RESEARCH ARTICLE

Cytotoxic Activity of the Cyclooxygenase Inhibitor Aspirin against Cholangiocarcinoma Cell Lines

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Abstract

Cyclooxygenase (COX) is the key enzyme responsible for prostanoids production, which plays an important role in inflammatory process and pathogenesis of several diseases including cancer. In this study, non-selective COX-2 inhibitor aspirin was investigated for its cytotoxic activity against cholangiocarcinoma (CCA) *in vitro.* The cytotoxicity against CCA cell line (CL-6) was performed using MTT assay. Median (range) IC_{50} values of aspirin and 5-FU were 748.0 (522.2-1,005.5) and 366.8 (306.8-410.9) μM, respectively. Results showed that nonspecific COX inhibitor exhibited potent activities against CCA. This implies that not only COX-2, but also COX-1 may also involve in carcinogenesis of CCA. The expression of *COX-1* gene was unchanged but that of *COX-2* was partially inhibited by 5-FU and aspirin. COX inhibitors particularly aspirin could potentially have a role in CCA chemotherapy, either as adjuvant or used in combination with conventional cytotoxic agents.

Keywords: cyclooxygenase inhibitor, cholangiocarcinoma, aspirin

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การศึกษาฤทธิ์ต้านเซลล์มะเร็งท่อน้ำดีของยาแอสไพรินซึ่งยับยั้งการทำงานของเอนไซม์ไซโคลออกซิจิเนส

วรรณา ชัยเจริญกุล, เกศรา ณ บางช้าง

ศูนย์แห่งความเป็นเลิศด้านเภสัชวิทยาและชีววิทยาระดับโมเลกุลของโรคมาลาเรียและมะเร็งท่อน้ำดี วิทยาลัยแพทยศาสตร์นานาชาติจุฬาภรณ์ มหาวิทยาลัยธรรมศาสตร์ (ศูนย์รังสิต) จังหวัดปทุมธานี ประเทศไทย

บทคัดย่อ

เอนไซม์ไซโคลออกซิจิเนส (Cyclooxygenase; COX) มีบทบาทสำคัญในกระบวนการสร้างสารพลอสตานอยด์ ซึ่งเป็นสาระสำคัญของกระบวนการอักเสบและพยาธิสภาพของโรคหลายชนิดรวมถึงโรคมะเร็ง การศึกษาครั้งนี้จะศึกษาฤทธิ์ต้านมะเร็งท่อน้ำดีในหลอดทดลองของแอสไพริน (aspirin) ซึ่งเป็นสารที่ยับยั้งเอนไซม์ไซโคลออกซิจิเนสแบบไม่จำเพาะ พบว่าความเข้มข้นของแอสไพรินและยา 5-FU ที่ฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี (CL-6) ได้ร้อยละ 50 เท่ากับ 748.0 (522.2-1,005.5) และ 366.8 (306.8-410.9) ไมโครโมล่าร์ ตามลำดับ ซึ่งแสดงให้เห็นว่าเอนไซม์ไซโคลออกซิจิเนส 1 น่าจะมีความสัมพันธ์กับการเกิดมะเร็งท่อน้ำดีด้วย แต่จากการศึกษาการแสดงออกของยีนที่ควบคุมการสร้างเอนไซม์ไซโคลออกซิจิเนสพบว่า การแสดงออกของยีนไซโคลออกซิจิเนส-1 ไม่มีการเปลี่ยนแปลง แต่ยีนไซโคลออกซิจิเนส-2 ถูกยับยั้งบางส่วนเมื่อได้สัมผัสกับแอสไพรินและ 5-FU ผลการศึกษาแสดงให้เห็นว่าแอสไพรินมีฤทธิ์ในการต้านมะเร็งท่อน้ำดี ซึ่งอาจจะนำไปใช้ร่วมกับยาอื่นเพื่อเพิ่มประสิทธิภาพของยา

