2007 년 8 월
박사학위논문

5-Fluorouracil 내성 대장암 세포에서 cyclooxygenase 2 에 의해 유도된 prostaglandin E_2의 upregulation

조선대학교 대학원

의 학 과

이 연 아

5-Fluorouracil 내성 대장암 세포에서 cyclooxygenase 2에 의해 유도된 prostaglandin E_2의 upregulation

- Upregulation of PGE_2 by cyclooxygenase 2 in colon cancer cells resistant to 5-fluorouracil -

2007년 8월 24일

조선대학교 대학원

의 학 과

이 연 아

5-Fluorouracil 내성 대장암 세포에서 cyclooxygenase 2에 의해 유도된 prostaglandin E_2의 upregulation

지도교수 민 영 돈

이 논문을 의학 박사학위신청 논문으로 제출함

2007년 4월

조선대학교 대학원

의 학 과

이 연 아

이연아의 박사학위논문을 인준함

위원장　송도병원이사장(의학박사) 이 종 균　인

위　원　의 과 대학교 교수　최 철 희　인

위　원　공 과 대학교 교수　조　훈　인

위　원　의 과 대학교 교수　김 경 종　인

위　원　의 과 대학교 교수　민 영 돈　인

2007 년 6 월

조선대학교 대학원

목 차

표 목 차

그 림 목 차

ABSTRACT

5-Fluorouracil 내성 대장암 세포에서 cyclooxygenase 2 에 의해 유도된 prostaglandin E_2 의 upregulation

조선대학교 대학원 의학과 외과학교실

이 연 아

지도교수: 민 영 돈

서론: 5- Fluorouracil (5-FU)은 결장직장암의 항암제로 가장 많이 사용되고 있으나, 그 효과는 내인성 또는 획득 내성 때문에 여전히 만족스럽지 못하다. 암세포에서 cyclooxygenase (COX)-2 의 발현은 세포고사에 저항을 보이는 것으로 알려져 있다. 이런 세포고사에 대한 저항은 내성세포에서 흔히 보이는 현상이다. 또한, COX-2 외에 microsomal prostaglandin (PG) E_2 synthase 1 (mPGES1)이 COX-2 와 연관되어 PGE_2 합성에 중요하며, PGE_2 를 주로 분해하는 효소로 알려진 15-PG dehydrogenase (15-PGDH)이 결장직장암의 병인에 중요한 역할을 하는 것으로 알려지고 있다. 따라서 본 연구는 대장암세포에서 COX-2 와 PGE_2 가 5-FU 에 대한 획득 내성에 중요한 역할을 하는지 알아보고자 하였다.

방법: 대장암세포주인 SNU-C5/WT 에 5-FU 의 농도를 14 μ M 에서부터 140 μ M 까지 점차 증량하면서 세포 배양하여 5-FU 에 내성을 보이는 대장암세포(SNU-C5/5FUR)을 배양하였다. 여러 약물에 대한 세포독성 실험을 MTT assay 을 이용하여

시행하였다. SNU-C5/WT 과 SNU-C5/5FUR 사이의 유전자의 발현 차이를 cDNA mircoarray assay 를 이용하여 비교하였다. COX-2, mPGES1, 15-PGDH, vascular endothelial growth factor (VEGF)의 발현은 real-time RT-PCR, RT-PCR, Western blot assays 등을 사용하였다. PGE_2 의 농도는 enzyme-linked immunosorbent assay (ELISA) 방법을 이용하여 측정하였다.

결과: SNU-C5/5FUR 세포주는 모세포주인 SNU-C5 (SNU-C5/WT)보다 5-FU 에 대해 6.7 배 더 내성을 보였다. COX-2 mRNA 와 단백질은 SNU-C5/WT 에서 보다 SNU-C5/5FUR 에서 더 높게 발현되었다. PGE_2 농도는 SNU-C5/5FUR 에서 유의하게 증가되었다($P < 0.01$). 그리고 meloxicam 투여 후에 PGE_2 levels 은 용량 의존적으로 감소되었다($P < 0.01$). 그러나 SNU-C5/WT 에서는 이러한 현상이 관찰되지 않았다. 또한 mPGES1 과 15-PGDH 발현은 SNU-C5/WT 에서 보다 SNU-C5/5FUR 에서 더 증가된 것이 관찰되었다. VEGF mRNA 의 발현은 SNU-C5/WT 에서 보다 SNU-C5/5FUR 에서 더 증가 되었으며, 이러한 up-regulation 은 meloxicam 투여 후 감소되는 것이 관찰되었다.

결론: 이러한 결과는 대장암세포에서 5-FU 의 획득내성과 관련하여 COX 2-derived PGE_2 가 관여한다는 것을 시사한다. 또한 대장암세포에서 PGE_2 합성을 감소시키는 약물 등은 혈관생성 억제 기전에 의해 5-FU 에 대한 내성 극복에 도움이 될 것으로 기대된다.

Ⅰ. Introduction

5-Fluorouracil (5-FU) is a widely used chemotherapeutic agent that inhibits cancer cell growth and initiates apoptosis by targeting thymidylate synthase (TS) and by direct incorporation of 5-FU metabolites into DNA and RNA[Longley DB et al. 2003]. 5-FU-based chemotherapy improves overall and disease-free survival of patients with solid cancers, including colorectal cancer. De novo and acquired chemoresistance is the major obstacle for the success of 5-FU-based chemotherapy. Overexpression of TS has been shown to be a major 5-FU resistance-inducing factor [Popat S, 2004].

However overexpression of TS does not account for all non-responding tumors in colorectal cancer patients treated with 5-FU [Johnston PG et al. 2004, Salonga D et al. 2003, Sobrero A et al. 2000]. Besides the overexpression of TS, high expression levels of dihydropyrimidine dehydrogenase (DPD) [Salonga D et al. 2003, Ishibiki Y et al. 2003], the genetic status of p53[Boyer J et al. 2004, Bunz F et al. 1999], NF-kB[Wang W et al. 2004, Wang W et al. 2003], DNA mismatch-repair genes[Meyers M et al. 2001], and cell cycle disturbance [Longley DB et al. 2003, Mirjolet JF et al. 2002] have been reported to be associated with 5-FU resistance. Thus, the resistant mechanisms of 5-FU have been thought to be multi-factorial and have not yet been fully understood.

Up-regulation of cyclooxygenase (COX)-2 expression is an early and key oncogenic event in human colon neoplasia, typifying 85% of colon cancers and 50% of colon adenomas [Brown JR & DuBois RN, 2005].

It has also been suggested that the overexpression of COX-2 in colorectal cancer is associated with tumor growth, angiogenesis, lymphatic invasion, and metastasis [Yamauchi T et al. 2002]. Nonsteroidal anti-inflammatory drugs (NSAIDs) targeting COX-2 have been shown to directly shrink the size of colon adenoma in some patients and mediate apoptosis in cancer cells [Williams CS et al. 1999, Fosslien E. 2000]. In mice, genetic inactivation of COX-2 similarly blocks development of murine intestinal adenomas [Subbaramaiah K & Dannenberg AJ. 2003, Jacoby RF et al. 2000].

