Cancer stem cell-suppressing effect of nonsteroidal anti-inflammatory drugs via cyclooxygenase-2-dependent and -independent pathways in colorectal cancer

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Department of Medicine

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Directed by Professor Won Ho Kim

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Chang Mo Moon

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CSC, cancer stem cell; CRC, colorectal cancer; NSAIDs, nonsteroidal anti-inflammatory drugs; PPARγ, peroxisome proliferator-activated receptor γ; COX, cyclooxygenase; 5-FU, 5-fluorouracil; PGE2, prostaglandin E2; IHC, immunohistochemistry; PE, phycoerythrin; FITC, fluorescein; SFM, serum-free medium; NOD/SCID, nonobese diabetic/severe combined immunodeficient; DMEM, Dulbecco's modified Eagle's medium; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine -*t*-butyl ester; JLK6, 7-amino-4-chloro-3-methoxyisocoumarin; PBS, phosphate buffered saline; BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; EGF, epidermal growth factor; bFGF, fibroblast growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; PPRE, PPAR-responsive element; CMC, carboxy methylcellulose; FAP, familial adenomatous polyposis; AOM, azoxymethane; SMAC, second mitochondria-derived activator of caspase

ABSTRACT

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Cancer stem cell (CSC) model assumes that a small subset of cells in tumors have the ability to initiate and sustain tumor growth. CSCs are resistant to many current chemotherapeutic agents and play a pivotal role in cancer relapse. In this study, we aimed to identify the effective agents to increase the sensitivity to chemotherapeutic agents by suppressing CSCs in human colorectal cancer (CRC).

Colosphere forming assay and flow cytometric analysis of CSC markers (CD133 and CD44) were performed to investigate the CSC suppressing effect of nonsteroidal anti-inflammatory drugs (NSAIDs), which are known having the activities of peroxisome proliferator-activated receptor γ (PPARγ) agonist and γ-secretase inhibitor as well as cyclooxygenase (COX) inhibitor. *In vitro* experiments using SW620 cells, CSC markers and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay were carried out after treatment of control, indomethacin, 5- fluorouracil (5-FU),

and combination of 5-FU and indomethacin. We also carried out nude mice xenograft experiments using 5-FU resistant SW620 cells and the same drug combination with *in vitro* cell line experiments. To investigate the underlying mechanisms, we measured changes of CSC population after treatment of COX-2 selective inhibitor (celecoxib), other NSAIDs (sulindac and aspirin) and combination of indomethacin and prostaglandin E2 (PGE2), and performed reporter assay using PPAR-responsive element (PPRE)-Luc. In xenograft experiments, the expressions of HES1 (Notch signaling marker), PPARγ and COX-2 as well as CD133 and CD44 were evaluated by immunohistochemical (IHC) stain.

As a result, NSAIDs, including indomethacin, sulindac and aspirin, celecoxib, γ-secretase inhibitor (DAPT), and PPARγ agonist (rosiglitazone) significantly decreased CD133+CD44+ cells and induced over 50% decrease in the number of colospheres compared to control of SW620 cells. Compared to the control (100%), the treatment of low dose indomethacin (12.5 μM) for 4 days significantly decreased CD133+CD44+ cells (72.1%, $P = 0.014$), treatment of low dose 5-FU (2.0 μM) for 4 days led to the significant increases of CD133+CD44+ cells (228.2%, $P = 0.014$), and this 5-FU induced increase of CD133+CD44+ cells was inhibited by combination with indomethacin for the same period (133.1%, $P = 0.021$). In MTT assay, there was no significant difference in cell survival between groups, and these CSC-inhibitory effects of indomethacin was reversed by PGE2 in a dose-dependent manner. Indomethacin treatment, as well as rosiglitazone, significantly increased PPRE transcriptional activity. In xenograft experiments, 5-FU treatment combined with indomethacin significantly reduced tumor growth compared to 5-FU alone treated group. In addition, the treatment of indomethacin alone and combination of 5-FU and indomethacin decreased the expression of CD133, CD44, COX-2 and HES-1, and increased PPARγ expression, compared to control and 5-FU alone treated mice, respectively.