คำสำคัญ: เอนไซม์ไซโคลออกซิจิเนส, แอสไพริน, มะเร็งท่อน้ำดี

Introduction

Cholangiocarcinoma (CCA), a malignant tumor that arises from any portion of the bile duct epithelium, is a serious public health in Thailand with increasing incidence and mortality especially in the northeast and north region.[1-2] Although the cholangiocarcinogenesis has not been clearly understood, evidences suggest that inflammation process may play an important role in the growth regulation of human CCA, which increases tumorigenic potential *via* prevention of cell apoptosis.[3-4] The increased level of cyclooxygenase (COX-2) in human CCA cells supports this hypothesis.[5] Cyclooxygenase (COX) enzyme exists in two isoforms, *i.e.,* COX-1 and COX-2, each of which displays distinct physiological profile.[5-6] COX-1 is constitutively expressed in most tissues and generates prostaglandins (PGs) for physiologic homeostasis, whereas COX-2 is inducible by both inflammatory and mitogenic stimuli resulting in increased PG synthesis in neoplastic and inflamed tissues. PGE_2 and PGD_2 are the two PGs which play important role as mediators of fever and immunosuppression.[7-8] Nonsteroidal anti-inflammatory drugs (NSAIDs) act *via* inhibiting COX enzymes and prostaglandin production. NSAIDs traditionally are grouped by their chemical characteristics. The objective of the study was to investigate the effect of aspirin on the growth control of CCA cells through determination of the expression of genes involved in PG synthesis.

Materials and Methods

Chemicals and reagents

Analytical grade ethanol and dimethyl sulfoxide (DMSO) were purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand). The cell culture medium RPMI-1640, fetal bovine serum (FBS), antibiotic-antimycotic, and 0.25% trypsin were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-fluorouracil (5-FU), aspirin and all primers were purchased from Sigma Aldrich (St. Louis, MO, USA). TRIzol™ reagent and SuperScript™ II RT were purchased from Invitrogen (Carlsbad, CA, USA). RQ1 RNase-Free DNase Kit was purchased from Promega (Madison, WI, USA). iTaq Universal SYBR Green Supermix was purchased from Bio-Rad (Hercules, CA, USA).

Cell line and cell culture

Human cholangiocarcinoma cell line (CL-6) was used in this study. CL-6 had been established and kindly provided by Associate Professor Dr. Adisak Wongkajornsilp, Department of Pharmacology, Faculty of Medicine (Siriraj Hospital), Mahidol University. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic. Cells were incubated at 37°C under humidified 5% CO_2 incubator.

Cytotoxic assay

The MTT colorimetric assay was used to investigate cytotoxic activities of 5-FU, and aspirin. The cells were seeded in a flat-bottomed 96-well microplate at a

density of 1×10^4 cells *per* well in culture medium. Following 24 h incubation and attachment, cells were treated with various concentrations of the 5-fluorouracil (5-FU) and aspirin for 48 h. Thereafter, cells were incubated with MTT reagent (20 µl of 5 mg/ml) at 37°C in 5% CO_2atmosphere for 4 h. The MTT dye was reduced by succinic dehydrogenase in the mitochondria of viable cells to formazan crystals. The supernatant was removed and DMSO (100 µl) was added and the absorbance was measured at 570 nm using a microplate reader (Varioskan Flash, Thermo Electron Corporation, Vantaa, Finland). Percent cell viability was expressed as the ratio of absorbance in the presence of test compound compared to that in the vehicle control. The results were generated from three independent experiments; each experiment was performed in triplicate. The IC_{50} value (concentration that inhibits cell growth by 50%) was calculated using Calcusyn™ software (Biosoft, Cambridge, UK).

Drug exposure

Prior to each experiment, CL-6 cells were grown until 80% confluence was reached. The cells were treated with 5-FU (400 µM) and aspirin (500 and 1,500 µM). The control cells were exposed to ethanol (final concentration less than 1%). Cells were harvested at 12, 24, and 48 h. To release cell from monolayer surface, the medium was aspirated out, and washed with 5 ml of PBS. Warm PBS was added and the monolayer cells were gently scraped using cell scraper. Cells were transferred to a 15 ml centrifuge tube, centrifuged at 10,000 × g (3 min, 4°C) and used immediately or stored at -80°C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from treated cells using TRIzol™ reagent according to the manufacturer's instruction. The concentration of RNA was quantitated by Nanodrop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was treated with RQ1 RNase-Free DNase Kit to remove contaminating genomic DNA. Then first-strand cDNA was synthesized from total RNA by reverse transcription reaction using oligo-dT primers and SuperScript™ II RT. cDNA was stored at –80°C until use.

