Leukotriene B4 Receptor Antagonist LY293111 Inhibits Proliferation and Induces Apoptosis in Human Pancreatic Cancer Cells

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ABSTRACT

Purpose: The effects of leukotriene (LT) B4 and its receptor antagonist LY293111 on proliferation and apoptosis of human pancreatic cancer cells were investigated, both in vitro and in vivo.

Experimental Design: Six human pancreatic cancer cell lines (MiaPaCa-2, HPAC, Capan-1, Capan-2, PANC-1, and AsPC-1) were used. Expression of LTB4 receptors, BLT1 and BLT2, was measured by reverse transcription-PCR. Cell proliferation was measured by [methyl-3H]thymidine incorporation and cell number counting. Extracellular signal-regulated kinase (ERK) 1/2 activation was measured by Western blotting. Apoptosis was assessed by morphology, terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay, and poly(ADP-ribose) polymerase cleavage. The effect of LY293111 on growth of AsPC-1 and HPAC cell xenografts was assessed in BALB/c nu/nu athymic mice.

Results: Both LTB4 receptor types were found to be expressed in human pancreatic cancer cells. The LTB4 receptor antagonist LY293111 caused both time- and concentration-dependent inhibition of proliferation of all six human pancreatic cancer cell lines studied. In contrast, LTB4 stimulated proliferation of these cell lines and induced ERK1/2 phosphorylation. The growth-stimulatory effect and ERK1/2 phosphorylation induced by LTB4 were inhibited by LY293111. Coincident with growth inhibition, LY293111 induced apoptosis in these pancreatic cancer cell lines, as indicated by morphology, TUNEL assay, and poly-(ADP-ribose) polymerase cleavage. In studies using AsPC-1 and HPAC cell xenografts in athymic mice, LY293111 treatment markedly inhibited tumor growth over a 24-day treatment period, as measured by both tumor volume and tumor weight. In situ tissue TUNEL assay showed massive apoptosis in LY293111-treated tumor tissues.

Conclusions: LTB4 can directly regulate the growth of human pancreatic cancer cells and control their survival. Additional studies will clarify the underlying mechanisms of LTB4-regulated pancreatic cancer cell growth and apoptosis. LTB4 receptor blockade and inhibition of the downstream signal pathway are likely to be valuable for the treatment of human pancreatic cancer.

INTRODUCTION

Pancreatic adenocarcinoma is characterized by poor prognosis and lack of response to conventional therapy. Although its incidence is lower than that of many tumor types, it ranks as the fourth leading cause of cancer death for both men (after lung, prostate, and colon cancer) and women (after lung, breast, and colon cancer) in the United States (1, 2). The 5-year survival rate for it is <2%, and the median survival after diagnosis is <6 months, hence pancreatic cancer poses one of the greatest challenges in oncology (3, 4).

Accumulating evidence has linked pancreatic cancer growth with dietary fat, especially intake of ω-6 PUFAs,3 such as linoleic acid and arachidonic acid, which enhance tumorigenesis through the production of eicosanoids (5, 6). On the other hand, ω-3 PUFAs such as eicosapentaenoic acid and docosahexaenoic acid, which are found in high concentrations in fish oils, have anticancer effects (5, 6). It is widely held that ω-3 PUFAs inhibit cancer cell growth and induce apoptosis by prevention of lipid peroxidation (7). There are two major metabolic pathways of arachidonic acid, the COX pathway and the LOX pathway. Early investigations into the role of arachidonic acid metabolism in cancer have focused mainly on the COX pathway, particularly that involving the inducible COX-2 enzyme, because of the epidemiological observation that the incidence of colonic cancer was significantly reduced in regular users of aspirin and other nonsteroidal anti-inflammatory drugs (8). However, several groups have shown that both the 5-LOX

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3 The abbreviations used are: PUFA, polyunsaturated fatty acid; ERK, extracellular signal-regulated kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PARP, poly(ADP-ribose) polymerase; COX, cyclooxygenase; LOX, lipoxygenase; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; TdT, terminal deoxynucleotidyl transferase; NDGA, nordihydroguaiaretic acid; BLT1, LTB4 receptor 1; BLT2, LTB4 receptor 2.
and 12-LOX pathways also play important roles in promoting tumor growth (9–11). Previous studies in our laboratory revealed that both 5-LOX and 12-LOX mRNA and protein are expressed in human pancreatic cancer cells but not in normal human pancreatic ductal cells (12). The products of 5-LOX and 12-LOX, 5-HETE and 12-HETE, stimulate growth of human pancreatic cancer cells through activation of the ERK1/2 and Akt/protein kinase B pathways (13). Ding et al. (12, 14) have also shown that the general LOX inhibitor NDGA, the 5-LOX inhibitor Rev-5901, and the 12-LOX inhibitor baicalein inhibit human pancreatic cancer cell proliferation and induce apoptosis.