Most actions of COX-2 are mediated by prostaglandin E_2 (PGE_2). COX-2 produces prostaglandin H_2 (PGH_2), and PGH_2 is converted to PGE_2 by cytosolic or membrane-associated PGE2 synthase (PGES). The inducible form of PGES is microsomal PGES1 (mPGES1; gene symbol PTGES), which has a marked affinity for coupling with COX-2. Induced expression of mPGES1 is observed in inflammatory conditions and cancers of the stomach, colon, endometrium, lung, and skin [Kamei D et al. 2003, Yoshimatsu K et al. 2001]. PGE_2 levels are regulated not only by its synthesis but also by its degradation. The key enzyme responsible for the biological inactivation of prostaglandins is NAD^+-linked 15-PGDH. And recent studies identified a tumor suppressor activity of 15-PGDH in various cancers [Backlund MG et al. 2005, Myung SJ et al. 2006].

Some studies reported that antitumor effect of 5-FU was increased with the combination treatment of COX-2 inhibitors [Mizutani Y et al. 2002, Wilgus TA et al. 2004]. However, little is known about the role of COX-2 in the mechanisms of the acquired resistance to 5-FU in colon cancer cells.

In the present study, I therefore investigated whether COX-2 might contribute to the acquired resistance to 5-FU in colon cancer cells. Furthermore, I examined whether mPGES-1 and 15-PGDH have been involved in colon cancer cells with acquired 5-FU resistance.

Ⅱ. Materials and Methods

Chemicals and antibodies

5-FU and meloxicam were purchased from Sigma Co. (ST. Louis, MO, USA). The primary antibodies for Western blotting assay were followings: COX-2 mouse monoclonal (1:500, Cayman, Ann Arbor, MI, USA); mPGES1 mouse monoclonal clone 6C6, (1:500, Cayman Chemical); 15-PGDH rabbit polyclonal (1:500, Nobus Biologicals, Littleton, CO, USA).

Cell culture

SNU C5 cell lines from the Cancer Research Center in Seoul National University (South Korea) were cultured at 37°C in a 5% CO_2 atmosphere using RPMI1640 medium (GibcoBRL, Gland Island, NY, USA) with 10% heat inactivated fetal bovine serum (Sigma). The cells were maintained as a suspension culture and then subcultured. The 5-fluorouracil-resistant SNU C5 subline SNU C5/5FUR was selected from the parental cell line, SNU C5, after chronic exposure to an intermittent dose schedule of 5-fluorouracil in order to allow the expression of the resistance phenotype. 5-fluorouracil was added starting from 1 x IC_{50} (The 50% inhibitory concentration), and the concentration was increased at a rate of 50%. Finally, the cells were cultured in a fixed 5-fluorouracil concentration (140 μM).

Cytotoxicity assay

The in vitro cytotoxicity of the drugs was determined by using the MTT assay [Pieters R et al. 1988]. Ninety-μl aliquots of the cell suspensions, at 1 x 10^5

cells/ml in a RPMI1640 medium containing 10% FBS, were seeded into a 96-well microplate which already contained 10 μl of a drug. The wells containing no drugs were used as a control of cell viability. A stock solution of 5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) was prepared in normal saline and then stored at -20 °C. After incubation of the cells at 37°C for 3 days, an aliquot of 10 μl of MTT solution was added to each well, shaken for 1 min, and the microplates were incubated for 5 hr. Formazan crystals were dissolved with dimethylsulfoxide (DMSO). The optical density of the wells was measured with a microplate reader at a wavelength of 540 nm. IC_{50} of a particular agent was defined as that drug concentration that causes a 50% reduction in the cell number versus the untreated control. The IC_{50} values were directly determined from the semilogarithmic dose-response curves. All experiments were carried out at least in triplicate.

cDNA microarray assay

TwinChip^{TM} Human-8K (Digital Genomics, Seoul, Korea) was used for this study. The total RNA was prepared from the cells by using a RNeasy midi kit (Quiagen, Hilden, Germany). The quality and integrity of the prepared total RNAs were confirmed with the use of an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA), and by spectrophotometry. Fluorescent labeled cDNA for the cDNA microarray analysis was prepared by the reverse transcription of the total RNA in

the presence of aminoallyl-dUTP. This was followed by the coupling of the Cy3 and Cy5 dyes (Amersham Pharmacia, Uppsala, Sweden) for SNU C5 and SNU C5/5FUR cells, respectively. The TwinChip^{TM} Human-8K cDNA microarray was hybridized with a mixture of the fluorescent labeled cDNAs from SNU C5 and SNU C5/5FUR cells at 58 °C for 16 hr and then they were washed. After the washing procedure, the DNA chips were scanned using a ScanArray Lite (Perkin-Elmer Life Sciences, Billerica, MA). The scanned images were analyzed with GenePix 3.0 software (Axon Instruments, Union City, CA) to obtain the gene expression ratio [Yang YH et al. 2002]. Logged gene expression ratios were normalized by a LOWESS regression. The genes were considered differentially expressed when the logarithmic gene expression ratios in two independent hybridizations were more than a 2-fold difference in the expression level.

Determination of mRNA levels by quantitative reverse transcription - Polymerase Chain Reaction (RT-PCR)

Quantitative Real-time RT- PCR

Extraction of mRNA from the colorectal cancer tissues and the corresponding normal tissues was performed according to the RNeasy proctocol (Qiagen, Hilden, Germany). One microgram of total RNA was reversely transcribed into cDNA in a volume of 20μ l with avian myeloma leukemia virus (AMV) reverse transcriptase and oligo dT primers (Promega, Madison, USA) according to the manufacture' s manual. Real-time PCR measurement of COX-2, mPGES1, and 15-PGDH cDNA

was performed with the Light Cycler Instrument (Roche) using the Fast Start DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). [Schulz C et al. 2003] For verification of the correct amplification product, PCRs were analyzed on a 2% agarose gel stained with ethidium bromide. The sequences of the primers are as follows: for β-actin, 5'-GACTATGACTTAGTTGCGTTA-3' and 5'-GTTGAACTCTCTACATACTTCCG-3'; for COX-2, 5'-ATGATCTACCCTCCTCAA-3' and 5'-GAACAACTGCTCATCAC-3'; for mPGES1, 5'- CAT CAA GAT GTA CGT GGT -3' and 5'- GGA GTA GAC GAA GCC C -3'; for 15-PGDH, 5'- TCT GTT CAT CCA GTG CGA TGT -3' and 5'- ATA ATG ATG CCG CCT TCA CCT -3'. Each reaction (20 μl) contained 4 μl cDNA (10 fold dilution), 2.5mM $MgCl_2$, 10 pmol of each primer and 2 μl of Fast Starter Mix (containing buffer, dNTPs, SYBR Green dye and Tag polymerase). The amplification of target genes were as follows: pre-denaturing at 95 °C for 10 min, 40 cycles of denaturing at 95 °C for 15 sec, annealing for COX-2 at 65°C; mPGES-1, cPGES-1 and 15-PGDH at 57°C; β-actin at 55°C for 5 sec, and extension at 72°C for 7 sec (β-actin for 21 sec). Melting curve analysis was performed to confirm production of a single product. Negative controls without template were produced for each run. Gene expression values (relative mRNA levels) are expressed as ratios (difference between the Ct values) between the gene of interest (COX-2, mPGES-1, cPGES-1 and 15-PGDH mRNA) and an internal reference gene (β-actin) that provide a normalization

factor for the amount of RNA isolated from a specimen. Analysis of data was performed using Light Cycler software version 4.0.