Quantification of mRNA gene expression

Gene expression was determined using real-time RT-PCR (CFX96™, BioRad Laboratories Inc., Hercules, CA, USA). The forward and reverse primers for the selected genes used in the study are shown in Table 1. The reaction mixture (20 µl) consisted of 1.5 µl cDNA, 10 µl of *iTaq* Universal SYBR Green Supermix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, and 6.5 µl of sterile double distilled water. The following PCR cycles were initiated: denaturation at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec, and annealing at 60°C for 1 min. No template control was used for each primer pair of each target gene, and each PCR was performed in duplicate. Ct values (threshold cycle) which is the intersection between an amplification and threshold line, was generated that reflected relative measure of the concentration of target in the PCR reaction.

The delta delta Ct method was used to calculate gene expression level relative to control (no treat) and housekeeping gene (GAPDH) was used for normalization of gene expression. The delta delta Ct (ΔΔCt) calculation for the relative quantification of target gene was as follows:

ΔCt (1) = [Ct(target gene) – Ct(GAPDH)]

ΔCt (2) = [Ct(control for target gene) – Ct(control for GAPDH]

ΔΔCt = ΔCt (1) – ΔCt (2)

Relative expression = $2^{-\Delta\Delta Ct}$

Table 1. Primer sequences for determination of mRNA gene expression

Primer Name	Sequence (5'-3')
COX1-F	AGC AGC TTT TCC AGA CGA CC
COX1-R	CGG TTG CGG TAT TGG AAC TG
COX2-F	CCA GCA CTT CAC GCA TCA GT
COX2-R	ACG CTG TCT AGC CAG AGT TTC AC
GAPDH-F	TCA ACG GAT TTG GTC GTA TT
GAPDH-R	CTG TGG TCA TGA GTC CTT CC

Results

In vitro cytotoxic activities of aspirin and 5-FU against CCA

Cytotoxic activities of aspirin and 5-FU against CL-6 were investigated. Median (range) IC_{50} values of aspirin and 5-FU were 748.0 (522.2-1,005.5) and 366.8 (306.8-410.9) μM, respectively.

mRNA gene expression

The expression of *COX-1* was comparable for both aspirin and 5-FU at various exposure times (Figure 1). The expression of *COX-2* was however, partially inhibited by 5-FU, aspirin (500 and 1,500 μM) (Figure 2). The expression of *COX-2* was decreased when compared with the non-exposed cells. But after that the expression of this gene was however, reversed to normal level after 48 hours of exposure.

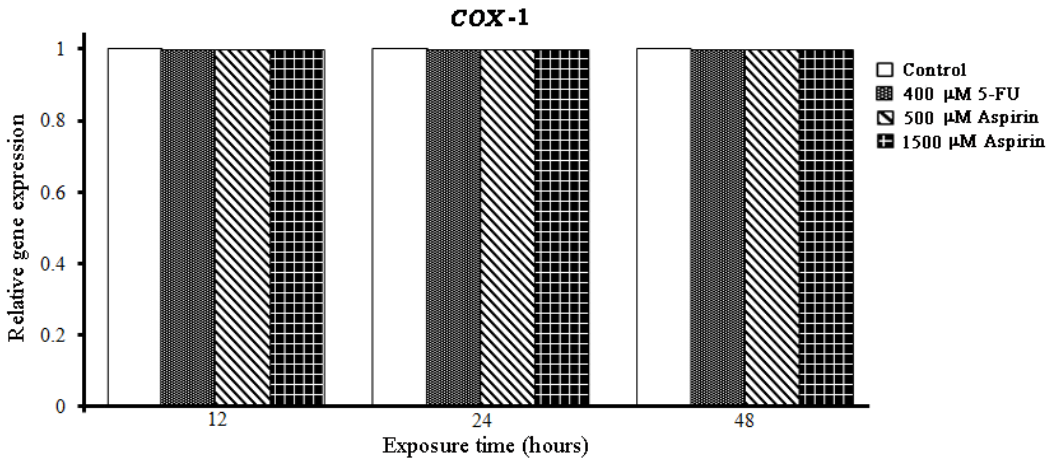

Figure 1. *COX-1* gene expression following exposure of CL-6 cells to 5-FU and aspirin (500 and 1500 µM).

