15-Lipoxygenase-1 mediates cyclooxygenase-2 inhibitor-induced apoptosis in gastric cancer

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Introduction

Apoptosis is an essential process for the development and tissue homeostasis of most organisms. Dysregulation of apoptosis, e.g. through bcl-2 expression, has been implicated in the pathogenesis of many diseases including gastric cancer (1). Accumulating evidence shows that apoptosis plays an important role in the chemopreventive effect of non-steroidal anti-inflammatory drugs (NSAIDs) in human colorectal and esophageal cancers and that non-steroidal anti-inflammatory drugs (NSAIDs) can therapeutically induce 15-LOX-1 expression to trigger apoptosis in those cancer cells. We found that a specific cyclooxygenase-2 (COX-2) inhibitor SC-236 similarly induced apoptosis in gastric cancer cells. In the present study, we tested whether SC-236 induced apoptosis through up-regulation of 15-LOX-1 in gastric cancer. We found that: (i) SC-236 inhibited growth of gastric cancer cells mainly by inducing apoptosis; (ii) SC-236 induced 15-LOX-1 expression and increased endogenous 13-S-HODE product, instead of 15-S-HETE during apoptosis; (iii) SC-236 did not affect expression of COX-1, COX-2, 5-LOX and 12-LOX; and (iv) 15-LOX-1 inhibition suppressed SC-236 induced apoptosis. These findings demonstrated that SC-236 induced apoptosis in gastric cancer cells via up-regulation of 15-LOX-1, and 13-S-HODE. These are potential and new targets for prevention and treatment of gastric cancer.

Materials and methods

Antibodies and reagents

Rabbit polyclonal antiserum to recombinant human 15-LOX-1, standard solutions of 13-S-HODE and linoleic acid were obtained from Cayman Chemical (Ann Arbor, MI). Proteinase inhibitors and caffeic acid were purchased from Calbiochem (La Jolla, CA). 13-S-HODE ELISA kits were from Oxford Biomedical Research (Oxford, MI), and 15-S-HETE ELISA kits were from Assay Designs (Ann Arbor, MI). Antibodies against caspase-3 and poly (ADP-ribose) polymerase were obtained from Pharmingen (San Diego, CA). Enhanced chemiluminescence (ECL) system was obtained from Roche Molecular Biochemicals (Mannheim, Germany).

Cell cultures and drug treatment

AGS was purchased from the American Type Culture Collection (ATCC, Rockville, MD). MGC803 was a kind gift from Professor D.M.Fan of Xijing Hospital, Xian, China. MKN45 and MKN28 were purchased from RIKEN (The Institute of Physical and Chemical Research), Cell Bank, Japan. Cells were cultured at 37°C in an atmosphere of 5% CO2 and 95% air in RPMI-1640 supplemented with 10% FBS, penicillin and streptomycin (Gibco BRL, Life Technologies, NY) and harvested for use as indicated. SC-236 (Searle, Skokie, IL) was freshly prepared in dimethyl sulfoxide (DMSO) before use. Vehicle control of DMSO (<0.1%) was included in the studies. When cells reached 60–80% confluence, they were treated with various concentrations of SC-236 as indicated. They were then cultured for times ranging from 12 to

Abbreviations: COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acids; HODE, hydroxyoctadecadienoic acid; 15-LOX, 15-lipoxygenase; NSAIDs, non-steroidal anti-inflammatory drugs.
The presence of 0.5% DMSO did not affect cell growth in repeated experiments. To further assess whether the effects of 15-LOX-1 inhibition resulted from the loss of 13-S-HODE production, additional experiments were carried out. Gastric cancer cells were treated with SC-236 (50 µM) with and without the addition of caffeic acid (4.4 µM), and then 13-S-HODE (155 µM) or linoleic acid (135 µM) was added, as described previously (23).

**RT-PCR analysis of COX-1 and COX-2 mRNA expression**

The expression of COX-1 and COX-2 was detected in various gastric cancer cells (AGS, MGC803, MNK45, MKN28). Cells were harvested and total RNA was extracted using Trizol Reagent (Gibco BRL). Two micrograms of RNA was reverse transcribed to cDNA by Thermoscript RT system reagent (Gibco BRL) according to manufacturer’s instruction. PCR was performed using 2 µl of resulting cDNA, 0.5 U Hotstart DNA polymerase, forward and reverse primers and dNTPs in a final volume of 50 µl. Forward (F) and reverse (R) primers used to detect COX-1 and COX-2 cDNAs in the amplification were: COX1-F, 5'-AATCTACCGGCGGCTTTGTTCTGGG-3'; COX1-R, 5'-CTGGCTCTGGGGCGGGATGC-3'; COX2-F, 5'-TATA CTA GAGCCCTCTTCCTGTGGCC-3'; and COX2-R, 5'-ACATCGGATACTCTGT TGTTGTTCCC-3'. Hotstart PCR was performed for 35 cycles with 95°C denaturation for 15 min (first cycle), 94°C denaturation for 30 s, 55°C annealing for 45 s, and 72°C elongation for 1 min and 10 min (final cycle). Amplification products for COX-1 and COX-2 were of the expected sizes (574 and 503 bp, respectively).

