

Full Paper

Blockade of Leukotriene B₄ Signaling Pathway Induces Apoptosis and Suppresses Cell Proliferation in Colon Cancer

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Abstract. We investigated whether leukotriene B₄ (LTB₄) and its signaling pathway play an important role in the progression of human colon cancer via a direct stimulation of cancer cell proliferation. Remarkable expression of LTB₄ receptor 1 (BLT1) in human colon cancer tissues was detected by immunohistochemistry, and Western blot analysis revealed the BLT1 expression in cultured human colon cancer cell lines, Caco2 and HT29. The 5-lipoxygenase inhibitor AA-861 and LTB₄-receptor antagonist U75302 showed negative effects on survival and proliferation of both Caco2 and HT-29 cells. The inhibition of cell proliferation is due to the apoptosis because nuclear condensation and increased annexin V expression were observed in the cells treated with AA-861 and U75302. Knockdown of BLT1 by small interfering RNA caused the suppression of BLT1 protein, resulting in the inhibition of cancer cell proliferation. Blockade of BLT1 by the receptor antagonist significantly suppresses the LTB₄-stimulated extracellular signal-regulated kinase (ERK) activation in colon cancer cells. These results indicate that the blockade of the LTB₄-signaling pathway induces apoptosis via the inhibition of ERK activation in colon cancer cells. The LTB₄-signaling pathway might be a new therapeutic target for colon cancer.

Keywords: colon cancer, leukotriene B₄ (LTB₄), 5-lipoxygenase (5-LOX), carcinogenesis, apoptosis

Introduction

Chronic inflammation of the colon and rectum has been implicated in dysplasia and colon carcinogenesis (1–3). In addition, chronic inflammation has been reported to promote the conversion of premalignant colonic adenoma cells into malignant adenocarcinomas in nude mice (4). Thus, chronic and excessive inflammatory conditions may lead to the formation of malignant adenocarcinoma in the colon. However, it is still unknown what kinds of inflammatory mediators are involved in colon carcinogenesis.

Leukotriene B₄ (LTB₄), a potent chemotactic lipid mediator in inflammation, plays important roles in

controlling inflammatory responses via LTB₄-receptor signaling. Two types of receptors, LTB₄ receptor 1 (BLT1) and receptor 2 (BLT2) are known, and BLT1 is mainly involved in inflammatory responses (5, 6). There are two major metabolic pathways for the arachidonic acid cascade: cyclooxygenase (COX) and lipoxygenase (LOX) pathways; LTB₄ is generated from arachidonic acid via the LOX pathway (6). The COX pathway, particularly including COX-2, has been widely studied because it is well known that the COX-2 and its metabolites play important roles in carcinogenesis in the colon (7–9). In contrast, little is known about the role of the LTB₄-signaling pathway on the carcinogenesis of colon cancer. Recently, several studies have demonstrated that inhibition of the 5-LOX pathway suppresses the proliferation of various cancer cells such as pancreatic, urological, and prostate cancer cells (10–12). In addition, Gregor et al. reported that combination of

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COX-2 and 5-LOX inhibition significantly decreased liver metastasis in ductal pancreatic cancer cells compared to single inhibition of COX-2 in Syrian hamster (13).

Based on the above evidences, we hypothesized that LTB₄ and its signaling pathway may play an important role in the progression of human colon cancer via a direct stimulation of cancer cell proliferation. To clarify the hypothesis, we showed the marked expression of BLT1 in both human colon cancer tissues and colonic cancer cell lines. In addition, we clearly showed that LTB₄ significantly stimulated the proliferation of colonic cancer cells, whereas the 5-LOX inhibitor AA-861 and selective LTB₄-receptor antagonist U75302 inhibited the proliferation and induced apoptosis. Furthermore, knockdown of BLT1 by small interfering RNA resulted in inhibition of cancer cell proliferation. These results presented in this study clearly suggest that the LTB₄-receptor signaling pathway directly controls the proliferation of tumor cells in the colon.

Materials and Methods

Reagents and antibodies

The polyclonal BLT1 receptor antibody was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The DAB substrate kit, 3,3-diaminobenzidine, and the Vectastain Elite ABC kit (rabbit IgG) for immunohistochemical staining were from Vector Laboratories (Burlingame, CA, USA). Mayer's hematoxylin solution was from Wako Pure Chemical Industries (Osaka). Fetal bovine serum (FBS) was from ICN Biomedicals (Aurora, OH, USA). AA-861, U75302, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bis-benzimide (Hoechst 33342), LTB₄, the Annexin-V FITC Apoptosis Detection kit, and the anti-phospho-ERK 1/2 were from Sigma Chemical Co. (St. Louis, MO, USA). The cell proliferation ELISA kit (BrdU, colorimetric) was purchased from Roche Diagnostics (Mannheim, Germany). Dye reagent concentrate for protein assay and Ready gels J were from Bio-Rad Laboratories (Hercules, CA, USA). The membrane optimized for protein transfer and the ECL Western Blot analysis system were from Amersham Biosciences Co. (Piscataway, NJ, USA). The anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) rabbit polyclonal antibody was from Trevigen, Inc. (Gaithersburg, MD, USA). Lipofectamine 2000 and The Stealth RNAi negative control kit were from Invitrogen Japan KK (Tokyo).