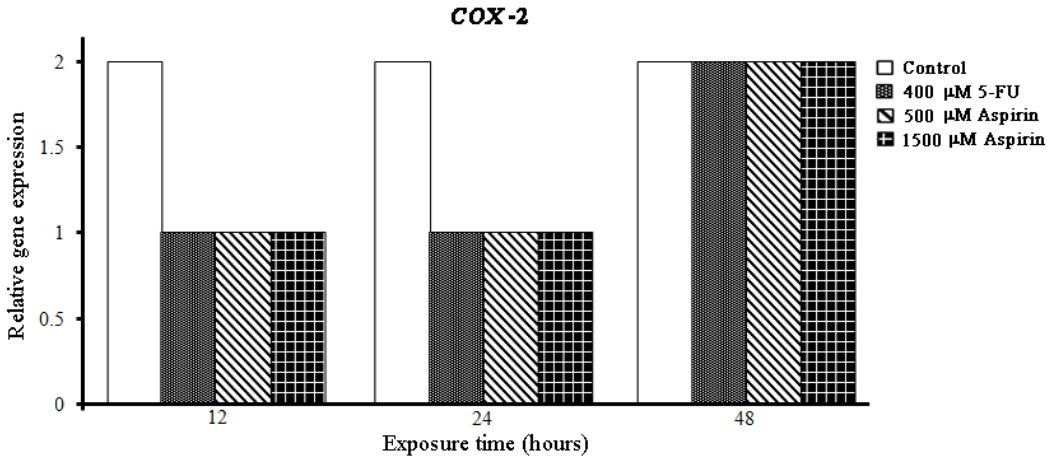

Figure 2. *COX-2* gene expression following exposure of CL-6 cells to 5-FU and aspirin (500 and 1,500 µM).

Discussion

The present study investigated the cytotoxic activities of the cyclooxygenase inhibitor aspirin (non-selective COX inhibitor) against CL-6 cell lines. The median (95%CI) IC_{50} value for 5-FU of366.8 (306.8-410.9) µM was similar to that previously reported (377 µM and 757 µM)[1,9], suggesting stability of phenotype expression of this cell. The cytotoxic activity of aspirin against cancer cell lines has been shown to be varied among cancer types. The IC_{50} values for MIA PaCa-2 (human pancreatic cell line) was 2,600 µM, whereas that for SW480, RKO, HT29 and HCT116 cell lines (colon cancer cells) were 100, 2,600 and 2,500-5,000 µM, respectively.[10-11] Results suggest relatively strong inhibitory potency of aspirin against the growth of various cancer cells.

In the present study, the analysis of gene expression was performed using quantitative real time PCR. The expression of *COX-1*gene was unchanged, whereas that of the *COX-2* was increased. This observation was in agreement with that was reported by Jongthawin and colleague[12] for the strong expression of *COX-2* but not the *COX-1* in CCA tissues. COX plays role in the synthesis of several arachidonic metabolites especially PGE_2 which is generated during chronic inflammation and development and progression of various human cancers including CCA.[13] Inhibition of COX reduces the production of PGE_2 which might result in CCA growth inhibition. Aspirin, a non-selective COX inhibitor, showed cytotoxic effect on CCA cell line, at least in part due to its inhibitory effect on *COX-2*, but not on the house-keeping gene *COX-1*.[14] Despite the fact that aspirin did not interfere with *COX-1* gene expression, it inhibited COX-1 enzyme function.

The expression of *COX-2* was decreased following exposure to aspirin and 5-FU. Results suggest the inhibitory effects of the non-selective cyclooxygenase inhibitor aspirin and 5-FU on *COX-2* expression. These inhibitory effects were reversible and were resumed after 48 hours. Altogether, available data suggest that CCA cell line might be resistant to both 5-FU and aspirin through unidentified mechanism that increases *COX-2* expression to normal level. Further study will be focused on identification of this underlying mechanism.

Conclusion

The non-selective COX inhibitor aspirin partially inhibited gene(s) involved in prostaglandin synthesis. The drug could potentially have a role in CCA chemotherapy, either as adjuvant or used in combination with conventional cytotoxic agents.

Acknowledgements

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