**MTT assay**

Cell proliferation was measured by MTT assay, Exponentially growing cells were plated into 96 well plates containing 5000 cells/well in 200 µl medium for 24 h. Then cells were treated with different concentrations of SC-236 for 24, 48 and 72 h. Twenty microliters of MTT stock solution (5 mg/ml) was added into each well, and cells were further incubated at 37°C for 4 h. The supernatant was replaced with 200 µl isopropanol to dissolve formazan production. The absorbance at wavelength 595 nm was measured with a micro-ELISA reader (Bio-Rad, Hercules, CA). The negative control well, into which only the medium had been added, was used for zeroing the absorbance. Each assay was performed three times in triplicate. The ratio of the absorbance of treated cells relative to that of the control cells were calculated and expressed as percentage of growth inhibition.

**Semi-quantitative RT–PCR analysis of 15-LOX-1 mRNA expression**

Total RNA was isolated from AGS and MGC803 cells after treatment with and without SC-236 for 12, 24, 48 and 72 h. Two micrograms of RNA was reverse transcribed to cDNA. PCR was performed using 2 µl of resulting cDNA, 0.5 U Hotstart DNA polymerase, reverse and forward primers and dNTPs in a final volume of 50 µl. Semi-quantification of 15-LOX-1 mRNA expression was determined by referring to the mRNA expression of a housekeeping gene, GAPDH. For amplification of the human 15-LOX-1 and GAPDH, the forward and reverse primer combinations were designed according to previous publication, 5'-GCTGGCGGCTTGGGAATCATCTC-3' and 5'-GGGGCCGGAAAATATCTCTCCTCA-3' (15-LOX-1) and 5'-GGTGAAGGTTCCGGGTAACG-3' and 5'-CAGAATTGCTGATGACCC-3' (GAPDH). PCR amplification was linear within the range of 25–40 cycles. PCR products were electrophoretically separated on 1% agarose gel and visualized under ultraviolet light.

**Western blotting analysis**

AGS and MGC803 were treated with and without SC-236 (50 µM) when reaching 60–80% confluency for 96 h. The cells were extracted with lysis buffer containing protease inhibitors (20 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40). The protein concentration was determined by bicinchoninic acid assay with bovine serum albumin (Sigma) as the standard. Equal aliquots of total cell lysates (40 µg) of each sample were solubilized in sample buffer and electrophoresed on 10% SDS–PAGE gel. The proteins were then transferred to polyvinylidene difluoride membranes (Millipore) using transfer buffer for 2 h. Non-specific binding was blocked with 10 mM phosphate buffered saline plus 0.05% Tween-20 containing 5% skimmed milk for 1 h at room temperature. The blots were probed with primary antibodies overnight at 4°C. The primary antibodies included monoclonal goat anti-human COX-1 antibody (1:1000) and goat anti-human COX-2 antibody (1:1000) (Santa Cruz, CA, USA); polyclonal rabbit anti-human 5-LOX serum (1:1500); polyclonal rabbit anti-human 12-LOX (1:500) (Cayman Chemical) and mouse monoclonal mouse anti-human actin antibody (1:1000) (Santa Cruz). Antigen-antibody complexes were visualized by the ECL system.

**Immunosay of endogenous 13-S-HODE and 15-S-HETE production**

AGS and MGC803 cells were cultured for 48 h after treatment with SC-236 and then lysed. 13-S-HODE and 15-S-HETE were extracted as described previously (20). In brief, the cells were homogenized in PBS at 4°C and centrifuged at 2100 g for 2 min. The solution was acidified to a pH of 3.5–4.0 with 0.2 M HCl solution. The organic phase of the solution was extracted with water-saturated ethyl acetate. Samples were dried completely in a centrifuge dryer, and reconstituted with dilution buffer (provided with the kits). Levels of 13-S-HODE and 15-S-HETE were measured according to the manufacturer’s protocol.

**Apoptosis assays**

Apoptosis was quantitatively evaluated by acridine orange staining examination to identify morphological changes. Single-cell suspensions were fixed in 1% formalin/PBS and stained with acridine orange (10 µg/ml, Sigma). A drop of the stained cell suspension was placed on a microscope slide. Cells were visualized under fluorescence microscope with blue-green filter. The apoptotic cells were defined as cells showing cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation. At least 300 cells were counted, and the percentage of apoptotic cells was determined.