RT-PCR analysis

RT-PCR was used to analyze the expression of mRNA for VEGF and β-actin (internal control). One microgram of total RNA was reversely transcribed into cDNA in a volume of 20μl with Taq polymerase and 10 pmole of each primer in a GeneAmp PCR2400 (Perkin-Elmer, Boston, MA, USA). For verification of the correct amplification product, PCRs were analyzed on a 2% agarose gel stained with ethidium bromide. For verification of the correct amplification product, PCRs were analyzed on a 2% agarose gel stained with ethidium bromide. The gels were documented using a Kodak Image Station 4000MM (Eastman Kodak, Rochester, NY, USA) and digitized using UN-SCAN-IT software (SilK scientific, Orem, UT). The sequences of the primers are as follows: for β-actin, 5'-GACTATGACTTAGTTGCGTTA-3' and 5'-GTTGAACTCTCTACATACTTCCG-3'; for VEGF, 5'-AYGCGGATCAAACCTCACCAAG-3' and 5'-ACAGCAGCGGGCACCAAC-3'. Each reaction (20 μl) contained 1.25 μl cDNA, 2.5mM $MgCl_2$, 10 pmol of each primer and Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT, USA). The amplification of target genes were as follows: pre-denaturing at 94°C for 5 min, 35 cycles of denaturing at 94°C for 30 sec, annealing for VEGF at 62°C (β-actin at 55°C) for 30 sec, and extension at 72°C for 1 min (β-actin for 21 sec).

Western blot analysis

Proteins were solubilized and then fractionated by SDS-PAGE. Western blotting was performed by a slight modification of the method first described by Towbin et al.[Towbin H et al. 1972]. Proteins were transferred onto a polyvinylidenefluoride (PVDF) membrane by electroblotting at a current of 60 V overnight. The membrane was incubated in 5% skim milk solution in TBST buffer for 2 hr at room temperature, washed, and then incubated with primary antibodies. The membrane was washed, and incubated with horseradish peroxidase-conjugated rabbit-antimouse IgG (diluted 1:2000, Sigma, ST. Louis, MO, USA) for 1 hr. The membrane was then stained using the detection reagent of the ECL detection kit (Amersham, Piscataway, NJ, USA). Protein concentration was determined with a Bio-Rad protein assay kit and standardized with bovine serum albumin.

Determination of PGE_2 levels by enzyme-linked immunosorbent assay (ELISA)

Prostaglandin E_2 levels in the culture medium were measured by radioimmunoassay using a general assay procedure adapted from Cetta and Goetz (Cetta F & Goetz FW, 1982). In preliminary studies, prostaglandin was recovered from culture medium with extraction fluid (ethyl acetate : isopropanol : 0.05 N HCl = 3 : 3 : 1) and the recovery rate was relatively constant (92 ± 3%, N = 9). When PGE_2 was assayed in increasing aliquots of unextracted medium (25, 50 and 100 μ L), potency estimates were parallel to a linearly transformed dose response

curve. Thus, the medium samples were assayed directly without extraction. Each sample was quantified using a liquid scintillation analyzer. Duplicate hormone standards (5 to 1000 pg) were included in each assay. The between and within assay coefficient of variants for PGE_2 were 7.3% and 6.5%, respectively.

Statistical analysis

Statistically significant difference among data sets of multiple groups were analyzed using ANOVA followed by Fisher's protected least significant difference. The SPSS version 12.0 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Ⅲ. Results

1. Sensitivity of SNU-C5/WT and SNU-C5/5FUR cells to 5-FU

The sensitivity of 5-FU on SNU-C5/WT and SNU-C5/5FUR cells was determined using MTT assay (Fig. 1). IC_{50} values against 5-FU of SNU-C5/WT and SNU-C5/5FUR cells were 17.8 μ M and 118.8 μ M, respectively, resulting in 6.7 times more resistant to 5-FU in SNU-C5/5FUR cells as compared with that in SNU-C5/WT cells.

2. mRNA expression profiles altered in SNU-C5/5FUR cells

The microarray data were classified into four categories by their function; signal transduction, immune response, transcription, and response to stress (Table 1 - 4). As shown in Table 1, interferon receptor 1 and prostaglandin E synthase were significantly increased, while vascular endothelial growth factor B, fibroblast growth factor binding protein 1, BRCA 1 associated gene, tumor necrosis factor receptor superfamily member 8 and 21 were significantly decreased. As shown in Table 2, leukotriene A4 hydrolase and Inhibitor of kappa light polypeptide gene enhancer in B-cells were significantly decreased. As shown in Table 3, transforming growth factor beta 1 induced transcript 4 was significantly increased, and Methyl CpG binding protein 2 (Rett syndrome), cyclin-dependent kinase 7 were significantly decreased. As shown in Table 4, kallikrein 6 and interferon

receptor 1 were significantly increased, while ubiquitin-like domain member 1, B-cell CLL/lymphoma 6 (BCL6) were significantly decreased.

3. Expression levels of COX-2, mPGES1, and 15-PGDH in SNU-C5/WT and SNU-C5/5FUR cells

On quantitative RT-PCR analyses, the expression levels of COX-2, mPGES1, and 15-PGDH were significantly increased in SNU-C5/5FUR than in SNU-C5/WT. On Western blotting assay, COX-2 expression was higher in SNU-C5/5FUR than in SNU-C5, whilst mPGES1 and 15-PGDH expression seemed similar in both cell lines (Fig. 2)

4. Effect of meloxicam on PGE_2 levels and sensitivity to 5-FU in SNU-C5/WT and SNU-C5/5FUR cells

PGE_2 levels present in the supernatant of the culture medium after incubation for 12 hours were determined using ELISA method. In the basal condition, PGE_2 levels were significantly higher in SNU-C5/5FUR than in SNU-C5 ($p < 0.01$). Furthermore, PGE_2 levels were significantly decreased in SNU-C5/5FUR cells in a dose-dependent manner ($P < 0.01$), but not in SNU-C5/WT with treatment of the COX-2 inhibitor meloxicam (Fig. 3).

To investigate whether or not meloxicam can overcome 5-FU resistance in SNU-C5/5FUR cells, MTT assay was carried out. Although at the concerntration of 500

μ M meloxicam enhanced sensitivity to 5-FU in both cell lines, at the lower concerntration meloxicam did not show any effect on 5-FU sensitivity in both cell lines (Fig. 4).

5. VEGF mRNA expression in SNU-C5/WT and SNU-C5/5FUR cells

In spite of up-regulation of PGE_2 levels in SNU-C5/5FUR cells, meloxicam showed little effect on overcoming the resistance to 5-FU under the circumstances of cell culture medium. Next, I investigated the expression of VEGF, which is the most powerful stimulator for angiogenesis that is important in tumor growth and metastasis. VEGF mRNA levels in SNU-C5/5FUR cells were higher than that in SNU-C5/WT cells. Meloxicam of 10 μ M to 500 μ M decreased VEGF mRNA levels in SNU-C5/5FUR cells in a dose-dependent manner, but paradoxically increased those in SNU-C5/WT cells in a dose-dependent fashion (Fig. 5).

Ⅳ. Discussion

In the current study, I demonstrate that the expression of COX-2, its main product, PGE2 and VEGF are up-regulated in SNU-C5/5FUR cells as compared with those in SNU-C5/WT. Furthermore, COX-2 inhibitor shows anti-angiogenic effect by blocking the levels of VEGF mRNA in the resistant cell, but not in the wild type colon cancer cells.