LTs constitute a class of potent biological mediators of inflammation and anaphylaxis. Their biosynthesis from 5-LOX is catalyzed by oxygenation of arachidonic acid in granulocytes, monocytes, and mast cells. LTB4 is produced from LTA4 through the LTA4 hydrolase, whereas LTC4, LTD4, and LTE4, which are formerly known as slow-reacting substance of anaphylaxis, are produced through a different pathway. LTC4 is first produced from LTA4 by LTC4 synthetase, and LTC4 can be further converted in turn to LTD4 and LTE4 by the successive elimination of glutamic acid and glycine residues (15, 16). LTB4 has been widely implicated in the pathogenesis of several inflammatory diseases, such as asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel disease (17). Although LTB4 is a final product of the 5-LOX pathway of arachidonic acid metabolism, its function in tumorigenesis has not been fully investigated. However, several pieces of evidence suggest that LTB4 may be as important as 5-HETE and 12-HETE in proliferation of cancer cells. Earashi et al. (18) found that LTB4 could reverse the inhibitory effect of NDGA on proliferation of the breast cancer cell line MDA-MB-231, whereas eicosapentaenoic acid and docosahexaenoic acid suppressed MDA-MB-231 cell growth and reduced the secretion of prostaglandin and LTB4. Okano et al. (19) showed that human melanoma cells express LTA4 hydrolase and generate LTB4 from LTA4. Bittner et al. (20) showed that LTB4 played an important role in glucocorticoid-induced inhibition of lymphoma growth, whereas dexamethasone treatment almost completely inhibited LT production. A study by EI-Hakim et al. (21) revealed a 10–30-fold increase in the level of LTB4 in oral squamous cell cancers compared with control normal tissues, suggesting a possible role of LTB4 in the pathogenesis of head and neck carcinoma. Cristina et al. (22) found that LTB4 stimulated the proliferation of colon cancer cell lines HT-29 and HCT-15 in a time- and concentration-dependent manner and that LTB4 receptors are expressed in colonic epithelial cells.

Based on the above evidence, the current study was aimed at investigating the importance of the LT4 metabolic pathway in pancreatic cancer cell growth and the effect of a selective LTB4 receptor antagonist, LY293111, on pancreatic cancer cell proliferation and apoptosis both in vitro and in vivo.

**MATERIALS AND METHODS**

**Materials.** DMEM, MEM, McCoy’s 5A medium, penicillin-streptomycin solution, trypsin-EDTA solution, proteinase K, propidium iodide, and RNase A were purchased from Sigma (St. Louis, MO). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). The APO-BRDU kits for TUNEL assay were from PharMingen (San Diego, CA). The DeadEnd Colorimetric Apoptosis Detection System for tissue sections was from Promega (Madison, WI). LTB4 and a selective LTB4 receptor antagonist, U75302, were from Cayman Chemicals (Ann Arbor, MI). The selective LTB4 receptor antagonist LY293111 was provided by Eli Lilly and Co. (Indianapolis, IN). The selective LTB4 receptor antagonist LY171883 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). [methyl-3H]Thymidine was from Amersham (Arlington Heights, IL). The monoclonal mouse phospho-ERK1/2 (Thr202/Tyr204) antibody and the monoclonal mouse ERK1/2 antibody were purchased from New England BioLabs (Beverly, MA). The monoclonal mouse antihuman PARP antibody was from Biomol Research Laboratories. All other chemicals were purchased from Sigma.

