Effects of Selective COX-Inhibitors and Classical NSAIDs on Endothelial Cell Proliferation and Migration Induced by Human Cholangiocarcinoma Cell Culture

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Objective: *Experiments were designed to explore cellular mechanisms and effects of NSAIDs on human umbilical vein endothelial cells (HUVEC) induced by human cholangiocarcinoma (HuCCA).*
Material and Method: *HUVEC were incubated with HuCCA or HuCCA-conditioned medium (CM) for various times to determine cell proliferation and migration. Expression of cyclooxygenase (COX) proteins was measured using immunoblotting technique. VSA (selective COX-1 inhibitor), NS-398 (selective COX-2 inhibitor), and aspirin were used as pharmacological tools to explore signaling mechanisms of HuCCA-CM-induced endothelial cell functions.*
Results: *HuCCA could significantly induce proliferation and migration of HUVEC. COX-2, but not COX-1, was increased. NS-398, but not VSA, could significantly inhibit HuCCA-CM-induced endothelial cell proliferation. HuCCA-CM-induced endothelial cell proliferations could be also inhibited by aspirin.*
Conclusion: *These findings suggest that HuCCA-CM-derived substances could induce HUVEC proliferation through COX-2 signaling mechanism. Classical NSAID and selective COX-2 inhibitors could also inhibit this step of HUVEC proliferation.*

Keywords: *Cholangiocarcinoma/ HUVEC/Proliferation/ COX-2/ NSAIDs*

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Human cholangiocarcinoma (HuCCA) is an epithelial neoplasm that originates from cholangiocyte and can occur at any place in the biliary tree. It is broadly classified into intrahepatic tumor and extrahepatic distal bile duct tumor[1]. Cholangiocarcinoma frequently occurs in Southeast Asia, especially in north-eastern Thailand, where more than 60% of liver tumors are HuCCA[2]. For growth and development, cancer tissues require angiogenesis system, which is the complex process involving an interplay between endothelial cells, soluble factors and extracellular matrix (ECM) components[3]. Endothelial cell proliferation and migration occur in the early stage of angiogenesis before the formation of a lumen and vessel wall maturation[4]. Endothelial cells in tumor have abnormalities in gene expression, require growth factors for survival and have defective barrier function to plasma proteins[5] and serve as gatekeepers governing extravasation of plasma, leukocytes and erythrocytes, and intravasation of tumor cells that form metastases[6].

Cholangiocarcinomas and the surrounding tissues may produce various factors that affect angiogenesis; depending on the balance of angiogenesis activators and inhibitors. There are a number of angiogenic factors expressed in HuCCA tissues, such as epidermal growth factor receptor (EGFR), erythroblastic leukemia viral oncogene homolog 2 (ErbB-2), and vascular endothelial growth factor receptor-2 (VEGFR-2), which are detected in biliary

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tract cancer[7]. These factors can exert marked effects on tumor growth *in vitro* and *in vivo*[7]. Cyclooxygenase (COX), also known as prostaglandin (PG) endoperoxide synthase, is an essential enzyme in the conversion of arachidonic acid to PGs. The isoforms of the enzyme are called COX-1 and COX-2 and the third recent discovered isoform COX-3[8]. COX-2 is strongly expressed in many kinds of tumors including bile duct tissue of HuCCA. Aberrant COX-2 expressions affect clinical-pathological parameters in both intrahepatic[9] and extrahepatic HuCCA[10].

COX-2 also has been found to be closely correlated with angiogenesis factors, such as VEGF, to induce tumor vascularization or enhance endothelial cell survival via Bcl-2 expression and Akt signaling[11]. Even though COX-2 may be involved in angiogenesis-induced cancer progression, its role in the mechanism of HuCCA is still not clearly known. The present study was designed to investigate the effect of HuCCA on human umbilical vein endothelial cell (HUVEC) proliferation and migration. COX inhibitors and classical NSAIDs were used as pharmacological tools to explore the signaling mechanisms by which HuCCA affected endothelia cell function.