TS, thymidylate phosphorylase, DPD suggested to be a resistance mechanism to 5-FU were not significantly changed in the microarray analysis of the present study. Also, NF-kB was not significantly changed in our microarray analysis. However, we used the microarray analysis as a screening method to detect the differential genes correlated with 5-FU resistance. So we believed that the results on the microarray analysis were not confirmative. It has been suggested that wild type p53 significantly improves response to 5-FU both in vitro and in vivo [Arango D, 2001]. In the present study, we used the colon cancer cell lines, SNU-C5 and its 5-FU resistant cell line, of which the former is known to have mutated p53 and complete loss of p53 function [Rand A, et al.1996].

It is known that COX-2 could be induced by a variety of stimuli including oncogenes (HER-2/neu), growth factors (EGF), tumor promoters (phorbol esters and bile acid), and chemotherapy [Subbaramaiah K, 2003]. Of various chemotherapeutic agents, microtubule-interfering agents (paclitaxel, vincristine,

etc) are known to induce COX-2 in cancer cells [Subbaramaiah K, 2000, Mathieu A, 2004]. However, it is yet inconclusive whether COX-2 is induced by 5-FU in 5-FU-resistant cancer cells. It has been reported that expression of COX-2 mRNA was increased by exposure to 5-FU or 5-FU combination chemotherapy in human solid tumors including esophageal, breast, ovarian, and colorectal tumors [Mercer SJ. et al. 2005]. In addition, significant increase of COX-2 has been reported in rectal cancer after preoperative chemoradiation [Debucquoy A, et al. 2006]. In the present study, I also demonstrate that COX-2 and its main product, PGE_2 levels are up-regulated in the colon cancer cell with acquired resistance to 5-FU (in SNU-C5/5FUR cells). This finding suggests that COX-2 inhibitors may play a beneficial role in overcoming the resistance of SNU-C5/5FUR cells to 5-FU.

Clinically, 5-FU is the main drug in the first line or in the second line or even in the third line chemotherapy of patients with colorectal cancer (CRC). In the first line chemotherapy or adjuvant therapy, 5-FU usually used combined with folic acid (leukovorin) in patient with CRC. In case of recurrent disease after treating with this regimen, 5-FU also used in combination with oxaliplatin (FOLFOX regimen) or irinotecan (FOLFIRI regimen). Theoretically, in that case it is likely to have little effect of 5-FU on the chemotherapy. So, it is needed something different to try to overcome the acquired resistance to 5-FU.

NSAIDs, targeting COX-2, is commonly prescribed drug in patients with colorectal cancer as well as in patient with other diseases such as arthritis and other inflammatory diseases. Although it has been well known that COX-2 inhibitors induce apoptosis in cancer cells and reduce the growth rate of established tumor, tumor regression is rarely observed. Hence, it is reasonable that COX-2 inhibitors are given in conjunction with standard anti-cancer agents rather than monotherapy. Irie et al. [Irie T et al. 2007] reported that celecoxib, selective COX-2 inhibitor synergistically potentiated the antitumor effect of 5-FU depending on IFN-gamma in vivo study with colon cancer cells. Similar finding has been reported in the Ultraviolet light B-induced skin tumors of mice [Wilgus TA et al. 2004]. Inconsistent those findings, we could not demonstrated that meloxicam, which is commonly used COX-2 inhibitor, exactly preferential COX-2 inhibitor, synergistically potentiated the antitumor effect of 5-FU in both the chemonaive colon cancer cells and acquired 5-FU resistant colon cancer cells.

Angiogenesis, the sprouting of capillaries from preexisting vasculature, occurs during embryonic development, wound repair, and tumor growth. Because it is thought to play a central role in human tumor development, inhibition of tumor-associated angiogenesis has been touted as a promising therapeutic strategy. In fact, it is generally believed that neovascularization is required for tumor to grow > 2-3 mm in size [Wang D and DuBois RN, 2004]. Chang et al.[Chang SH, 2004] reported that PGE_2 stimulated that the expression angiogenic regulatory genes in

mammary tumor cells isolated from COX-2 transgenic mice, defining COX-2-derived PGE2 as a potent inducer of angiogenic switch during mammary cancer progression. In the present study, consistent to that report, up-regulation of VEGF mRNA was observed in the colon cancer cell with the acquired resistance to 5-FU and this up-regulation was recovered with the treatment of COX-2 inhibitor, suggesting that COX-2 derived PGE2 may also play an important role in angiogenic switch during the acquired resistance of colon cancer to 5-FU. Furthermore, in the present study, we demonstrated that meloxicam exerted anti-angiogenic effect through inhibition of PGE_2 synthesis at a low dose (10μ M), which is comparable to clinically therapeutic levels.

In this study, however, there was an unexpected result. In SNU-C5, which is chemonaive colon cancer cell and is little expressing VEGF and COX-2 in the basal state, up-regulation of VEGF was observed after treatment of meloxicam. We could not interpret this result exactly. Similar to this result, Ueno et al. [Ueno T et al. 2006] reported that treatment with COX-2 inhibitor, celecoxib, decreased serum VEGF levels at an early time and increased VEGF levels in serum and plasma at a late time in breast cancer patients. So further studies are necessary to elucidate how COX-2 inhibitors play a role in production of VEGF in COX-2 not expressing cancer cells or tissues.

Ⅴ. Conclusions

These results suggest that upregulation of COX-2 derived PGE_2 may be involved in the acquired 5-FU resistance in colon cancer cells. Targeting to decrease PGE_2 level may benefit in overcoming 5-FU resistance through the anti-angiogenic effect in colon cancer cells.

References

1. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanism of action and clinical strategies. Nat Rev Cancer 2003;3:330-338.

2. Popat S, Matakidou A, Houlston RS. Thymidylate synthase expression and prognosis in colorectal cancer: a systematic review and met-analysis. J Clin Oncol 2004;22:529-536.

3. Johnston PG, Benson AB, Catalano P, Rao MS, O' Dwyer PJ, Allegra CJ. Thymidylate synthase protein expression in primary colorectal cancer: lack of correlation with outcome and response to fluorouracil in metastatic disease sites. J Clin Oncol 2004;21:815-819.

4. Salonga D, Dannenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 2000;6:1322-1327.

5. Sobrero A, Kerr D, Glimelius B, Van Cutsem E, Milano G, Pritchard DM, Rougier P, Aapro M. New directions in the treatment of colorectal cancer: a look to the future. Eur J Cancer2000;36:559-566.

6. Ishibiki Y, Kitajima M, Sakamoto K, Tomiki Y, Sakamoto S, Kamano T. Intratumoral thymidylate synthase and dihydropyrimidine dehydrogenase acitivities are good predictors of 5-fluorouracil sensitivity in colorectal cancer. J Int Med

Res 2003;31:181-187.

7. Boyer J, McLean EG, Aroori S, Wilson P, McCulla A, Carey PD, Longley DB, Johnston PG. Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. Clin Cancer Res 2004;10;2158-2167.

8. Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW, Vogelstein B. Disruption of p53 in human cancer cells alters the response to therapeutic agents. J Clin Invest 1999;104:263-269.

9. Wang W, Cassidy J, O' Brien V, Ryan KM, Collie-Duguid E. Mechansistic and predictive profiling of 5-fluorouracil resistance in human cancer cell line. Cancer Res 2004;64:8167-8176.

10. Wang W, McLeod HL, Cassidy J. Disulfiram-mediated inhibition of NF-kappaB activity enhances cytotoxicity of 5-flurorouracil in human colorectal cancer cell lines. Int J Cancer 2003;104:504-511.

11. Meyers M, Wagner MW, Hwang HS, Kinsella TJ, Boothman DA. Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle response. Cancer Res 2001;61:5193-5201.