**Human Pancreatic Cancer Cell Lines and Cell Culture.** Six human pancreatic cancer cell lines were used: MiaPaCa-2 and HPAC (poorly differentiated), Capan-1 and Capan-2 (well differentiated), and Panc-1 and AsPC-1 (pleomorphic). These cell lines were purchased from American Type Culture Collection (Manassas, VA). HPAC and MiaPaCa-2 were grown in DMEM, Panc-1 and AsPC-1 were grown in MEM, and Capan-1 and Capan-2 were grown in McCoy’s 5A medium. Cells were plated as monolayers in the medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% O2 and 5% CO2 at 37°C. The cells were regularly seeded into 75-cm2 flasks with media changes every other day. For experiments, cells were grown to 70% confluence, digested with trypsin-EDTA, and plated in either 12- or 24-well plates at a concentration of 50,000 cells/ml as appropriate.

**Reverse Transcription-PCR Analysis of BLT1 and BLT2 mRNA Expression in Pancreatic Cancer Cells.** RNA was isolated from MiaPaCa-2 and AsPC-1 cells and leukocytes separated from human whole blood as described previously using RINazol B (TEL-TEST Inc., Friendswood, TX). RNA was reversed transcribed into cDNA using random hexamer and reverse transcriptase according to the manufacturer’s protocol (Perkin-Elmer GeneAmp kit; Perkin-Elmer, Foster City, CA). After the reverse transcription reaction, cDNA was amplified to determine both BLT1 and BLT2 mRNA expression using their specific primers. Primers for BLT1 are 5’-CACCCTTCCTTTTCCCTTCA-3’ (forward) and 5’-CAGGTTGAGTGGTTTTCCTG-3’ (reverse).

**Fig. 1** Expression of LTB4 receptor (BLT1 and BLT2) mRNA in human pancreatic cancer cells. Total RNA was isolated from MiaPaCa-2 and AsPC-1 pancreatic cancer cells as well as leukocytes. The RNA was reverse transcribed and then amplified by PCR using specific primers. The PCR products were separated on a 1% agarose gel with ethidium bromide and visualized under UV light. The PCR product for BLT1 is 830 bp, and the PCR product for BLT2 is 312 bp. A representative gel from three separate experiments is shown.
TTCGTTTAACCT-3' (reverse). Primers for BLT2 are 5'-AGGCTGGAGACTCTGACCGCTTTCG-3' (forward) and 5'-GACGTAGAGCACCGGGTTGACGCTA-3' (reverse). The PCR products for BLT1 and BLT2 are 830 and 321 bp, respectively. The PCR profile was 94°C for 30 s, 58°C (for BLT1) or 68°C (for BLT2) for 30 s, and 72°C for 30 s for 30 cycles. The final PCR products were separated on a 1% agarose gel with ethidium bromide and visualized under UV light.

**DNA Synthesis by [methyl-3H]Thymidine Incorporation.** Cells were plated in either 12- or 24-well plates at a concentration of 50,000 cells/well. After reaching 50% confluence, they were incubated in serum-free medium for 24 h, which was then replaced with fresh serum-free medium with or without the appropriate treatments. After the required period of culture, cellular DNA synthesis was assayed by adding 0.5μCi [methyl-3H]thymidine/well and incubating cells for another 2 h. Then the cells were washed twice with PBS, fixed with 10% trichloroacetic acid for 30 min, and solubilized by adding 250 μl of 0.4 M NaOH to each well. Radioactivity, indicating incorporation of [methyl-3H]thymidine into DNA, was measured by adding scintillation mixture and counting on a scintillation counter (LKB RackBeta; Wallac, Turku, Finland).

**Cell Proliferation Assay.** Cells were regularly seeded into 12-well microplates and incubated at 37°C for 24 h. Cells were then cultured in serum-free medium for another 24 h and treated in fresh serum-free medium with or without 250 nM LY293111 for 24, 48, and 72 h. At the end of each time period, the cells were trypsinized to produce a single cell suspension, and the cell number in each well was determined using a Z1-Coulter Counter (Luton, Bedfordshire, United Kingdom).

**Morphological Changes Using Light Microscopy.** Pancreatic cancer cells grown in 75-cm² flasks were treated with different concentrations of LY293111 for different periods of time. Cells were then viewed using light microscopy, and digital images were taken with a Kodak DC120 zoom digital camera (Eastman Kodak Co., Rochester, NY).