Material and Method

Reagent

Dulbecco's Modified Eagle's Medium (DMEM), medium 199 (M199) and fetal bovine serum (FBS) were purchased from HyClone, USA. Nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin and selective COX-1 inhibitor, valeryl salicylate (VSA), were purchased from Sigma, USA, and NS-398, (the selective COX-2 inhibitor), were purchased from Cayman Chemical, Australia.

HuCCA cell culture

Primary HuCCA was prepared from biliary fluid aspirated by endoscopic retrograde cholangio-pancreatography (ERCP) of a HuCCA patient as previously described[12]. Primary antibodies raised against pan cytokeratin (clone AE1/AE3, Zymed®) were used to specifically distinguish biliary epithelial cells from hepatocytes[13,14]. Goat secondary antibodies were conjugated with horseradish peroxidase. Cancer cells were cultured in T-75 flasks with DMEM containing 15% FBS, 100 unit/ml penicillin G sodium, and 100 µg/ml streptomycin at 37°C until nearly confluent before use. Conditioned medium of cholangiocarcinoma (HuCCA-CM) was obtained by incubating cells with serum-free DMEM for 24 h.

HUVEC cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from newborn umbilical cords of healthy pregnant women's placentas as previously described[15]. Then HUVECs were cultured 37°C to the third passage in 96-well and 6-well culture plates with M199: EC medium (1:1) containing 10% FBS, 100 unit/ml penicillin G sodium, and 100 µg/ml streptomycin. The medium of third passage was replaced with HuCCA-CM for 3, 6, and 24 h. Aspirin, VSA, and NS-398 were used to explore the role of COX in endothelial cell proliferation and migration.

MTT assay

HUVEC proliferation was measured using MTT assay, modified from that of Mosmann[16]. MTT in free-serum culture medium (2 mg/ml) was added to attach cells in 96-well plate, which was incubated for 1 h at 37°C. Medium was discarded and intracellular MTT-formazan was solubilized with 100 µl of DMSO for 5 min at room temperature. Absorbance in each well was determined at 595 nm using a spectrophotometer. The effects of HuCCA-CM on endothelial cell functions of various times are reported as % proliferation compared with untreated control.

Crystal violet staining assay

HUVEC proliferation was also measured using a crystal violet staining assay. Fifty microlitre of 0.05% crystal violet solutions in methanol were added to each well for 15 min at room temperature. The plate was rinsed twice with PBS and 200 µl of 0.5% sodium dodecyl sulfate (SDS) in 50% methanol were added to solubilize the stained cells. The plate was incubated for 60 min at 37°C before determining absorbance at 595 nm using a spectrophotometer. Results are shown as% proliferation compared with untreated control.

Western blot analysis

After treatment, HUVECs were extracted for analysis of COX-1 and COX-2 expression using specific antibodies. In brief, samples were boiled with an equal volume of gel loading buffer. The sample proteins were loaded into 7.5% separating gel and electrophorised for 2 h at constant voltage. The separated protein bands were transferred onto nitrocellulose membrane. Membrane was incubated with blocking solution (5% low-fat milk in washing buffer) and then with primary selective monoclonal antibodies for COX-1 and -2 proteins. Membrane was washed and incubated with anti-mouse IgG conjugated

to alkaline phosphatase (Sigma, USA). Immunoblots were developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) (Sigma, USA) for 5 min. Equal amounts of protein (19 μg/lane) were loaded in each lane. Results are expressed as densitometry unit ($x10^3$) of each protein band. Experiments were conducted with cell extracts from three separate batches of cells.

