether or not it would, as an antagonist, prevent TC cell growth.

The present study revealed CysLT₁R to be strongly expressed in TC tissue. We also found, by RT-PCR, that CysLT₁R was expressed in the TC NEC-8 cell line. RT-PCR determined CysLT₁R expression to be down-regulated by 100 μM CysLT₁R antagonist.

Additionally, co-incubation of NEC-8 with CysLT₁R antagonist was demonstrated to stop the growth of NEC-8 cells and to potentially inhibit cell growth in a dose-dependent manner, determined by the MTT assay. These results indicate that CysLT₁R is essential for the cell growth of NEC-8 cells. The mechanism by which the CysLT₁R antagonist suppressed growth in NEC-8 cells required clarification. To address this issue, we examined whether or not apoptosis was involved in growth suppression in these cancer cells. The CysLT₁R antagonist (100 μM) strongly induced early apoptosis in NEC-8 cells, as shown by flow cytometry. Our results indicate that apoptosis may be involved in mechanisms related to the CysLT₁R antagonist, thus preventing cell growth in NEC-8 cells. These findings therefore provide initial confirmation that, through apoptosis, the CysLT₁R antagonist inhibits TC cell growth.

Regarding CysLT₁R and colon cancer, Ohd *et al* reported that CysLT₁R was overexpressed in human colorectal cancer and was significantly correlated with COX-2 and 5-LOX. The expression of CysLT₁R was higher in high-grade and early-stage carcinoma, suggesting typical differences in colon cancer (6). Furthermore, survival time was slightly shorter in patients



SPANDIDOS PUBLICATIONS -intensity CysLT₁R staining than in those with low-staining (7). We also reported that CysLT₁R was

overexpressed in human prostate cancer and renal cell carcinoma, and that its expression was higher in high-grade compared to low-grade cancer. Furthermore, the CysLT₁R antagonist inhibited prostate cancer and renal cell carcinoma cell growth through apoptosis (17,18). These reports suggest that there are numerous relationships between CysLT₁R and various types of cancers, and that the CysLT₁R antagonist can prevent cell growth in other types of cancer besides TC.

In conclusion, our study provides evidence that the cell growth and apoptosis of TC cells are among the pathways or mechanisms related to CysLT₁R. Growth inhibition of TC cells by blocking CysLT₁R was associated with the induction of apoptosis. Thus, the CysLT₁R antagonist provides a novel approach to anticancer therapies.

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