Tissue samples

Ten samples of colon adenocarcinoma tissue were

randomly selected from the surgical specimens of patients who underwent surgery at Yokohama City University Hospital after obtaining their informed consent. Ten normal colon tissues were also obtained from patients as controls. All specimens were processed for histology by conventional methods, and 4- μ m tissue sections were prepared from paraffin blocks and mounted on glass slides.

Immunohistochemistry for LTB₄ receptor

After deparaffinization, the slides were immersed in methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to quench endogenous peroxidase activity. The slides were then washed in phosphate-buffered saline (PBS) and incubated for 20 min at room temperature with PBS containing 1.5% normal goat serum. Next, the slides were incubated for 18 h at 4°C with the primary antibody. The primary antibody against BLT1 (rabbit, polyclonal) was used at dilutions of 1:400 in PBS. The slides were then washed again in PBS and incubated for 30 min at room temperature with biotinylated secondary antibody against rabbit primary antibody. The biotinylated secondary antibody was used at dilutions of 1:200 in PBS containing 1% normal goat serum. Next, the slides were washed in PBS, and after incubating for 30 min at room temperature with the Vectastain ABC reagent, the brown immunoperoxidase reaction was developed with the DAB substrate kit. Counterstaining was performed with Mayer's hematoxylin solution. To ensure the specificity of the primary antibodies, other tissue sections were processed without primary antibodies, as controls.

Cell culture and treatment with reagents

The human colon adenocarcinoma cell lines Caco2 and HT-29 cells were cultured in Dulbecco's modified Eagle medium and McCoy's 5A medium, respectively, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Cells were grown as monolayers in 10-cm culture dishes and incubated at 37°C under a humidified atmosphere of 5% CO₂ in air.

Cell survival and cell proliferation analysis

Cell survival and cell proliferation were determined by the tetrazolium dye (MTT) assay and 5-bromo-2'-deoxyuridine (BrdU) incorporation assay, respectively. Cells were plated in 96-well, flat-bottom microtiter plates at a density of 2×10^4 cells/well and allowed to attach. Twenty-four hours later, the cells were incubated in medium in the absence or presence of the specified experimental conditions as indicated in the figure captions. After treatment, the medium was removed. For MTT assay, serum-free medium containing MTT was

added to each well, and the cells were incubated for another 4 h. The crystalline precipitate of the formazan product generated by the living cells was dissolved in 20% SDS for 18 h at room temperature, and the absorbance of the solution was read on the ELISA reader at 595 nm. For the BrdU incorporation assay, 10 μ M BrdU was added to each well, and the cells were incubated for 2 h to label them with BrdU. The labeling medium was then removed, and cells were fixed and incubated with anti-BrdU-POD solution for another 90 min. After the antibody-conjugate was removed, cells were washed three times. The substrate solution (tetramethyl-benzidine) was then added to each well, and incubation was continued at room temperature until color development. The peroxidase reaction was stopped by adding 25 μ l of 1M H₂SO₄ to each well and mixing thoroughly. The absorbance of the samples was measured in the ELISA reader at 450 nm, and the reference wavelength was 690 nm.

Western blot analysis

To analyze of LTB₄-receptor expression, Caco2 and HT-29 cells were scraped out into medium and pelleted by centrifugation at 1200 rpm for 10 min. The supernatant was removed, and the cells were resuspended in Chaps cell extract buffer. The cell suspensions were frozen and thawed three times and then centrifuged at 14,000 rpm for 20 min. To analyze extracellular signal-regulated kinase (ERK) activation, cells were lysed in lysis buffer [50 mM Tris (pH 7.5), 100 mM EDTA, 0.5% Triton X-114, 0.5% Triton X-100, 10 μ l/ml protease inhibitor cocktail, and 1 mM PMSF] by incubating for 20 min at 4°C. The protein concentration was determined with dye reagent concentrate. The samples were heated with SDS sample buffer at 95°C – 100°C for 5 min. After cooling on ice, the samples were centrifuged for 5 min. The samples were then loaded onto 12% SDS-polyacrylamide gel, and the proteins were electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk solution and incubated with primary antibodies overnight at 4°C. The primary antibodies were used at dilutions of 1:1000 in 3% dry milk. The membranes were washed three times in TBS and 0.05% Tween 20 and incubated with secondary antibody for 1 h at room temperature. After washing three times in TBS and 0.05% Tween 20, the blots were visualized with an ECL Western Blot analysis system.