In addition, cells were harvested 72 h after treatment and then lysed. DNA was extracted from an equal number of cells, precipitated, electrophoresed on a 1% agarose gel, and examined after being stained with ethidium bromide, as described previously (25).

**Statistical analyses**

All experiments were performed in triplicate and were repeated at least three times. Mean values ± SEM were calculated to represent the three experiments. Statistical difference was determined by Student’s t-test, or one-way ANOVA. A P value of <0.05 was considered significant.

**Results**

COX-1 and COX-2 mRNA expression in gastric cancer cells

COX-1 and COX-2 expression at the mRNA level were examined by RT–PCR analysis in the four gastric cancer cell lines, AGS, MGC803, MKN28 and MKN45. COX-1 mRNA was expressed constitutively in all four cell lines (Figure 1A). COX-2 mRNA was expressed in AGS, MKN28 and MKN45 cells, but had a low expression in MGC803 cells (Figure 1B).

**Effect of SC-236 on growth of AGS and MGC803**

To evaluate the effect of SC-236 on the growth of AGS and MGC803, SC-236 at various concentrations were added to the culture medium for 24, 48 and 72 h. MTT assay showed that SC-236 inhibited cell growth in a time-dependent manner in both AGS (Figure 2A) and MGC803 (Figure 2B) cells when SC-236 was added at a concentration of 25 µM or higher (all P < 0.05, one-way ANOVA). Moreover, there was also a dose-dependent inhibitory effect (all P < 0.05, one-way ANOVA). Compared with the control, significant inhibition of cell growth was observed for both cell lines 48 h after SC-236 at concentrations of 50 and 100 µM (all P < 0.05). The concentration of SC-236 for further experiments was chosen at 50 µM based on the MTT assay results.
Cox-2 inhibitor induced apoptosis in gastric cancer

Fig. 2. Dose- and time-response to SC-236 treatment in gastric cancer cells. Treatment with SC-236 at concentrations from 12.5 to 100 µM resulted in a dose- and time-dependent inhibition in cell growth of AGS (A) and MGC803 (B). The values were expressed as means ± SEM from three independent experiments. *P < 0.05 versus corresponding control group.

Fig. 3. Effect of SC-236 on 15-LOX-1 mRNA expression. Cells were treated with 50 µM SC-236 for 12, 24, 48 and 72 h. 15-LOX-1 mRNA expression was absent in the untreated cells and began detectable at 12 h. SC-236 caused an increase of 15-LOX-1 mRNA in a time-dependent manner in AGS (A) and MGC803 (B) cells. The figure is a representative of three different experiments.

Effect of SC-236 on 15-LOX-1 mRNA expression
We detected 15-LOX-1 mRNA expression induced by SC-236 (50 µM) in both AGS and MGC803 cells at different time points. 15-LOX-1 mRNA was undetectable by RT-PCR in untreated cells. However, SC-236 at the concentration of 50 µM caused a significant increase in expression of 15-LOX-1 mRNA, which began 12 h after initial treatment, and was in a time-dependent manner in both AGS (Figure 3A) and MGC803 (Figure 3B) cells.

Fig. 4. Effect of SC-236 on COX-1, COX-2, 5-LOX and 12-LOX protein expression. Cells were treated with 50 µM SC-236 for 6, 12, 24, 48 and 72 h. As shown with western blot, SC-236 did not change the expression of COX-1, COX-2, 5-LOX and 12-LOX protein level up to 72 h in AGS (A) and MGC803 (B) cell lines. The figure is a representative of three different experiments.

Effect of SC-236 on COX-1, COX-2, 5-LOX and 12-LOX protein expression
To investigate the effect of SC-236 on other arachidonic acid metabolites, we examined the expression of COX-1, COX-2, 5-LOX and 12-LOX proteins in both AGS and MGC803 cells at different time intervals. Western blots showed that COX-1, COX-2, 5-LOX and 12-LOX protein expression remained unchanged after SC-236 treatment at the concentration of 50 µM for up to 72 h (Figure 4).

Effect of SC-236 on 13-S-HODE and 15-S-HETE production
Endogenous 13-S-HODE was below detectable level in untreated AGS and MGC803 cells. SC-236 increased levels of endogenous 13-S-HODE to 8.69 ± 1.07 and 13.15 ± 4.52 (ng/µg protein) in AGS and MGC803 cells, respectively, at 48 h. Addition of caffeic acid suppressed the levels of 13-HODE to an undetectable level. The values were expressed as means ± SEM from three independent experiments. Bars, ± SEM. *Represents values below the lowest detection level of 5 ng/well.