In conclusion, NSAIDs could selectively reduce the colon CSCs and suppress 5-FU induced increase of CSCs through COX-2-dependent and -independent pathways, such as PPARγ and Notch pathway. These suggest that NSAIDs could play an important role of adjunctive treatment with conventional chemotherapy in CRC.

Key Words: cancer stem cell; colorectal cancer; nonsteroidal anti-inflammatory drugs

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I. INTRODUCTION

Colorectal cancer (CRC) remains the fourth leading cause of cancer-related deaths in the world despite the emergence of novel anti-cancer therapies.[1] About 25% of patients were initially diagnosed with metastatic disease and they need to receive systemic chemotherapy.[2] However, the current 5-year survival for patients with metastatic CRC is still less than 10% and most of patients lose their lives.[3] In addition, a significant number of patients that present localized disease at initial diagnosis eventually recur to metastatic disease derived from the residual microscopic malignancy.[4] In spite of the improvement of

chemotherapeutic agents and emergence of promising biologic agents, it is discouraging that none of these modalities is fundamentally curative for advanced CRC. Conventional therapies, including chemotherapy and radiotherapy, target the rapidly dividing tumor cells and suppress the tumor growth. However, the limitation of these agents is to develop of therapy-resistant cancer cells and fail to eradicate disease.[5, 6] Therefore, many researchers have become interested in the cause of these resistant cancer cells. Recent evidence has suggested that a small subset of cells, which are isolated on the basis of phenotypic and molecular characteristics, are referred as cancer stem cells (CSCs)[7, 8] and they have a pivotal role in tumor initiation, growth and recurrence.[8-11] Conventional cytotoxic agents are not curative because CSCs are relatively quiescent and slowly proliferative. "Cancer stem cell hypothesis" assumes that they have the capacity to self-renew and differentiate into the different cell types.[7]

In CRC, the existence of CSCs has been demonstrated in several experimental studies. Subcutaneous injection of colon cancer CD133+ cells created tumors in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice that resembled the primary tumor, whereas CD133− cells failed to induce tumor formation.[10] Another investigators also reported that a small set of CRC cells (EpCAM high/CD44+ epithelial cells) have the tumor-initiating properties *in vivo*.[12] These studies showed that small numbers of cancer cells with expression of CSC markers can create and propagate CRC unlike the majority of other cancer cells. Moreover, in the study to evaluate the relationship between CSC and chemo-resistance in CRC, CSCs were enriched in the residual tumors following classical chemotherapy and still had the capability to generate tumors.[13] Likewise, in CRC cell lines, treatment with 5-FU or oxaliplatin increased the proportion of CD133+CD44+ cells *in vitro*.[14] Based on these results, CSCs have been becoming recognized as a specific target to obtain complete elimination of CRC.[15-17] Many researchers have been attempting to

identify the underlying signal pathways associated with CSCs because understanding of CSC behavior can lead to the effective targeted therapies. To date, IL-4 signaling transduction pathway,[18] the sonic hedgehog signaling,[19] and Notch pathway[20] has been suggested to play a role in CSCs of CRC. However, it has not been elucidated the definite relevant mechanisms and the targeted agents with anti-CSC effects.

Therefore, the purpose of this study is to identify the effective agents for suppressing CSCs to increase the anti-cancer effect of chemotherapeutic agents and the relevant mechanisms in human CRC.