Hoechst 33342 staining for nuclear condensation

Apoptotic cells were detected by nuclear staining with Hoechst 33342 as described previously (14). Briefly, cells were seeded in 24-well plates at a density of 10⁵

cells/well and allowed to attach. After 24 h, the cell culture medium was replaced with serum-free medium. The cells were then treated for 48 h with AA-861 (30 μ M) serum-containing medium, and both detached and attached cells were harvested with 0.25% trypsin/EDTA. The cells were then pelleted by centrifugation at 1,000 rpm for 10 min, and after fixation in 4% paraformaldehyde, they were stained with 5 μ g/ml of Hoechst 33342.

Flow cytometry for detection of apoptotic cells

HT-29 cells (2×10^6) were seeded in 6-cm culture dishes, and after incubation at 37°C for 48 h, the cells were treated for 24 h with AA-861 (30 μ M) or U75302 (5 μ M) in serum-containing medium. The cells were then scraped out into medium, and after pelleting by centrifugation at 3000 rpm for 10 min, the supernatants were removed, and the pelleted cells were resuspended in binding buffer. Annexin V-FITC conjugate and propidium iodide (PI) solution was added to each cell suspension, and the samples were incubated at room temperature for 10 min in the dark. Cell fluorescence was measured immediately with a FACScan flow cytometer from BD Biosciences (San Jose, CA, USA). At least 12,000 cells were examined in the gated region used for calculation. Dual parameter cytometric data were analyzed by using CellQuest software from BD Biosciences.

LTB₄-receptor siRNA transfection experiments

Stealth small interfering RNAs (siRNA) corresponding to BLT1 genes were obtained and annealed commercially (Invitrogen Japan KK). The following LTB₄-receptor gene-specific sequences were chemically synthesized: stealth siRNA-LTB₄R sense 5'-UACUCCACACCACAAAGCUGUUGCC-3' and antisense 5'-GGCAACAGCUUUGUGGUGUGGAGUA. Non-specific siRNA, stealth RNAi negative control kit, was used as a negative control. LTB₄-receptor siRNA was transfected into Caco2 and HT-29 cells with Lipofectamine 2000 reagent. To put it briefly, 2×10^5 cells were plated in 6-cm culture dishes and incubated in serum-containing medium without antibiotics for 24 h. Lipofectamine 2000 was diluted in Opti-MEM I medium without serum and incubated for 5 min. LTB₄-receptor siRNA and negative control siRNA were each diluted in Opti-MEM I medium without serum and added to the diluted Lipofectamine 2000. The mixture was incubated for 20 min at room temperature to allow the siRNA/negative control siRNA: Lipofectamine 2000 complexes to form. An 80- μ l aliquot of complexes was added to each dish, and cells were incubated for another 48 h without replacement of the medium.

Finally, LTB₄-receptor expression in treated cells was analyzed by Western blotting as described above.

Statistical analyses

Statistical comparisons were made using Student's *t*-test or Scheffe's method after an analysis of variance (ANOVA). The results were considered significantly different at $P < 0.05$.

Results

Expression of LTB₄ receptor in human colonic cancer tissues and cultured cell lines

The immunohistochemical studies revealed the strong expression of BLT1 in all of the human colon cancer tissues (10 of 10 samples positive, Fig. 1A). The staining was seen not only in lymphocyte-infiltrated areas but also in adenocarcinoma cells. In contrast, the expression was not seen in any of the normal colon tissues (none of 10 samples are positive). BLT1 expression was also detected in the tissues from most adenomas (8 of 10 samples are positive), but the staining intensity was much weaker than that in the cancer tissue (data not shown).

Western blot analysis revealed that strong expression of BLT1 protein was observed in established human colon cancer cell lines, Caco2 and HT-29 (Fig. 1B). These results for expression of the BLT1 in both colon cancer tissues and cultured cancer cell lines indicate that the LTB₄-receptor signaling pathway may play an important role in colon cancer.