Endothelial cell migration assay

HUVEC migration assay was modified from that of Meadows et al 2001[17]. Migration assays were conducted using HUVEC in BD Falcon™ HTS FluoroBlok™ 24-Multiwell co-culture system (BD Biosciences). A suspension of HuCCA (1.5×10^4 cells) in 15% FBS- DMEM was added to each bottom well and grown to near confluency for about 3 days before use. HUVECs (5×10^4 cells) were placed into the top chamber of FluoroBlock, containing an insert of PET membrane with 8.0 μm pore, in 250 μl of EC:M199 (1:1) medium without FBS and antibiotics. HUVECs and HuCCA were co-cultured at 37°C with 5% CO_2 for 24 h. Then the insert wells were placed into a new 24-well plate, which contained 500 μl of calcein AM solution (10 μg/ml in PBS) in each well. HUVECs that migrated through the pores of the membrane were labeled with calcein AM solution for 90 min. Migrated cells were detected using a fluorescence plate reader with bottom reading capability at excitation/emission wavelength of 485/530 nm. The effects of HuCCA on HUVEC migration of various times are shown as % migration compared with untreated control.

Statistic of analysis

ANOVA or Student's unpaired t-test as appropriate was used to determine the significance of difference between means. P-value of less than 0.05 is considered as statistically significant. Results are expressed mean ± SD of at least three independent experiments.

Results

Effects of HuCCA-CM on HUVEC proliferation and COX expression

Pooled HuCCA cells from 4 cases of Siriraj Hospital, Thailand were cultured with serum-free DMEM for 24 h to obtained HuCCA-CM. HuCCA-CM was incubated with HUVEC for various times. HuCCA-CM could significantly induce HUVEC proliferation at 3, 6 and 24 h (140.72 ± 3.60%, 194.03 ± 5.55% and 255.22 ± 13.22% respectively) (Fig. 1). HuCCA-CM could not induce COX-1 expression (1.1, 1.0 and 1.1 fold at 3, 6 and 24 h, respectively) but COX-2 expression was induced 9, 15 and 28 fold at 3, 6 and 24 h, respectively (Fig. 1).

Effects of COX inhibitors on HUVEC proliferation and COX expression

VSA, a selective COX-1 inhibitor, could not significantly inhibit HuCCA-CM induced HUVEC proliferation using both MTT and crystal violet staining assay (Fig. 2). However, NS-398, a selective COX-2 inhibitor, significantly inhibited HUVEC proliferation induced by HuCCA-CM using MTT (1-100 μg/ml) and crystal violet staining assay (10-100 μg/ml). Aspirin, a classical NSAID, could significantly inhibit HuCCA-CM-induced HUVEC proliferation by MTT (250-1000 μg/ml) and crystal violet staining assay (500-1000 μg/ml) (Fig. 3).

VSA (100 μg/ml) had no effect on HUVEC COX-2 expression induced by HuCCA-CM, but NS-398 (100 μg/ml) and aspirin (100 μg/ml) inhibited induction of COX-2 expression (Fig. 4).

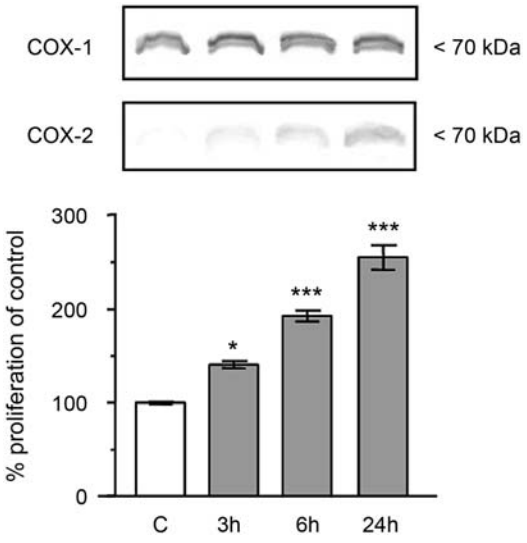

Fig. 1 Effects of conditioned medium from human cholangiocarcinoma cell culture (HuCCA-CM) on endothelial cell proliferation. HUVECs were treated with HuCCA-CM for 3, 6 and 24 h. Cell proliferation was measured using MTT assay. COX-1 and COX-2 protein expression in HUVEC treated with HuCCA-CM were determined by immunoblotting. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; compared with control (c)