Fig. 5. Effect of SC-236 on endogenous 13-S-HODE production. 13-S-HODE was below detectable level in untreated AGS and MGC803 cells. SC-236 increased levels of endogenous 13-S-HODE to 8.69 ± 1.07 and 13.15 ± 4.52 (ng/µg protein) in AGS and MGC803 cells, respectively, at 48 h. Addition of caffeic acid suppressed the levels of 13-HODE to an undetectable level. The values were expressed as means ± SEM from three independent experiments. Bars, ± SEM. *Represents values below the lowest detection level of 5 ng/well.

Apoptosis induced by SC-236 and reversed by caffeic acid
Quantitative examination by acridine orange staining showed that SC-236 induced apoptosis in a dose- and time-dependent manner.
Apoptosis assays after treatment with SC-236 and caffeic acid. (A) Apoptosis was induced by SC-236 in AGS (A) and MGC803 (B) cells in a dose- and time-dependent manner. This was confirmed by DNA gel electrophoresis (C). Caffeic acid suppressed apoptosis induced by SC-236. The values were expressed as means ± SEM from three independent experiments.

Supplementation with 13-S-HODE restored apoptosis in gastric cancer cells treated with SC-236 and CAF
13-S-HODE restored apoptosis in AGS and MGC-803 cells treated with SC-236 and CAF. However, at an equal concentration (135 µM), LA, the parent compound of 13-S-HODE, failed to restore apoptosis in AGS and MGC-803 cells treated with the SC-236 and CAF (Figure 7).

Discussion
In our present study, we found that the COX-2 specific inhibitor, SC-236, increased 15-LOX-1 expression in gastric cancer cells and induced apoptosis dependent on 15-LOX-1 activity. Basal 15-LOX-1 expression is absent in all four human gastric cancer cell lines tested, suggesting that 15-LOX-1 down-regulation occurs in tumorigenesis. We showed that SC-236 up-regulated 15-LOX-1 expression and increased 13-S-HODE production in gastric cancer cells.

The crucial role of 15-LOX-1 up-regulation in SC236-induced apoptosis was demonstrated by the blockage of apoptosis via inhibition of 15-LOX-1. Because SC236-induced up-regulation of 15-LOX-1 increased the formation of 13-S-HODE but not of 15-S-HETE (the other major product of 15-LOX-1), it is clear that the effects of 15-LOX-1 up-regulation on apoptosis are mediated through 13-S-HODE. These findings agree with previous reports that linoleic acid is the preferred substrate for human 15-LOX-1, which predominantly produces 13-S-HODE (19,20). The selective inhibition of the enzymatic activity of 15-LOX-1 with caffeic acid also inhibits formation of 13-S-HODE in gastric cancer cells. This inhibition of 13-S-HODE formation by 15-LOX-1 inhibits apoptosis. More importantly, addition of 13-S-HODE overcomes the block on apoptosis induced by caffeic acid, indicating that 13-S-HODE is the mediator of apoptosis in gastric cancer. Therefore, 15-LOX-1 and 13-S-HODE are the crucial pathways in SC-236-induced apoptosis in gastric cancer cells. The effects observed in SC-236, however, would be regarded as specific to this compound only, and need to be studied in other COX-2 inhibitors to see if it can be generalized.

The mechanism for 15-LOX-1 up-regulation by NSAIDs remains unknown. Regulation of 15-LOX-1 expression has been observed at the transcriptional and post-translational levels (26). Acetylation by histone acetyltransferase CREB-binding protein/p300 of STAT6 is required for transcriptional activation of the 15-LOX-1 (27). Interleukin-4 (IL-4) induces expression of 15-LOX-1 in various mammalian cells via STAT6 signaling transduction pathway (27,28).

The reported link between 13-S-HODE and peroxisome proliferator-activated receptor-γ activation may be another signal transduction pathway involved in NSAID-induced apoptosis in gastric cancer. 15-LOX-1 metabolites down-regulate PPAR-γ via up-regulating MAPK activity and increasing PPAR-γ phosphorylation (29).

15-LOX-1 is a specific and crucial molecular target for inducing apoptosis, whereas other lipoxygenases block apoptosis and promote cell proliferation through the formation of HETE products, thereby promoting tumorigenesis (30,31).
We have reported that 12-LOX inhibition induced apoptosis in human gastric cancer cells (32). Our present study indicated that 15-LOX-1 is up-regulated in NSAID-induced apoptosis. We also showed in gastric cancer cells the unique role of 15-LOX-1 in promoting apoptosis through the production of 13-S-HODE, thus potentially inhibiting tumorigenesis. These results identify novel molecular targets for the study of chemoprevention and treatment of gastric cancer.

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