12. Mirjolet JF, Didelot C, Barberi-Heyob M, Merlin JL. G(1)/S but not G(0)/G(1)cell fraction is related to 5-fluorouracil cytotoxicity. Cytometry 2002;48;6-13.

13. Brown JR, DuBois RN. COX-2: a molecular target for colorectal cancer

prevention. J Clin Oncol 2005;23:2840-2855.

14. Yamauchi T, Watanabe M, Kubota T, Hasegawa H, Ishii Y, Endo T, Kabeshima Y, Yorozuya K, Yamamoto K, Mukai M, Kitajima M. Cyclooxygenase-2 expression as a new marker for patients with colorectal cancer. Dis Colon Rectum 2002;45:98-103.

15. Williams CS, Mann M, DuBois RN: The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999;18:7908-7916.

16. Fosslien E. Biochemistry of cyclooxygenase (COX-2) inhibitors and molecular pathology of COX-2 in neoplasia. Crit Rev Clin Lab Sci 2000;37:431-502.

17. Subbaramaiah K, Dannenberg AJ. Cyclooxygenase2: a molecular target for cancer prevention and treatment. Trends Pharmacol Sci 2003;24:96-102.

18. Jacoby RF, Seibert K, Cole EC, Kelloff G, Lubet RA. The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. Cancer Res 2000;60:5040-5044.

19. Kamei D, Murakami M, Nakatani Y, Ishikawa Y, Ishii T, Kudo I. Potential role of microsomal prostaglandin E synthase-1 in tumorigenesis. J Biol Chem 2003;278:19396-19405.

20. Yoshimatsu K, Golijanin D, Paty PB, Soslow RA, Jakobsson PJ, DeLellis RA, Subbaramaiah K, Dannenberg AJ. Inducible microsomal prostaglandin E synthase is overexpressed in colorectal adenoma and cancer. Clin Cancer Res 2001;12:3971-3976.

21. Backlund MG, Mann JR, Holla VR, Buchanan FG, Tai HH, Musiek ES, Milne GL, Katkuri S, DuBois RN. 15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer. J Biol Chem 2005;280:3217-3223.

22. Myung SJ, Rerko RM, Yan M, Platzer P, Guda K, Dotson A, Lawrence E, Dannenberg AJ, Lovgren AK, Luo G, Pretlow TPP, Newman RA, Willis J, Dawson D, Markowitz SD. 15-Hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. Proc Natl Acad Sci USA 2006;103:12098-12102.

23. Mizutani Y, Kamoi K, Ukimura O, Kawauchi A, Miki T. Synergistic cytotoxicity and apoptosis of JTE-522, a selective cyclooxygenase-2 inhibitor, and 5-fluorouracil against bladder cancer. J Urol 2002;168:2650-2654.

24. Wilgus TA, Breza TS Jr, Tober KL, Oberyszyn TM. Treatment with 5-fluorouracil and celecoxib displays synergistic regression of ultraviolet light B-induced skin tumors. J Invest Dermatol 2004;122:1488-1494.

25. Pieters R, Huismans DR, Leyva A, Veerman AJ. Adaptation of the rapid automated tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia, Cancer Lett 1988;41:323-332.

26. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation, Nucleic Acids Res 2002;30:15.

27. Cetta F, Goetz FW. Ovarian and plasma prostagrandin E and F levels in brook trout (Savelinus frontinalis) during pituitary-induced ovulation. Biol Reprod 1982;

27:1216- 1221.

28. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biotechnology 1972;24:145-149.

29. Arango D, Corner GA, Wadler S, Catalano PJ, Augenlicht LH. c-myc/p53 interaction determines sensitivity of human colon carcinoma cells to 5-fluorouracil in vitro and in vivo. Cancer Res 2001;61:4910-4915.

30. Rand A, Glenn KS, Alvares CP, White MB, Thibodeau SM, Karnes WE. p53 function loss in a colon cancer cell line with two missense mutations (218leu and 248trp) on separate alleles. Cancer Lett 1996;98:183-191.

31. Mathieu A, Remmelink M, D' Haene N, Penant S, Gaussin J, Ginckel RV, Darro F, Kiss R, Salmon I. Development of a chemoresistant orthotopic human nonsmall cell lung carcinoma model in nude mice: analyses of tumor heterogeneity in relation to the immunohistochemical levels of expression of cyclooxygenase-2, ornithine decarboxylase, lung-cancer resistance protein, prostaglandin E synthetase, and glutathione-S-transferase (GST)-alpha, GST-mu, GST-pi. Cancer 2004;101:1908-1918.

32. Mercer SJ, Di Nicolantonio F, Knight LA, Gabriel FG, Whitehouse PA, Sharma S, Fernando A, Bhandari P, Somers SS, Toh SK, Cree IA; NHS Collaborative Research Programme on Predictive Oncology. Rapid up-regulation of

cyclooxygenase-2 by 5-fluorouracil in human solid tumors. Anticancer Drugs 2005:16:495-500.

33. Debucquoy A, Goethals L, Geboes K, Roels S, McBride WH, Haustermans K. Molecular responses of rectal cancer to preoperative chemoradiation. Radiother Oncol 2006;80:172-177.

34. Irie T, Tsujii M, Tsuji S, Yoshio T, Ishii S, Shinzaki S, Egawa S, Kakiuchi Y, Nishida T, Yasumaru M, Iijima H, Murata H, Takehara T, Kawano S, Hayashi N. Synergistic antitumor effects of celecoxib with 5-fluorouracil depend on IFN-gamma. Int J Cancer 2007;Epub ahead of print.

35. Wang D, DuBois RN. Cyclooxygenase 2-derived prostaglandin E2 regulates the angiogenic switch. Proc Natl Aca Sci USA 2004;101:415-416.

36. Chang SH, Liu CH, Conway R, Han DK, Nithipatikom K, Trifan OC, Lane TF, Hla T. Role of prostaglanding E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. Proc Natl Acad Sci USA 2004;101:591-596.

37. Wilgus TA, Breza TS Jr, Tober KL, Oberyszyn TM. Treatment with 5-fluorouracil and celecoxib displays synergistic regression of ultraviolet light B-induced skin tumors. J Invest Dermatol 2004;122:1488-1494.

38. Ueno T, Chow LW, Toi M. Increases in circulating VEGF levels during COX-2 inhibitor treatment in breast cancer patients. Biomed Pharmacother 2006;60:277-279.

39. Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stiumulate the transcription of cyclooxygenase-2. J Biol Chem 2000;275:14838-14845.