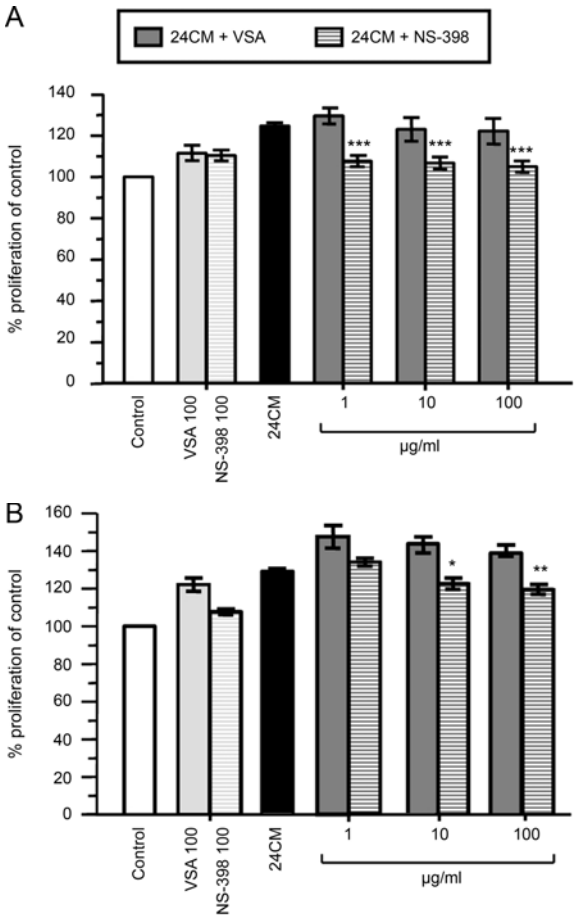

Fig. 2 Effects of valeryl salicylate (VSA) and NS-398 on HuCCA-CM induced HUVEC proliferation. HUVEC were treated with HuCCA-CM for 24 h. Cell proliferation was measured using MTT (A) and crystal violet staining (B) assays. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; compared with HuCCA-CM-induced condition (24CM)

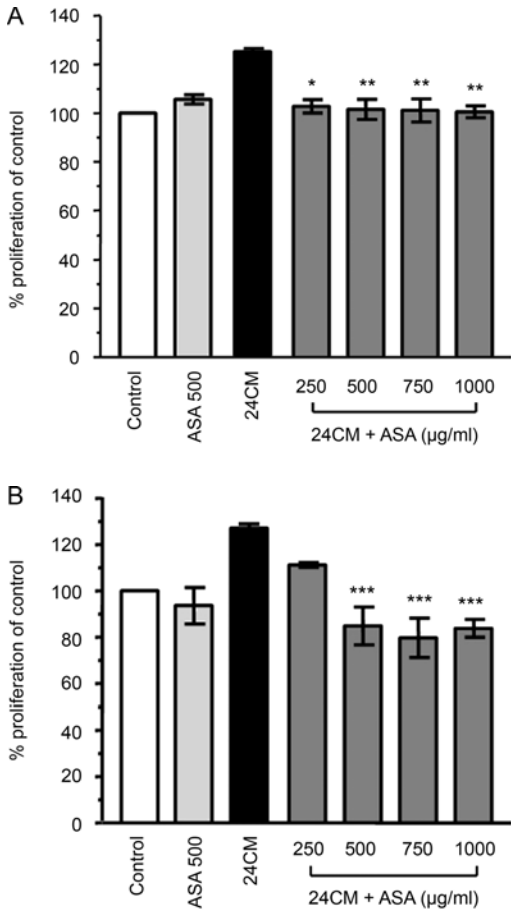

Fig. 3 Effect of aspirin (ASA) on HuCCA-CM induced HUVEC proliferation. HUVEC were treated with HuCCA-CM for 24 h. Cell proliferation was measured using MTT (A) and crystal violet staining (B) assays. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; compared with HuCCA-CM-induced condition (24CM)