**TUNEL Assay.** Cells were treated with LY293111 in serum-free medium for the appropriate period of time and then digested with trypsin-EDTA, washed twice with ice-cold PBS, and fixed in 10% paraformaldehyde on ice for 20 min. Cells were then washed with PBS, permeabilized with 70% ethanol for at least 4 h, washed again with PBS, and incubated with 2.5 units of TdT enzyme and 100 pmol of bromo-dUTP in DNA labeling solution for 1 h at 37°C. Cells were then rinsed twice in PBS and incubated in 10% normal horse serum for 50 min. The cells were then incubated with a rabbit anti-digoxigenin antibody for 45 min, washed with PBS, and finally incubated with the BCIP/NBT solution for visualization of the TUNEL staining. Digital images were taken with a Kodak DC120 zoom digital camera (Eastman Kodak Co., Rochester, NY).

**Table 1.** Concentration-dependent growth inhibition induced by LY293111 in human pancreatic cancer cells at 24 h

<table>
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<th>Cells</th>
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<td>103.6 ± 8.9</td>
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<td>87.3 ± 9.8</td>
<td>51.2 ± 7.9</td>
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<td>8.9 ± 3.3</td>
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<tr>
<td>PANC-1</td>
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<td>43.5 ± 11.2</td>
<td>12.4 ± 3.7</td>
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* P < 0.05.
** P < 0.001.
*** P < 0.01.

Fig. 2 Effects of different concentrations of LY293111 on proliferation of (A) MiaPaCa-2 and (B) AsPC-1 human pancreatic cancer cells after 24 h, as measured by [methyl-3H]thymidine incorporation. Time course effects of 500 nM LY293111 on proliferation of (C) MiaPaCa-2 and (D) AsPC-1 human pancreatic cancer cells after 6, 12, and 24 h. Results are expressed as a percentage of control. *, P < 0.05 compared with control; **, P < 0.01 compared with control; ***, P < 0.001 compared with control. Data represent results from four separate experiments.
PBS and resuspended in 0.1 ml of fluorescein labeled antibrornodeoxyuridine antibody solution in the dark for 30 min. Then, 0.5 ml of propidium iodide/RNase A solution was added, and the cells were analyzed by flow cytometry at 488 nm excitation.

**Western Blotting.** For analysis of ERK1/2 phosphorylation, cells were either treated with LTb4 and LY293111 alone or pretreated with LY293111 for 2 h and then treated with LTb4 for 10 min. For measurement of PARP cleavage, cells were treated with 250 nM LY293111 for 6, 12, 24, and 48 h. After treatment, cells were scraped into lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM sodium vanadate, 1.0 mM sodium fluoride, 100 mM NaCl, 2.0 mM phosphate substrate, 1% NP40, 0.5% sodium deoxycholate, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25.0 μg/ml pepstatin, 2.0 mM EDTA, and 2.0 mM EGTA] at 4°C. Cell lysates were clarified by microcentrifugation at 12,000 × g after incubation on ice for 25 min. The supernatants were recovered, and their protein concentrations were measured using Bio-Rad protein assay reagent. Equivalent amounts of cell lysate protein (30 μg) were resolved by 15% SDS-PAGE. Proteins were transferred to nitrocellulose membranes by electroblotting using a Bio-Rad semidry transfer blotting apparatus. Membranes were subsequently blocked in Tris-buffered saline and 0.1% Tween 20 and then incubated with appropriate antibodies (phospho-ERK1/2 and ERK1/2, 1:2000 dilution; PARP, 1:1000 dilution) in 5% nonfat milk overnight at 4°C. The membranes were then detected by chemiluminescence, and bound protein-antibody complexes were then incubated with horseradish peroxidase-conjugated goat antimouse secondary antibodies at a dilution of 1:2000 at 4°C. The membrane-bound protein-antibody complexes were then incubated with horseradish peroxidase-conjugated goat antimouse secondary antibodies at a dilution of 1:2000 for 2 h at room temperature. The membranes were then detected by chemiluminescence, and light emission was captured on Kodak X-ray films (Eastman Kodak Co.).

**Animal Studies.** Athymic nude mice (BALB/c nu/nu, 5-week-old females) were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Mice were acclimatized to the animal facility for 1 week before receiving xenografts. Three million AsPC-1 or HPAC human pancreatic cancer cells were injected into the flanks of nude mice, and once visible tumors were evidenced 4–5 days after injection, the animals were divided equally into two groups (6 animals/group) and treated with LY293111 (250 mg/kg/day) or control vehicle (sterile PBS, PBS) by daily gavage. Animal weight and tumor size were recorded every 3 days. The formula used for tumor volume is as follows: volume = (length) × (width) × (length + width)/2 × 0.526. After 24 days of treatment, the animals were euthanized, the tumors were dissected carefully, and tumor weights were measured.