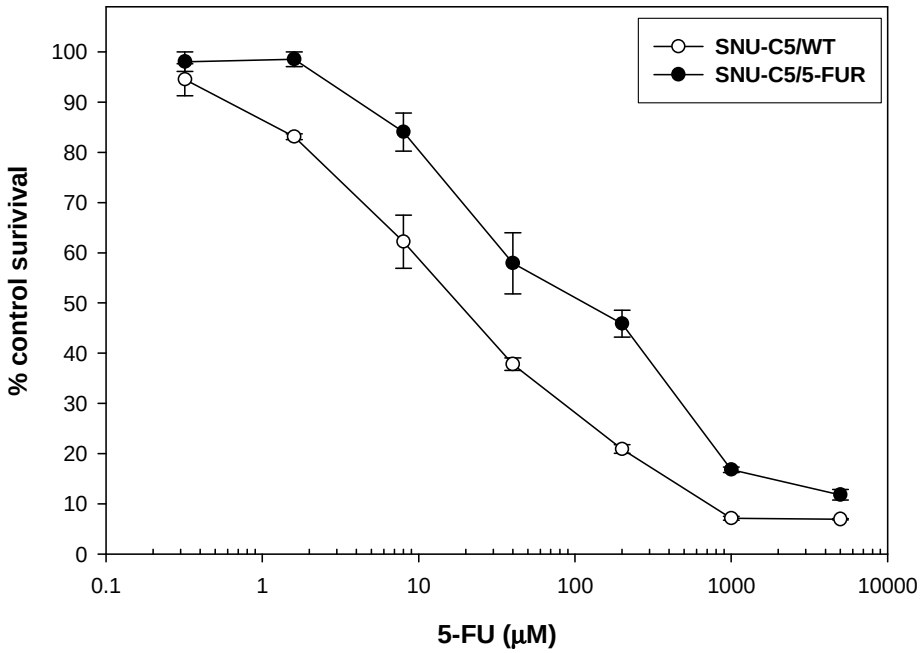

Figure1. Cytotoxic effect of 5-FU in SNU-C5 and SNU-C5/5FUR. Cells were pretreated with various concentrations of 5-FU for 3 days, and then cell viability was measured by MTT assay.

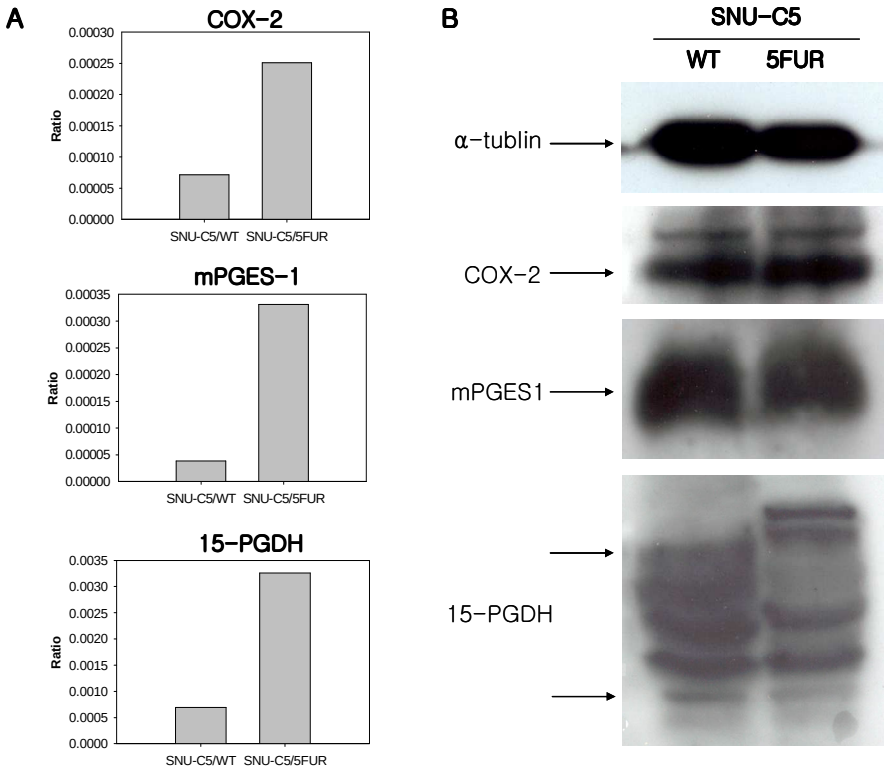

Figure2. (A) mRNA expression patterns of COX-2, mPGES1, and 15-PGDH in SNU-C5 and SNU-C5/5FUR determined by quantitative RT-PCR. (B) Protein expression patterns of COX-2, mPGES1, and 15-PGDH in the two cells determined by Western blott assay. Equivalent amounts (100μ g) of total protein extract from the both cells were separated by SDS-PAGE and immunoblotted with each antibody described in " Materials and methods".

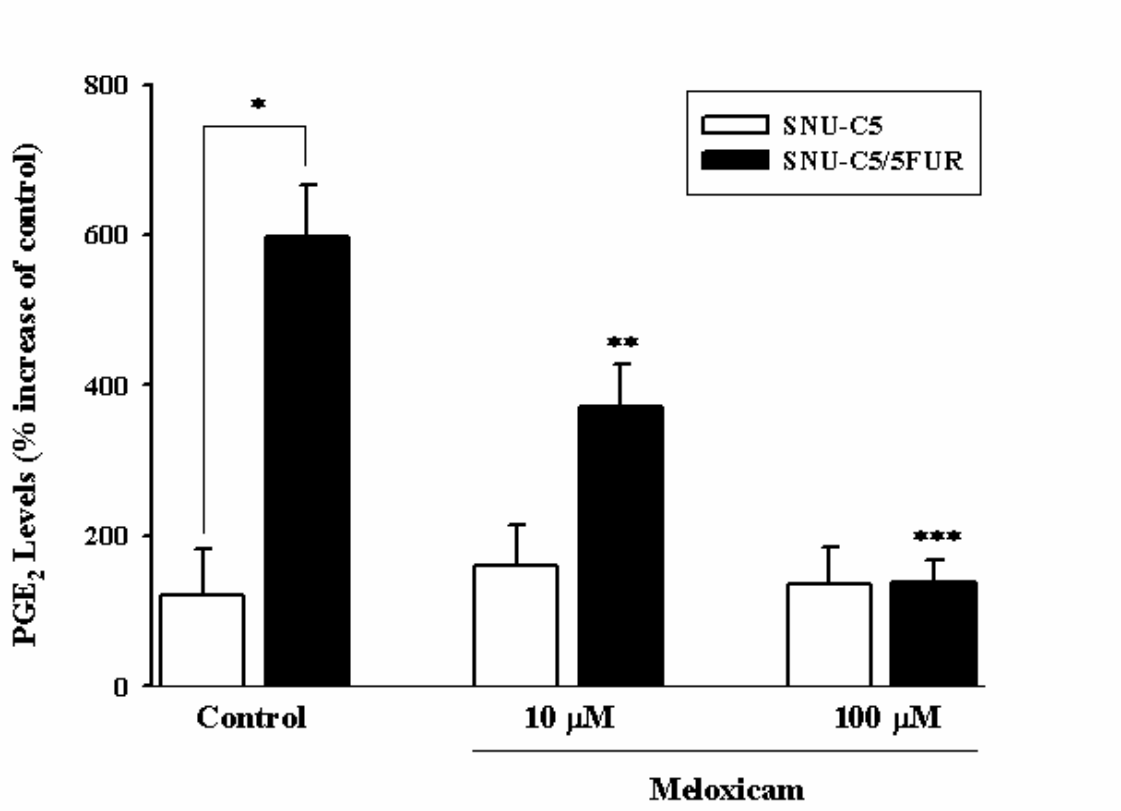

Figure3. Comparison of PGE_2 levels between SNU-C5 and SNU-C5/5FUR. PGE_2 levels present in the supernatant of the culture medium were determined by ELISA using PGE_2 monoclonal antibody (* compare PGE_2 levels between SNU-C5 and SNU-C5/5FUR at basal state; ** compare PGE_2 levels after 12hours treatment of meloxicam (10 μ M) with control in SNU-C5/5FUR. *** compare PGE_2 levels after meloxicam (100 μ M) with control in SNU-C5/5FUR, all of which P value are less than 0.01.)