Effects of HuCCA and COX-inhibitors on migration of HUVEC

HuCCA co-cultured with HUVEC for 24 h significantly induced HUVEC migration through the pores of the insert membrane when compared with control (Fig. 5). However, VSA, NS-398 and aspirin had no effect on HUVEC migration induced by HuCCA.

Discussion

The process of angiogenesis plays an important role in tumor growth and metastasis. Tumor can induce angiogenesis by releasing several angiogenic and antiangiogenic factors to regulate each step of process through different intracellular signaling mechanisms. CM harvested from many kinds of tumor, human pulmonary squamous cell carcinoma, pulmonary small cell carcinoma and gastric adenocarcinoma, is able to enhance angiogenic activity of endothelial cells[18]. Thus, tumor CM is widely used as in *in vitro* angiogenesis model[19]. The present study has shown that CM from primary culture of HuCCA induced COX-2 expression and cell proliferation of HUVEC. Experiments in HuCCA cell lines by Ogasawara et al[20] have shown that HuCCA-CM accelerates EC proliferation by expressing both bFGF and VEGF. In other cancer types, Liang and

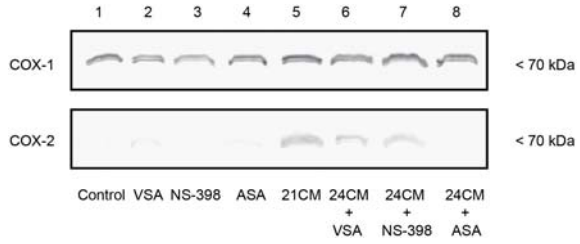

Fig. 4 Effects of VSA, NS-398 and aspirin (ASA) (100 μg/ml) on COX-1 and COX-2 protein expression in HUVEC treated with HuCCA-CM for 24 h (24CM). COX-1 and COX-2 was detected by Western blotting

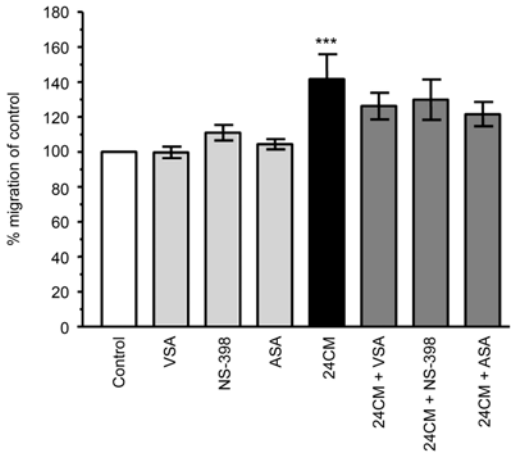

Fig. 5 Effects of human cholangiocarcinoma cell culture (HuCCA) on HUVEC migration. HUVEC migration was measured using BD Falcon™ HTS FluoroBlok™ 24-Multiwell co-culture system. VSA, NS-398 and aspirin (ASA) (100 μg/ml) were used to explore the effects of COX inhibitors on HuCCA induced endothelial cell migration. ***, $p \leq 0.001$; compared with control

Hyder[21] have also shown that CM from progestin-treated breast tumor cells can promote VEGF-induced EC proliferation in a paracrine manner. CM of fibroblasts cultured with bladder cancer CM also stimulates cancer cell invasion with more increased hepatocyte growth secretion than CM of cultured fibroblasts alone.