**In Situ Tissue TUNEL Assay.** Paraffin-embedded tissue sections were deparaffinized in xylene for 5 min, rehydrated in gradient ethanol, and fixed with 4% paraformaldehyde. After permeabilizing tissues with proteinase K, they were refixed with 4% paraformaldehyde. The sections were then incubated with TdT enzyme and biotinylated nucleotide mix after endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Finally, the sections were incubated with 3,3′-diaminobenzidine and chromogen for 10 min and viewed microscopically with digital images recorded as described above.

**Statistical Analysis.** Data were analyzed by ANOVA with Dunnett’s or Bonferroni’s corrections for multiple comparisons, as appropriate. This analysis was performed with the Prism software package (GraphPad, San Diego, CA). Data were expressed as mean ± SE.

**RESULTS**

**Expression of BLT1 and BLT2 mRNA in Pancreatic Cancer Cells.** Two G protein-coupled receptors for LTb4 have been identified and cloned thus far, namely, BLT1 and BLT2. They have been shown to be different in their binding profile to LTb4 and also in their tissue distributions. BLT1, a high-affinity LTb4 receptor, is exclusively expressed in leukocytes, with a little expression in thymus and spleen (23, 24). BLT2, a low-affinity receptor for LTb4, is expressed relatively...
ubiquitously, with the highest expression in spleen, followed by ovary, liver, and leukocytes (25, 26). BLT2 open reading frame is present in the promoter region of BLT1, thus weaving these two receptors tightly at the both genomic and functional levels (25). Our results with reverse transcription-PCR have shown that the mRNA for both types of LTB4 receptors is expressed in MiaPaCa-2 and AsPC-1 pancreatic cancer cells as well as leukocytes separated from human blood, which serve as a positive control (Fig. 1).

The Selective LTB4 Receptor Antagonist, LY293111, Inhibits Proliferation of Human Pancreatic Cancer Cells.

The selective LTB4 receptor antagonist LY293111 caused both a concentration-dependent [Fig. 2, A and B; F(5,30) = 77.30, \( P < 0.001 \) for MiaPaCa-2; F(5,30) = 53.73, \( P < 0.0001 \) for MiaPaCa-2 and (D) AsPC-1 cells. Results are expressed as a percentage of control. *, \( P < 0.05 \) compared with control; **, \( P < 0.01 \) compared with control; ***, \( P < 0.001 \) compared with control. Data represent results from four separate experiments.

Fig. 4 Effects of a selective LTB4 receptor antagonist, U75302, on thymidine incorporation in (A) MiaPaCa-2 and (B) AsPC-1 pancreatic cancer cells. Effects of a selective LTD4 antagonist, LY171883, on thymidine incorporation in (C) MiaPaCa-2 and (D) AsPC-1 cells. Results are expressed as a percentage of control. *, \( P < 0.05 \) compared with control; **, \( P < 0.01 \) compared with control; ***, \( P < 0.001 \) compared with control. Data represent results from four separate experiments.

Fig. 5 Concentration-dependent effects of LTB4 on thymidine incorporation in (A) MiaPaCa-2 and (B) AsPC-1 human pancreatic cancer cells. Time-course effects of 100 nM LTB4 on thymidine incorporation in (C) MiaPaCa-2 and (D) AsPC-1 human pancreatic cancer cells. Results are expressed as a percentage of control. *, \( P < 0.05 \) compared with control; **, \( P < 0.01 \) compared with control; ***, \( P < 0.001 \) compared with control. Data represent results from four separate experiments.
AsPC-1 and time-dependent [Fig. 2, C and D; F(3,20) = 331.1, P < 0.0001 for MiaPaCa-2; F(3,20) = 328.8, P < 0.0001 for AsPC-1] inhibition of thymidine incorporation in both MiaPaCa-2 and AsPC-1 human pancreatic cancer cells. Similar effects were seen in all of the other cell lines studied (Tables 1 and 2). LY293111 inhibited proliferation by at least 50% at a concentration of 250 nM and by >95% at 1000 nM at 24 h. The inhibition of DNA synthesis and proliferation was followed by a decrease in cell number over a 3-day period with 250 nM LY293111. The cell number of untreated cells doubled about every 24 h, whereas the cell number of treated cells remained unchanged or decreased slightly (Fig. 3, A and B). To confirm the involvement of LTB4 receptors in mediating the effect of LY293111 on human pancreatic cancer cell proliferation, we also treated the cells with another selective LTB4 receptor antagonist, U75302, and with a selective LTD4 antagonist, LY171883. U75302 inhibited the proliferation of both MiaPaCa-2 and AsPC-1 human pancreatic cancer cells but did so less potently than LY293111 as expected from the lower receptor affinity of this drug (Fig. 4, A and B). Inhibition of proliferation of approximately 50% was seen with 5 μM U75302. In contrast, the selective LTD4 antagonist LY171883 had no effect on the proliferation of either of the human pancreatic cancer cell lines tested (Fig. 4, A and D).