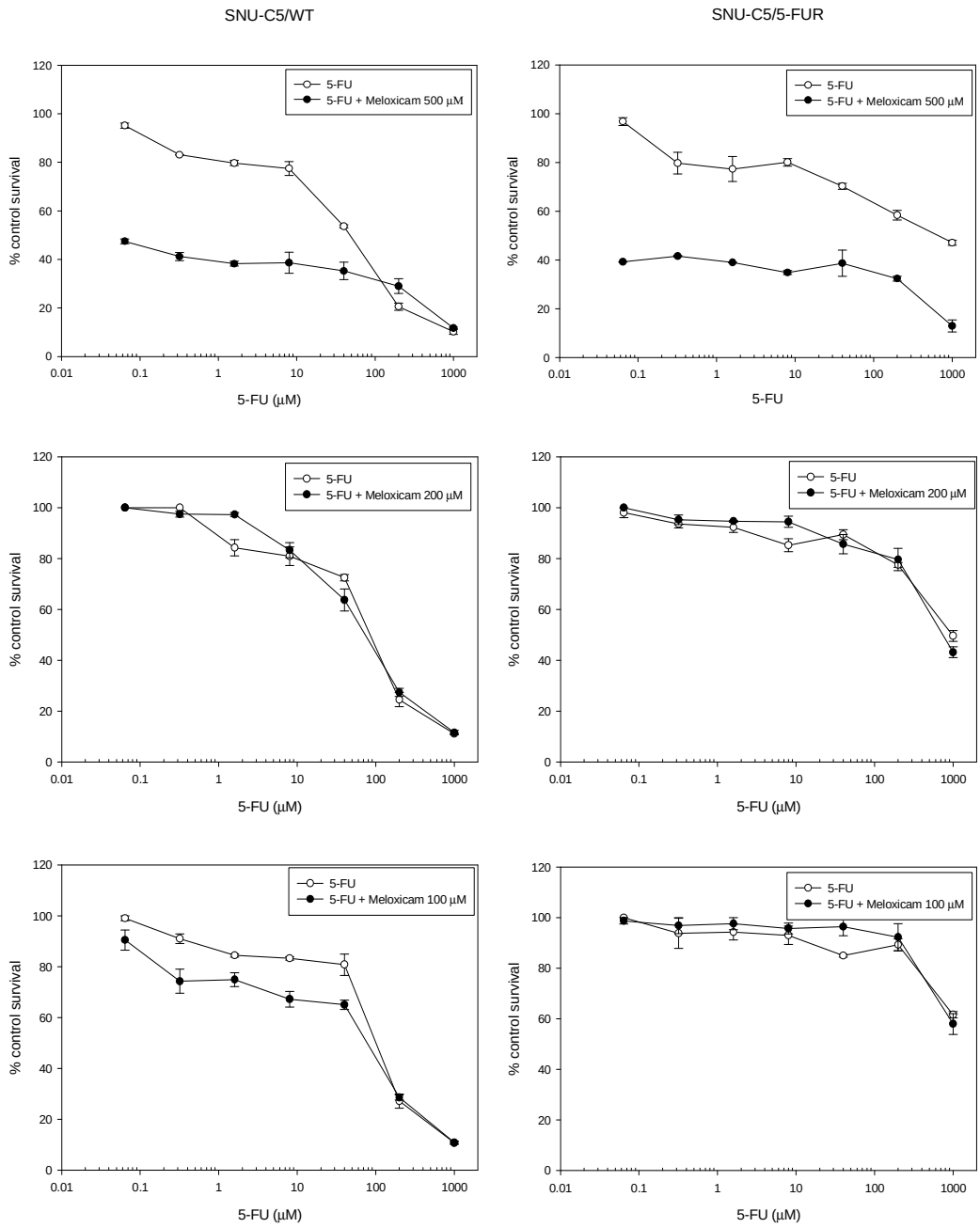

Figure4. Cytotoxic effect of 5-FU alone and/or in combination with meloxicam in SNU-C5 and SNU-C5/5FUR. MTT assay was used to analyze cell viability.

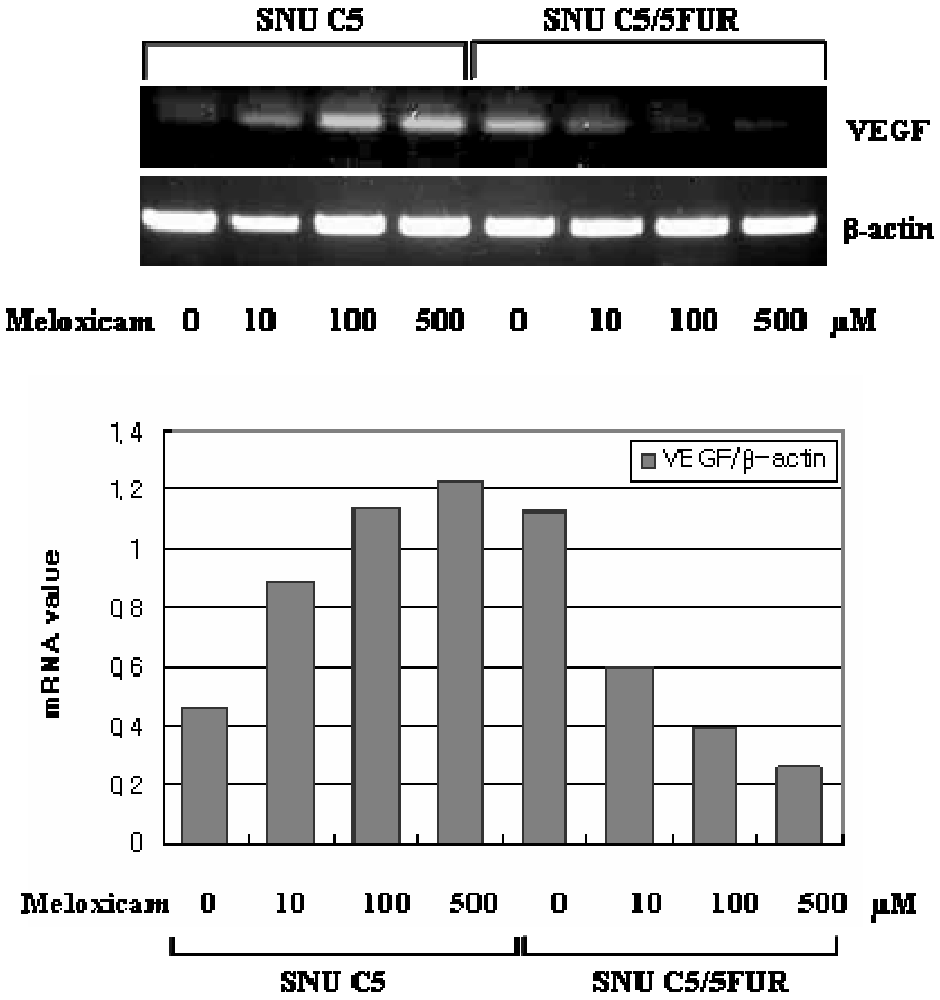

Figure5. Expression patterns of VEGF mRNA in SNU-C5 and SNU-C5/5FUR at basal state and after treatment of meloxicam (10, 100 and 500 μ M) for 24 hours by RT-PCR method.