After EC proliferation occurs, EC migration is one of the important steps in early angiogenesis. Neovascularization requires cell migration, which depends on the assembly of protease-protein complexes at the migrating cell front to degrade the underlying basement membrane and invade into the stroma of the neighboring tissue[23]. EC migration is controlled by a complex of effective regulators for further cell adhesion and tube formation[23]. Many different cytokines are involved in the regulation of chemotactic endothelial cell migration during angiogenesis. The three major promoters of this type of actin-based motility are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiopoietins[24]. bFGF or VEGF could also induce TXA_2 synthesis, which stimulates HUVEC migration[25]. The migration of HUVEC stimulated by HuCCA was studied by using a co-culture system. The co-culture system restores *in vitro* cell-cell interaction signals that are presented *in vivo* by mimicking the physiological situation. The present results demonstrated that HuCCA-CM significantly induced HUVEC migration.

HuCCA-CM induced COX-2 but not COX-1 expression in HUVEC. Elevated COX-2 expression is common in several types of cancers and is strongly related with poor prognostic characteristics and an unfavorable outcome of patients[17]. Möbius C et al[27] suggested that inhibitors of COX-2 and 5-lipoxygenase (5-LOX) may offer a potential additional adjuvant therapeutic approach to HuCCA. COX-2 is related closely also with multisteps of cancer-derived angiogenesis. COX-2 and their metabolites could directly induce VEGF[28], MMP-2[29] and TGF-β[30].

Itatsu et al[31] recently proposed a novel signaling pathway in HuCCA in which TNF-α induces activation of COX-2 and PGE_2 via tumor necrosis factor receptor-1 (TNF-R1), followed by up-regulation of MMP-9 via the PGE_2 ($EP_{2/4}$) receptor. However, the mechanism of COX-2 derived angiogenesis processes in HuCCA remains unclear. Thus, the present study investigated the role of COX-2 in EC proliferations and migrations induced by HuCCA-CM by using VSA, selective COX-1 inhibitor and NS-398, selective COX-2 inhibitor as pharmacological tools. The results demonstrated that VSA could not significantly inhibit proliferation of HUVEC induced by HuCCA-CM. VSA has been described as an irreversible selective inhibitor of COX-1 by acylating the hydroxyl side chain of the active side serine[32] and could inhibit cytokine-stimulated PGE_2 production[33]. These results suggest that COX-1 may not be involved in HuCCA-induced early steps of angioge- nesis.

NS-398 potently inhibits COX-2 activity and reduces PGE_2 production[34]. NS-398 inhibits

bFGF-induced COX-2 expression in microvascular endothelial cell[34]. In *in vivo* model, NS-398 inhibits VEGF-dependent corneal angiogenesis[35] and urokinase-type plasminogen activator (uPA) mRNA in colon cancer implanted mouse[36]. From the presented studies, NS-398 had a significant inhibitory effect on HUVEC proliferation and COX-2 expression induced by HuCCA-CM. These results suggest that COX-2 is strongly involved with endothelial proliferation activated with HuCCA-CM.

Aspirin is one of the most widely used class of medication NSAIDs for treatment pain, arthritis, cardiovascular diseases and more recently, in the prevention of colon cancer and Alzheimer's disease[37]. In angiogenesis, there are evidences showing that aspirin significantly inhibits normal human dermal microvascular endothelial cells (HuDMEC) and HUVEC proliferation[38]. From the present studies, aspirin ($\geq$ 500 μg/ml) significantly inhibited proliferation of HUVEC induced by HuCCA-CM. Aspirin (100 μg/ml) also could inhibited COX-2, but not COX-1 expression induced by HuCCA-CM. Thus, classical NSAIDs may be used as a pharmacological tool to reduce or prevent HuCCA growth.