Effect of LTB₄ on the proliferation of colon cancer cells

To investigate the effect of LTB₄ signaling on the proliferation of colon cancer cells, we applied LTB₄ itself to HT-29 and Caco2 cells. As shown in Fig. 2, LTB₄ stimulated the proliferation of both Caco2 and HT-29 cells (Fig. 2: A and B, respectively). Stimulated proliferation by LTB₄ was time-dependent (data not shown), although it seemed to show a bell-shaped dose-response curve. Namely, the proliferation rate of HT-29 cells was 137%, 128%, and 119 % at the concentration of 10^{-12} , 10^{-10} , and 10^{-8} M, respectively (Fig. 2A). Similar results were observed when Caco2 cells were treated with LTB₄ (Fig. 2B). However, the stimulatory effect of LTB₄ on cancer cell proliferation was not so dramatic in our present study.

Effect of 5-LOX inhibition on the survival and proliferation of colon cancer cells

As supplementation of LTB₄ itself showed weak stimulation of cell proliferation, we therefore investigated the effect of the inhibition of LTB₄ synthesis. As

the 5-LOX is a critical enzyme to synthesize LTB₄, we examined the effect of the specific 5-LOX inhibitor AA-861 on the survival of colon cancer cells (15). Treatment with AA-861 significantly decreased both Caco2 and HT-29 cell survivals in a dose-dependent manner (Fig. 3A). In addition, we investigated the effect of 5-LOX inhibition by AA-861 on colon cancer cell proliferation using the BrdU incorporation assay. As shown in Fig. 3B, AA-861 showed a dose-dependent inhibition of proliferation on Caco2 and HT-29 cells proliferation. These results indicated that 5-LOX metabolites, especially LTB₄, might play important roles of colon cancer cell survival and proliferation.

Effect of the LTB₄-receptor blockade on human colon cancer cell survival and proliferation

To clarify whether the inhibition of proliferation observed in the cancer cells treated with 5-LOX inhibitor is due to the reduction of LTB₄ and its receptor signaling, we investigated the effect of the selective BLT1 antagonist U75302 on colon cancer cell proliferation and survival. As shown in Fig. 3C, U75302 dose-dependently decreased the survival of both Caco2 and HT-29 cells. Similarly, U75302 inhibited the proliferation both of Caco2 and HT-29 cells as assessed by the BrdU incorporation assay (Fig. 3D). These results clearly indicated that the blockade of LTB₄-receptor signaling induced the suppression of cell survival and proliferation.

Induction of colon cancer cell apoptosis by 5-LOX inhibitors and BLT1 antagonist

To determine whether the 5-LOX-induced inhibition of cell proliferation and decrease in cell survival were attributable to apoptosis, we investigated nuclear condensation, a specific morphological change associated with apoptosis, by chromatin staining with Hoechst 33342 on colon cancer cells treated with AA-861. As shown in Fig. 4A, Hoechst 33342 staining clearly showed the nuclear condensation in HT-29 cells treated with AA-861. To confirm the apoptotic effect of AA-861, we also performed flow cytometric analysis by double staining with Annexin V-FITC and PI. As shown in Fig. 4B, the percentage of Annexin V-positive/PI-negative cells, that is, apoptotic cells, was increased to 24.7% when cells were treated with AA-861.

Similar results about the chromatin condensation and Annexin V expression were observed when cells were treated with the BLT1 antagonist U75302 (Fig. 4: A and B). Based upon the results observed in our present study, it is strongly indicated that the blockade of LTB₄-receptor signaling caused apoptosis resulting in the decrease in cell survival.