Table1. Significantly affected genes related to the signal transduction in SNU-C5/5FUR

Title	Gene. Symbol	Fold	Cytoband
Increased genes			
Corticotropin releasing hormone receptor 1	CRHR1	2.4	17q12-q22
Regulator of G-protein signalling 2, 24kDa	RGS2	2.2	1q31
Interferon (alpha, beta and omega) receptor 1	IFNAR1	2.2	21q22.1
Rho guanine nucleotide exchange factor (GEF) 7	ARHGEF7	2.0	13q34
A kinase (PRKA) anchor protein (gravin) 12	AKAP12	2.0	6q24-q25
Prostaglandin E synthase	PTGES	2.0	9q34.3
Dimethylarginine dimethylaminohydrolase 1	DDAH1	2.0	1p22
Decreased genes			
Lymphotoxin beta receptor (TNFR superfamily, member 3)	LTBR	2.0	12p13
Glutamate receptor, ionotropic, N-methyl D-aspartate 2D	GRIN2D	2.0	19q13.1-qter
Gamma-aminobutyric acid (GABA) A receptor, beta 1	GABRB1	2.0	4p12
Ribosomal protein L17	RPL17	2.0	18q21
Rho GTPase activating protein 4	ARHGAP4	2.0	Xq28
Epithelial cell transforming sequence 2 oncogene	ECT2	2.0	3q26.1-q26.2
EPH receptor B2	EPHB2	2.0	1p36.1-p35
Nuclear receptor subfamily 4, group A, member 1	NR4A1	2.1	12q13
Vascular endothelial growth factor B	VEGFB	2.1	11q13
Receptor (calcitonin) activity modifying protein 1	RAMP1	2.2	2q36-q37.1
Tumor-associated calcium signal transducer 2	TACSTD2	2.2	1p32-p31
Fibroblast growth factor binding protein 1	FGFBP1	2.3	4p16-p15
Rho family GTPase 3	ARHE	2.5	2q23.3
BRCA1 associated protein	BRAP	2.5	12q24
TXK tyrosine kinase	TXK	2.8	4p12
Fibroblast growth factor receptor 4	FGFR4	2.9	5q35.1-qter
Endothelin 1	EDN1	3.1	6p24.1
A disintegrin and metalloproteinase domain 9 (meltrin gamma)	ADAM9	3.1	8p11.23
Tumor necrosis factor receptor superfamily, member 8	TNFRSF8	3.2	1p36
Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	IKBKE	3.3	1q32.1
Tumor necrosis factor receptor superfamily, member 21	TNFRSF21	3.6	6p21.1-12.2
Transmembrane 4 superfamily member tetraspan NET-5	NET-5	6.5	12p13.33-13.32

Table2. Significantly affected genes related to the immune response in SNU-C5/5FUR

Title	Gene. Symbol	Fold	Cytoband
Increased genes			
Corticotropin releasing hormone receptor 1	CRHR1	2.4	17q12-q22
Hypothetical protein FLJ35429	FLJ35429	2.4	6p21.32
Keratin 1 (epidermolytic hyperkeratosis)	KRT1	2.0	12q12-q13
Prostaglandin E synthase	PTGES	2.0	9q34.3
Decreased genes			
Lymphotoxin beta receptor (TNFR superfamily, member 3)	LTBR	2.0	12p13
CCAAT/enhancer binding protein (C/EBP), beta	CEBPB	2.0	20q13.1
Leukotriene A4 hydrolase	LTA4H	2.3	12q22
Proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	PSME1	2.6	14q11.2
Dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	DPP4	2.6	2q24.3
Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	IKBKE	3.3	1q32.1
B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	3.4	3q27

Table3. Significantly affected genes related to the transcription in SNU-C5/5FUR

Title	Gene. Symbol	Fold	Cytoband
Increased genes			
Oligodendrocyte lineage transcription factor 2	OLIG2	2.9	21q22.11
Regulatory factor X, 2 (influences HLA class II expression)	RFX2	2.7	19p13.3-p13.2
Snf2-related CBP activator protein	SRCAP	2.5	16p11.2
SPTF-associated factor 65 gamma	STAF65 (gamma)	2.4	2pter-p25.1
Transforming growth factor beta 1 induced transcript 4	TGFB1I4	2.0	13q14
Decreased genes			
Chromobox homolog 7	CBX7	2.0	22q13.1
Activating transcription factor 2	ATF2	2.0	2q32
Telomeric repeat binding factor (NIMA-interacting) 1	TERF1	2.0	8q13
CCAAT/enhancer binding protein (C/EBP), beta	CEBPB	2.0	20q13.1
Nuclease sensitive element binding protein 1	NSEP1	2.0	1p34
Nuclear receptor subfamily 4, group A, member 1	NR4A1	2.1	12q13
Transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	TCF2	2.1	17cen-q21.3
High-mobility group box 2	HMGB2	2.1	4q31
Methyl CpG binding protein 2 (Rett syndrome)	MECP2	2.1	Xq28
Protein phosphatase 1, regulatory subunit 10	PPP1R10	2.2	6p21.3
Transcription factor binding to IGHM enhancer 3	TFE3	2.5	Xp11.22
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	SMARCB1	2.5	22q11.23
Cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)	CDK7	2.6	5q12.1
Zinc finger protein 45	ZNF45	2.6	19q13.2
BTB and CNC homology 1, basic leucine zipper transcription factor 1	BACH1	2.8	21q22.11
B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	3.4	3q27
Sterol regulatory element binding transcription factor 2	SREBF2	5.6	22q13

Table4. Significantly affected genes related to the response to stress in SNU-C5/5FUR

Title	Gene. Symbol	Fold	Cytoband
Increased genes			
Kallikrein 6 (neurosin, zyme)	KLK6	3.8	19q13.3
Interferon (alpha, beta and omega) receptor 1	IFNAR1	2.2	21q22.1
Keratin 1 (epidermolytic hyperkeratosis)	KRT1	2.0	12q12-q13
Prostaglandin E synthase	PTGES	2.0	9q34.3
Decreased genes			
RAD21 homolog (S. pombe)	RAD21	2.0	8q24
CCAAT/enhancer binding protein (C/EBP), beta	CEBPB	2.0	20q13.1
Nuclease sensitive element binding protein 1	NSEP1	2.0	1p34
Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	HERPUD1	2.1	16q12.2-q13
High-mobility group box 2	HMGB2	2.1	4q31
Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1	SERPIND1	2.2	22q11.2
Leukotriene A4 hydrolase	LTA4H	2.3	12q22
Putative translation initiation factor	SUI1	2.3	17q21.2
Cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)	CDK7	2.6	5q12.1
Polymerase (DNA directed), delta 1, catalytic subunit 125kDa	POLD1	2.6	19q13.3
Defensin, alpha 4, corticostatin	DEFA4	2.7	8p23
Connective tissue growth factor	CTGF	3.3	6q23.1
B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	3.4	3q27
Tumor rejection antigen (gp96) 1	TRA1	4.0	12q24.2-q24.3

저작물 이용 허락서

학 과	의학과	학 번	20057457	과 정	박사
성 명	한글 이 연 아 한문 李 娟 婀 영문 Lee Yeon Ah				
주 소	광주광역시 서구 풍암동 금호 2 차 201 동 1502 호				
연락처	E-mail : zeten33@naver.com				
논문제목	한글: 5-fluorouracil 내성 대장암 세포에서 cyclooxygenase 2 에 의해 유도된 Prostaglandin E_2 의 upregulation				
	영문: Upregulation of PGE_2 by cyclooxygenase 2 in colon cancer cells resistant to 5-fluorouracil				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2007 년 8 월 일

저작자: 이 연 아 (인)

조선대학교 총장 귀하