In co-cultures of HuCCA and HUVEC, VSA, NS-398 and aspirin (100 μg/ml) did not decrease HUVEC migration. Danial et al[39] showed that endothelial cell migration and corneal angiogenesis could be inhibited by selective COX-2 inhibitor, VU08, and by TXA_2 receptor antagonist, SQ29548. TXA_2, a COX-2 product may function as a critical intermediary of angiogenesis. Ashton and Ware[40] demonstrated that VEGF-induced EC differentiation and migration are linked with the expression of thromboxane A_2 receptor (TP isoform) on EC. All these findings suggest that HuCCA may be involved in inducing endothelial migration through COX pathway. However, the COX pathway is only one of a number of migration regulators. The role of selective COX inhibitors and NSAIDs on EC migration must be studied further.

In summary, HuCCA-CM induced HUVEC proliferation and migration, which are important for the early steps of tumor-derived angiogenesis. EC proliferation was induced through COX-2 signaling pathway as NS-398, a selective COX-2 inhibitor, inhibited both HUVEC proliferation and COX-2 protein expression induced by HuCCA-CM. The classical NSAID, aspirin, also inhibited HuCCA-CM induced proliferation of HUVEC indicating that of may be useful as a pharmacological drug for tumor-induced angiogenesis.

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บทบาทของ selective COX-inhibitors และ classical NSAIDs ต่อการสร้างหลอดเลือดใหม่ในระยะเริ่มต้นที่ถูกกระตุ้นโดยเซลล์มะเร็งท่อทางเดินน้ำดี

สุขฤทัย บุญมาไสว, ประวิทย์ อัครเสรีนนท์, กิติรัตน์ เตชะไตรศักดิ์, อธิวัฒน์ ถาวร, ศิริกุล โชติวุฒมากร, ทิชาภรณ์ ปาโล

วัตถุประสงค์: *เพื่อศึกษากลไกและผลของ selective COX-2 inhibitors และclassical NSAIDs ต่อการสร้างหลอดเลือดใหม่ในระยะเริ่มต้นที่ถูกกระตุ้นโดยเซลล์มะเร็งท่อทางเดินน้ำดี*

วัสดุและวิธีการ: *เซลล์เยื่อบุผนังหลอดเลือดถูกเพาะเลี้ยงร่วมกับเซลล์มะเร็งท่อทางเดินน้ำดี หรือ conditioned medium จากเซลล์มะเร็งท่อทางเดินน้ำดีในช่วงเวลาต่าง ๆ เพื่อศึกษาการเพิ่มจำนวน (proliferation) และการเคลื่อนที่ (migration) ของเซลล์เยื่อบุผนังหลอดเลือด ศึกษาการแสดงออกของโปรตีน cyclooxygenase (COX) โดยใช้เทคนิค immunoblotting และศึกษาบทบาทของ VSA (selective COX-1 inhibitor), NS-398 (selective COX-2 inhibitor) และ aspirin เพื่อวิเคราะห์กลไกการทำงานของเซลล์เยื่อบุผนังหลอดเลือดในภาวะที่ถูกกระตุ้นดังกล่าว*

ผลการศึกษา: *จากการศึกษาพบว่าเซลล์มะเร็งท่อทางเดินน้ำดีสามารถกระตุ้นการเพิ่มจำนวน และการเคลื่อนที่ของเซลล์เยื่อบุผนังหลอดเลือด รวมทั้งกระตุ้นการแสดงออกของโปรตีน COX-2 การใช้ NS-398 และ aspirin ยกเว้น VSA สามารถยับยั้งการเพิ่มจำนวนของเซลล์เยื่อบุผนังหลอดเลือดที่ถูกกระตุ้นด้วยเซลล์มะเร็งท่อทางเดินน้ำดี*

สรุป: *conditioned medium จากเซลล์มะเร็งท่อทางเดินน้ำดี สามารถกระตุ้นการเพิ่มจำนวนของเซลล์เยื่อบุผนังหลอดเลือด โดยผ่านกลไกของ COX-2 signaling mechanism และการใช้ classical NSAIDs และ selective COX-2 inhibitors สามารถยับยั้งการทำงานของเซลล์เยื่อบุผนังหลอดเลือดในระยะเริ่มต้นนี้ได้*