**LTB4 Stimulates Human Pancreatic Cancer Cell Proliferation.** Because blocking LTB4 receptor inhibits proliferation of pancreatic cancer cells, we hypothesized that LTB4 may be a growth factor for human pancreatic cancer growth. The effects of LTB4 on proliferation of human pancreatic cancer cells were investigated. LTB4 stimulated proliferation in both a concentration-dependent [Fig. 5, A and B; F(3,11) = 95.57, P < 0.0001 for MiaPaCa-2; F(3,11) = 53.99, P < 0.0001 for AsPC-1] and time-dependent [Fig. 5, C and D; F(3,11) = 88.34, P < 0.0001 for MiaPaCa-2; F(3,11) = 129.4, P < 0.0001 for AsPC-1] manner in MiaPaCa-2, AsPC-1, and all of the other pancreatic cancer cell lines studied (Table 3). LY293111 was able to inhibit the stimulatory effect of LTB4 on human pancreatic cancer cell proliferation when cells were treated with a combination of 100 nM LTB4 and 250 nM LY293111 (Fig. 6, A and B).

**Fig. 6** Effect of LY293111 on LTB4-stimulated proliferation of (A) MiaPaCa-2 and (B) AsPC-1 human pancreatic cancer cells. Cells cultured in triplicate in 12-well plates in serum-free medium were treated with either 100 nM LTB4 or 250 nM LY293111 or a combination of both for 24 h. Results are expressed as a percentage of control. * compared with control; #, compared with LTB4 alone. Data represent results from three separate experiments.

**Table 3** Concentration-dependent growth stimulation induced by LTB4 in human pancreatic cancer cells at 24 h

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<td>123.8 ± 5.7a</td>
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<td>312.3 ± 11.6c</td>
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<td>Capan-1</td>
<td>100</td>
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<td>136.4 ± 10.5a</td>
<td>226.7 ± 12.3b</td>
<td>313.7 ± 14.6c</td>
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<tr>
<td>PANC-1</td>
<td>100</td>
<td>106.7 ± 3.7</td>
<td>189.6 ± 13.7a</td>
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a P < 0.05.
b P < 0.01.
c P < 0.001.

LTB4-induced phosphorylation of ERK1/2 after 10 min of treatment. LTB4-induced ERK1/2 phosphorylation was inhibited by pretreatment with LY293111 in both MiaPaCa-2 and AsPC-1 pancreatic cancer cells (Fig. 7). Total ERK1/2 was also measured to confirm that ERK1/2 protein content did not change after treatment. These results suggested that ERK1/2 phosphorylation is involved in LTB4-stimulated pancreatic cancer cell proliferation.

**LY293111 Induces Apoptosis in Human Pancreatic Cancer Cells.** LY293111 treatment induced dramatic morphological changes in human pancreatic cancer cells within 6 h after commencing treatment. Over time, the treated cells became rounded and exhibited membrane blebbing, chromatin condensation, and nuclear fragmentation and finally detached from the microplate (Fig. 8). These morphological changes have been previously interpreted as reflecting apoptosis.