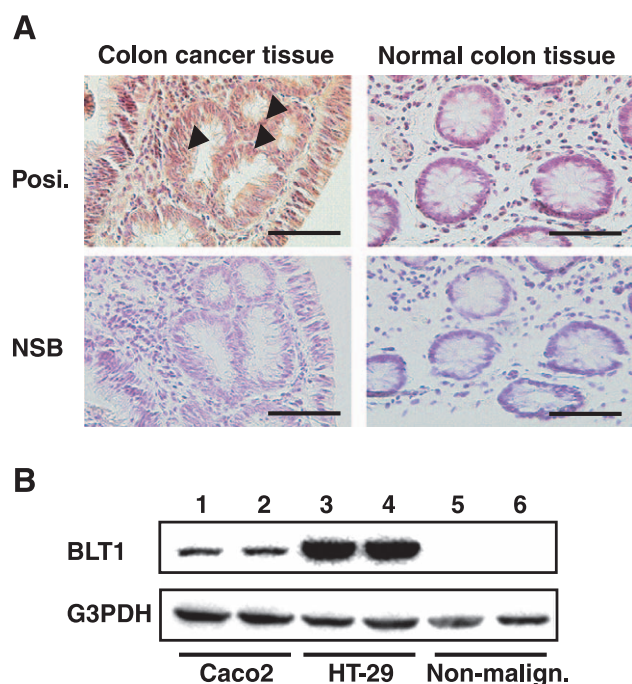


Fig. 1. Expression of LTB_4 receptor in human colon cancer tissues and cultured cell lines. A: Human colonic adenocarcinoma with strong positive staining (left panel, brown color, arrowheads) and the normal colon tissue (right panel). Lower two panels represent non-specific bindings (NSB). Counterstaining was performed with Mayer's hematoxylin solution. Scale bar: $100\ \mu\text{m}$. B: Expression of LTB_4 -receptor 1 protein (BLT1) in human colon cancer cell lines. Lanes 1 and 2: Caco2, Lanes 3 and 4: HT-29, Lanes 5 and 6: neuronal cells differentiated from neural stem cells (non-malignant transformation). G3PDH is an internal control to adjust the sample loading.

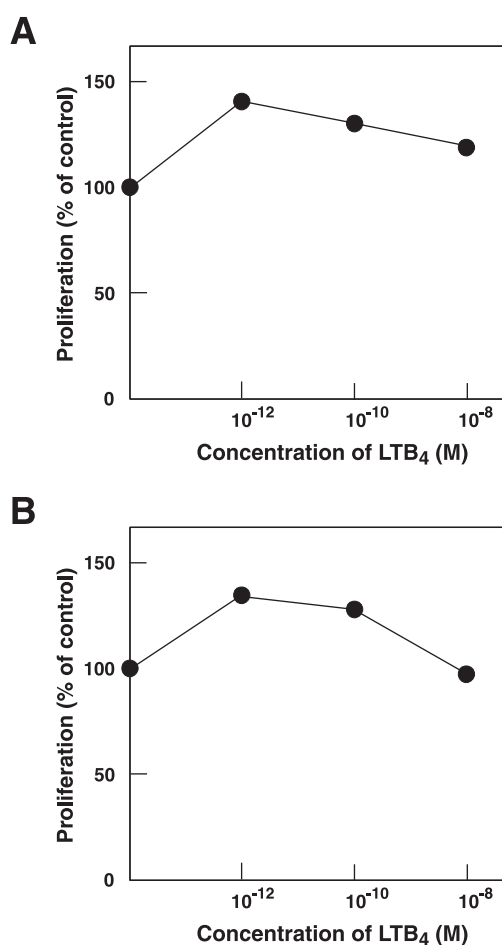


Fig. 2. Effect of LTB_4 on the proliferation of human colon cancer cells, HT-29 (A) and Caco2 (B). Cells were treated with 10^{-8} – 10^{-12} M of LTB_4 for 48 h. BrdU incorporation was used to determine cell proliferation rate. The results are each expressed as a percentage of the proliferation rate of the control. Each data point is expressed as the mean from 3 independent experiments.

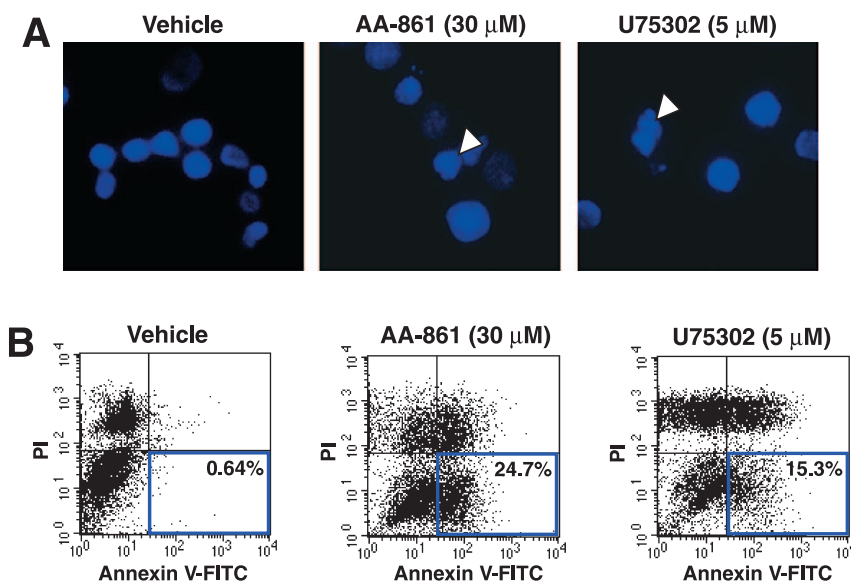


Fig. 4. Induction of colon cancer cells apoptosis by AA-861 and U75302. A: Evaluation of apoptosis by Hoechst 33342 staining. HT-29 cells were treated with $30\ \mu\text{M}$ of AA-861 or $5\ \mu\text{M}$ of U75302 for 48 h at 37°C . Both detached and attached cells were harvested, and stained with Hoechst 33342. The nuclear morphology of cells was observed by fluorescence microscopy to evaluate cell apoptosis. $400\times$ original magnification. Apoptotic cells are indicated by arrowheads. B: Flow cytometric analysis with Annexin V-FITC/PI staining of human colon cancer cells. Numbers indicate the percentage of early apoptotic cells (Annexin V-FITC positive, PI negative) in bottom right quadrant (Blue squares). Typical data are representative of 3 independent experiments.