LY293111-induced apoptosis was also evaluated by the TUNEL assay, in which DNA strand breaks can be detected by the incorporation of fluorescence-labeled dUTP in the presence of TdT enzyme. Treatment of both MiaPaCa-2 and AsPC-1 cells with 250 and 500 nM LY293111 for 24 h greatly increased apoptosis from 1.5% to 17.4% and 59.3% in MiaPaCa-2 cells and from 0.4% to 8.9% and 48.3% in AsPC-1 cells, respectively (Fig. 9). Again, similar results were obtained in the other pancreatic cancer cell lines.

To further investigate LY293111-induced apoptosis, cleavage of the caspase-3 substrate PARP was measured by Western blotting. Cleavage of the M, 116,000 PARP protein into a M, 85,000 fragment by activated caspase-3 is a hallmark of apoptosis. LY293111 induced time-dependent cleavage of PARP.
after 6, 12, 24, and 48 h in both MiaPaCa-2 and AsPC-1 cells (Fig. 10).

**LY293111 Inhibits Growth of AsPC-1 and HPAC Cancer Cell Xenografts and Induces Apoptosis in Vivo.** LY293111 not only inhibited proliferation of human pancreatic cancer cells in vitro but also inhibited growth of human pancreatic cancer xenografts in athymic mice. In experiments with AsPC-1 and HPAC human pancreatic cancer cells, LY293111 markedly inhibited tumor growth as measured by both tumor volume (Fig. 11, A and B) and tumor weight (Fig. 11, C and D). Furthermore, 50% of control animals showed s.c. metastases, but none were seen in the treated animals. There was no significant difference in body weight between the control and LY293111-treated animals throughout the entire treatment period (Fig. 11, E and F), although the untreated control animals were less active because of large tumor burden.

Tissue sections from tumor xenografts were subjected to in situ tissue TUNEL assay for the detection of apoptosis. In the control tumors, few apoptotic cells were seen. In contrast, localized apoptosis was visualized by the dark brown staining of the fragmented nuclei, especially in the middle of the tumors in LY293111-treated animals (Fig. 12). This indicates that LY293111 inhibits tumor growth in vivo by inducing tumor cell apoptosis.

**DISCUSSION**

Our previous studies and those of others have confirmed the importance of the LOX metabolic pathways of arachidonic acid in the proliferation of cancer cells (9–14). Both 5-LOX mRNA and 12-LOX mRNA are expressed in pancreatic cancer cells, but not in normal pancreatic ductal cells. 5-HETE and 12-HETE stimulate human pancreatic cancer cell proliferation, and blockade of either pathway using specific enzyme inhibitors blocks proliferation and induces apoptosis. The effects of LTs such as LTB4, LTC4, or LTD4 have not previously been investigated in pancreatic cancer cells. We hypothesized that one of these active metabolites derived from the 5-LOX pathway was likely to be important in the proliferation of pancreatic cancer cells. The present studies confirmed the expression of both types of LTB4 receptor mRNA in human pancreatic cancer cells. We also showed that LTB4 stimulated proliferation of human pan-
creatic cancer cells, whereas the selective LTB4 receptor antagonist, LY293111, inhibited proliferation and induced apoptosis in these cells both in vitro and in vivo. Furthermore, this growth-stimulatory effect of LTB4 is inhibited by LY293111, confirming the specificity of this action on LTB4 receptors. The results also indicate that the LTD4 pathway is not involved in pancreatic cancer growth because blockade of this pathway using a selective LTD4 antagonist had no effect on cell proliferation.