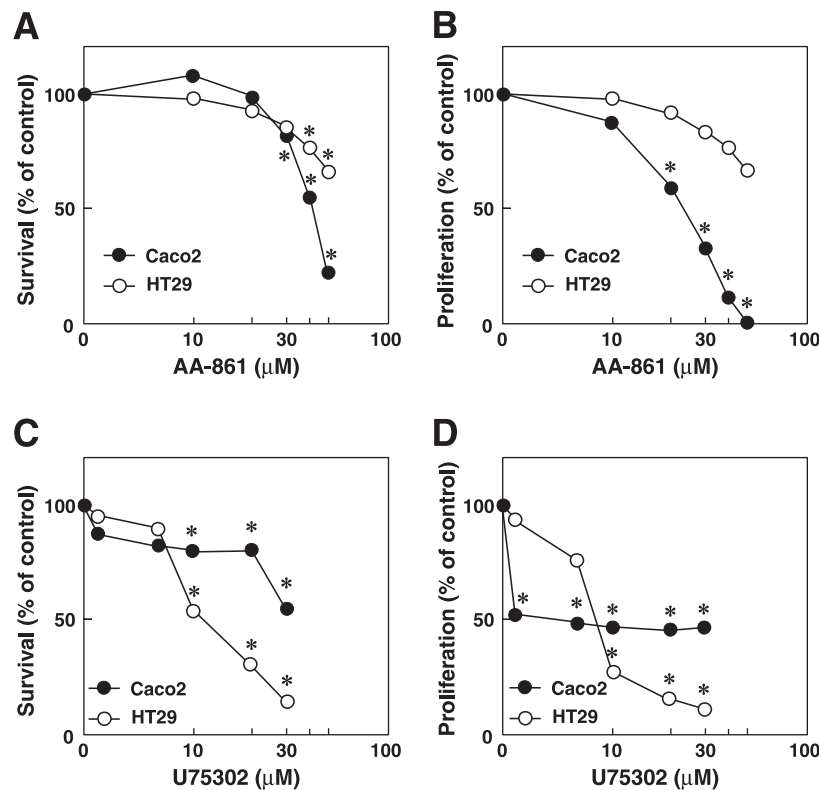


Fig. 3. Effects of AA-861 or U75302 on the survival and proliferation of colon cancer cells. A and B: Effect of AA-861 on human colon cancer cell survival (A) and proliferation (B). C and D: Effect of U75302 on human colon cancer cell survival (C) and proliferation (D). Cells were incubated with AA-861 or U75302 for 48 h at 37°C. The results are each expressed as a percentage of the control. Each data point is expressed the mean \pm S.E.M. from 3 independent experiments. * $P < 0.05$.

Inhibition of LTB₄-receptor expression by RNAi in colon cancer cell lines suppresses cell proliferation

To confirm the inhibitory effect of LTB₄-receptor signaling blockade on the cell proliferation, we applied siRNA corresponding to BLT1 genes. The gene-specific sequence of siRNA down-regulated BLT1 expression in both Caco2 (Fig. 5A) and HT29 (data not shown) cells. Compared with the negative control group, siRNA suppressed BLT1 expression by 77.15% at 20 nM in Caco2 cells. The decrease in BLT1 expression by siRNA caused the reproducible inhibition of Caco2 cell proliferation compared to the negative control (Fig. 5B).

Effect of the blockade of BLT1 on LTB₄-induced activation of ERK

To investigate the mechanisms of the inhibition of proliferation by blockade of LTB₄ signaling, we examined whether LTB₄ signaling is involved in the activation of the ERK pathway in colon cancer cells. LTB₄ itself induced phosphorylation of ERK 15 min after the treatment in both Caco2 (Fig. 6: A and B) and HT-29 cells (data not shown). The LTB₄-induced phosphorylation of ERK was reproducibly inhibited when

cells were treated with the BLT1 antagonist U75302. These results indicate that ERK activation elicited by LTB₄ signaling via BLT1 may be involved in LTB₄-induced stimulation of colon cancer cell proliferation.

Discussion

Several studies reported that inhibition of the 5-LOX pathway suppresses the proliferation of various cancer cells (10 – 13). Since the major metabolite in the 5-LOX pathway is LTB₄, the involvement of LTB₄ in carcinogenesis in various organs has also been indicated (16, 17). In addition, overproduction of LTB₄ in human colon cancer tissue and rat esophageal adenocarcinomas (18, 19) and LTB₄-mediated proliferation of colon cancer cells were reported (20, 21). Thus, many fragmentary studies indicate the critical role of the LTB₄-signaling pathway on the proliferation of colon cancer cells, but stronger evidences, such as direct proof of LTB₄-receptor expression in colon cancer tissue and knock-down of the LTB₄ receptor itself by the siRNA approach, are required for definitive proof.

In the present study, we clearly demonstrated the

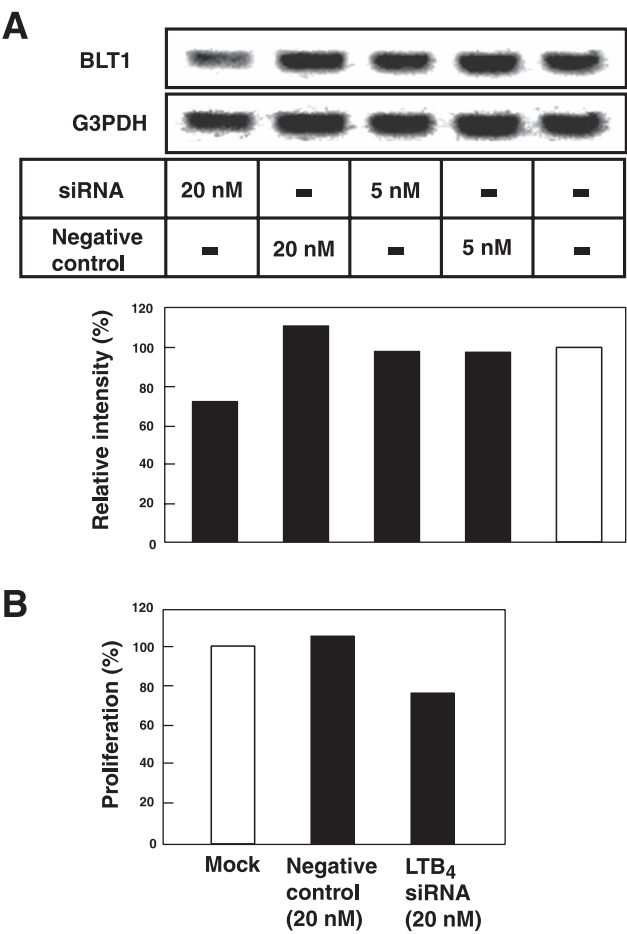


Fig. 5. Knockdown of LTB₄-receptor expression by siRNA in colon cancer cell lines (A) and inhibition of proliferation (B). The Stealth siRNA demonstrated successful knockdown of the LTB₄-receptor 1 (BLT1) expression in Caco2 cells at 20 nM and resulted in the inhibition of cell proliferation. The data are the mean from 3 independent experiments.

strong expression of BLT1 in the cancerous regions of human colon tissues, but not in the normal regions. BLT1 expression was also detected in the tissues from adenomas, but the staining intensity was much weaker than that in the cancer tissues. These results strongly indicate the involvement of the LTB₄-receptor signaling pathway in the colon cancer. BLT1 expression was also confirmed in the cultured human colon cancer cell lines Caco2 and HT-29, but not in non-malignant neural stem cells. It is well known that the LTB₄ receptor, BLT1, is almost exclusively expressed in peripheral leukocytes and to a much lesser extent in the thymus and spleen (5, 6). However, Western blot analysis revealed that strong expression of BLT1 protein was observed in established human colon cancer cell lines, Caco2 and HT-29. These findings and previous reports indicate that the LTB₄-receptor signaling pathway may be required for cancer