LTs, together with prostaglandins, thromboxanes, and lipoxins, are the major constituents of a group of biologically active oxygenated fatty acids known as eicosanoids. LTs have been implicated in the pathogenesis of several inflammatory diseases, such as asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel disease. Whereas there is increasing interest in the role of COX and LOX pathways in tumorigenesis, few studies have focused on the relationship between LT metabolism and cancer development. A study by Cristiana et al. (22) found that both LTB4 and 12(R)-HETE stimulated proliferation of the colonic cancer cell lines HCT and HT29 in a time- and concentration-dependent manner. Qiao et al. (27) studied the effect of several prostaglandins and LTB4 on the proliferation of the human colon adenocarcinoma cell lines SW1116 and HT29 and found that selective prostaglandins and LTB4 stimulated growth. Our results confirm that LTB4 plays an important role in the proliferation of human pancreatic cancer cells. Treatment with 100 nM LTB4 for 24 h almost doubled the thymidine incorporation in all of the human pancreatic cancer cell lines tested. LTB4 also induced marked activation of ERK1/2, and this effect was inhibited by LY293111, providing further evidence that LTB4 may be an important growth factor for pancreatic cancer. The actions of LTB4 appear to be mediated by a specific G protein-coupled receptor, BLT1, originally termed BLT. BLT1 is highly expressed in leukocytes and expressed to a much lesser extent in other tissues (23, 24). Recently, another LTB4 receptor, termed BLT2, was discovered. BLT2 is related to BLT1, with an amino acid identity of 45.2%. BLT2 is expressed almost ubiquitously and is a low-affinity receptor for LTB4 (25, 26). In the current study, we have shown the mRNA expression of both BLT1 and BLT2 receptors for LTB4 in human pancreatic cancer cells. Immunohistochemistry in our previous study has also revealed that LTB4 receptors are expressed in human pancreatic cancer tissues, but not in normal human pancreas from multiple-organ donors (28). Whereas its effects on BLT1 are well characterized, at this time, it is not known whether LY293111 has any effect on the BLT2 receptor. Which receptor mediates the growth-stimulatory effect of LTB4 on human pancreatic cancer cells needs to be further investigated.

Apoptosis, or programmed cell death, is an intrinsic cell suicide mechanism that plays an important role in the development and maintenance of healthy tissues. It is morphologically distinct from necrotic cell death. Deregulation of this cell death pathway occurs in cancer, autoimmune disease, and neurodegenerative disorders. In recent years, emphasis has focused on
the importance of apoptosis as the mechanism by which chemotherapy and irradiation kill cancer cells. A reduction in apoptosis favors malignant transformation and proliferation of cancer cells, whereas most chemotherapeutic agents induce apoptosis (29, 30). Our studies and those of others have shown that LOX inhibitors such as NDGA, baicalein, and Rev-5901 can all inhibit proliferation and induce apoptosis in cancer cells. The present study shows that LY293111 also induces pancreatic cancer cell death through induction of apoptosis. Distinctive morphological changes associated with apoptosis were evident after LY293111 treatment, including membrane blebbing and nuclear fragmentation. Cleavage of nuclear DNA into 180–200-bp internucleosomal fragments is considered a hallmark of the late stage of apoptosis. The 3’-end break of double-stranded DNA during apoptosis can also be detected by incorporation of fluorescence-labeled dUTP in the presence of TdT enzyme and analyzed by flow cytometry (31). Treatment with low concentrations of LY293111 (250 and 500 nm) induced substantial apoptosis within 24 h in all of the human pancreatic cancer cell lines investigated. We also found a time-dependent cleavage of PARP after LY293111 treatment, which indicated the activation of the caspase cascade induced by LY293111.

Effects of LY293111 were also examined in vivo. Athymic mice bearing s.c. transplanted human pancreatic cancer cells have proven to be an effective model for studying in vivo effects of anticancer treatments (32, 33). Oral administration of LY293111 at a dose of 250 mg/kg/day dramatically inhibited tumor growth compared with that seen in vehicle-treated control animals during the 24-day treatment period. There did not appear to be any toxic effects of LY293111 at the dose used in these animals. All animals tolerated the treatment very well, and there was no significant change in body weight between control and treated animals. LY293111 treatment also induced massive apoptosis in the xenografted tumors, as shown by in situ tissue


TUNEL assay, indicating that induction of apoptosis is likely to be responsible for the tumor growth inhibition in these mice. The difficulty of early diagnosis of pancreatic cancer and the lack of therapeutic options for pancreatic cancer have prompted the search for alternative treatments. The finding of the importance of LTB4 in the proliferation of pancreatic cancer, coupled with the effectiveness of its receptor antagonist in blocking proliferation and inducing apoptosis, provides a promising avenue for therapeutic intervention in this devastating disease. The LTB4 antagonist activity of LY293111 (34, 35) was evaluated previously in clinical testing (36, 37). The current study showed that the LTB4 receptor antagonist, LY293111, markedly inhibited proliferation and induced apoptosis of human pancreatic cancer cells both in vitro and in vivo. LY293111 also inhibited the growth-stimulatory effect of LTB4. To our knowledge, this is the first report of anticancer effects of an LTB4 receptor antagonist. LY293111 may be valuable in the treatment of human pancreatic cancer.

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REFERENCES

Leukotriene B4 and Pancreatic Cancer


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