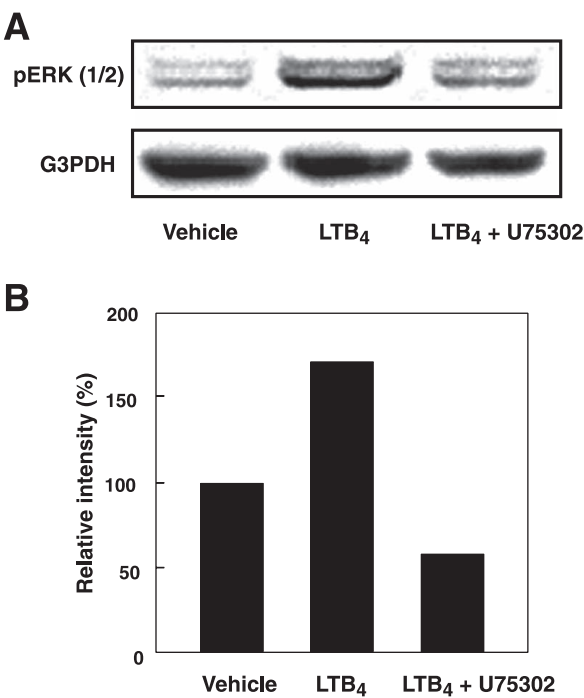


Fig. 6. Effect of U75302 on LTB₄-induced activation of ERK. A: Typical photographs of LTB₄-induced phosphorylation of ERK (pERK) 15 min after the treatment in Caco2 cells. B: Relative intensity of the results from panel A. The percentages compared to the vehicle control after the correction by G3PDH, an internal control, are expressed. The data are the mean from 3 to 5 independent experiments.

cell growth and invasion.

Based on the above evidences, we hypothesized that LTB₄ and its signaling pathway may play an important role in the progression of human colon cancer via a direct stimulation of cancer cell proliferation. To clarify this hypothesis, we showed that LTB₄ itself increased the growth of both Caco2 and HT-29 cells. However, the stimulation of cell proliferation by LTB₄ itself was not so dramatic. One of the reasons for our results might be the instability of LTB₄ itself in culture conditions (5, 6) or the endogenous LTB₄ level might be already high enough to stimulate the proliferation. We, therefore, applied a 5-LOX inhibitor and BLT1 antagonist to suppress the LTB₄ level and block the receptor, respectively. Both of the 5-LOX inhibitor and BLT1 antagonist showed dramatic inhibition of cell survival and proliferation. These results clearly indicate that endogenously synthesized LTB₄ and its receptor pathway play the critical role in cell survival and proliferation of colon cancer, and the blockade of the signaling pathway causes the apoptotic cell death. In fact, nuclear condensation and Annexin V expression, markers of apoptosis, were observed when cells were treated with the 5-LOX inhibitor and BLT1 antagonist. Similar results have

been reported for the treatment with an LTB₄-receptor antagonist, LY293111, on apoptosis in human cancer cells (10, 22).

The K_i value of U75302 derived from binding assay is much lower than the IC₅₀ derived from culture cell proliferation and survival. The binding assay data was obtained using tissue membrane prepared from guinea-pig lung; that is, the most sensitive material was used the in vitro binding assay. In contrast, we used cultured cells in our present study, and cell culture medium contains fetal bovine serum and other reagents. We think, therefore, it is not surprising to see the difference between the K_i value derived from the binding assay and the IC₅₀ of cultured cell proliferation.

Although our data strongly suggested that the blockade of LTB₄-receptor signaling pathway suppressed cancer cell growth, definitive evidence is still required. Therefore, we determine whether knockdown of the LTB₄ receptor by the RNA interference approach can suppress the proliferation of colon cancer cells. Our results demonstrated that successful knockdown of the BLT1 by siRNA resulted in the inhibition of cell proliferation. These results, together with previous reports, strongly suggest that the LTB₄-receptor signaling pathway plays a critical role in the cell survival and proliferation of colon cancer cells.

However, it is still unclear how LTB₄-receptor signaling stimulates colon cancer cell proliferation. Several mechanisms for stimulation of cell proliferation by LTB₄-receptor signaling have recently been suggested. Induction of ERK phosphorylation by LTB₄ has been reported in human pancreatic cancer cells (10), and exogenous LTB₄ has been reported to stimulate ERK in rat fibroblasts (23). In the present study, we also demonstrated that LTB₄ induced phosphorylation of ERK in both Caco2 and HT-29 cells. In addition, LTB₄-induced phosphorylation of ERK was inhibited by the treatment with the BLT1 antagonist U75302. The MAPK/ERK signaling pathway is known to be important for growth in many cell types (24, 25). Activation of ERK occurs through phosphorylation of threonine and tyrosine residues, and activated ERK dimer can regulate targets in the cytosol and the nucleus where it phosphorylates a variety of transcription factors resulting in regulation of gene expressions (24, 25). Therefore, our results indicate that the stimulation of cell proliferation by LTB₄ may be mediated by the activation of the ERK pathway via BLT1.

In conclusion, strong expression of the LTB₄ receptor was observed in colon cancer tissues and cultured cancer cell lines. Blockade of the LTB₄-receptor-signaling pathway significantly decreased cell survival and proliferation of colon cancer cells via induction of

apoptosis. Our findings have revealed the role of LTB₄ in the colon cancer cells and provide a new approach to prevent carcinogenesis in the colon.

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