II. MATERIALS AND METHODS

1. *In vitro* cell line study

A. Cells and cell culture

The human CRC cell lines (SW620, SW480, Caco-2, Colo205, HT-29, WiDr, HCT116, LoVo, DLD-1, RKO and HCT-15) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, U.S.A), and L-glutamine (Life Technologies, Carlsbad, CA, U.S.A). All cells were maintained in 5% CO_2 incubator at 37℃. In the xenograft mouse experiment of this study, we used 5-fluorouracil (5-FU) resistant cell line. This cell line was developed by escalating doses of 5-FU serially in SW620 cells with modification of prior studies.[14, 21] Briefly, cells were exposed to an initial dose of 10 μM 5-FU for 72 hr and cultured in a drug-free condition during the defined period. After cells recovered from the prior dose of 5-FU, the 5-FU concentration was escalated into the double dose

serially and it finally came to 1.0 mM.

B. Drugs and antibodies

Indomethacin, *N*- [*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine -*t*-butyl ester (DAPT), butyrate, sulindac, aspirin, celecoxib, 5-FU, and prostaglandin E2 (PGE2) were purchased from Sigma (St. Louis, MO, U.S.A). 7-amino-4-chloro-3-methoxyisocoumarin (JLK6), rosiglitazone, and trichostatin-A were purchased from Tocris Bioscience (Minneapolis, MN, U.S.A) and Cayman Chemical (Ann Arbor, MI, U.S.A). Antibodies used for flow cytometry, Western blotting, or immunohistochemical (IHC) analysis were as follows: mouse anti-CD133 (Cell Signaling Technology, Danvers, MA, U.S.A), mouse anti-CD44, anti-HES1, and anti-peroxisome proliferator-activated receptor γ (PPARγ) (Santacruz, Delaware, CA, U.S.A), anti-cyclooxygenase-2 (COX-2) (Invitrogen, Camarillo, CA, U.S.A), phycoerythrin (PE)-conjugated anti-CD133, PE-conjugated mouse-IgG1 (Miltenyi Biotec, Bergisch Gladbach, Germany), fluorescein (FITC)-conjugated anti-CD44 antibody (BD Biosciences, Franklin Lakes, NJ, U.S.A)

C. Flow cytometric analysis

Flow cytometeric analysis of CSC markers (CD133 and CD44) were performed to investigate the CSC suppressing effect of each agents (indomethacin, DAPT, JLK6, butyrate, rosiglitazone, and trichostatin-A). We also measured CD133+CD44+ cells after 4 day-treatment of control, indomethacin (12.5 μM), 5-FU (2 μM), and combination of 5-FU and indomethacin. To evaluate the effect of indomethacin reversed by PGE2, the change of CD133+CD44+ cells were measured after treatment of control, indomethacin (50 μM) alone, combination of indomethacin and PGE2 (0, 5, 10 μM) with/without 5-FU (2 μM). The prepared cells were detached by accutase (Millipore, Billerica, MA,

U.S.A), and washed with phosphate buffered saline (PBS), and then resuspended in FACS buffer (1x PBS, 1% bovine serum albumin (BSA), 2 mM ethylene diamine tetraacetic acid (EDTA)). Primary antibodies were added and incubated for 10 min on ice. Samples were then washed and analyzed by using BD LSRII (BD Biosciences, Franklin Lakes, NJ, U.S.A) coupled to a computer with data analysis software (BD FACS Diva software).

C. Colosphere culture assay

The sphere-forming ability as a cancer stem cell activity was evaluated by colosphere culture assay as described in prior studies.[22, 23] Firstly, to select the adequate colon cancer cell line, we performed the colosphere culture in various colon cancer cell lines (SW620, SW480, Caco-2, Colo-205, HT-29, WiDr, HCT116, LoVo, DLD-1, RKO, HCT-15). For evaluation of CSC-inhibitory effect of candidate agents, the selected cells (SW620) were plated with 2,000 cells/well in 24-well ultra-low adhesive plates (Corning Incoporated, NY, U.S.A) in serum-free medium (SFM) in the presence of each agents. Candidate agents included nonsteroidal anti-inflammatory drugs (NSAIDs) (indomethacin, sulindac, aspirin, and celecoxib), γ-secretase inhibitor (DAPT, JLK6), and PPARγ agonist (rosiglitazone). This SFM was DMEM-F12 supplemented with B27 (Life Technologies, Carlsbad, CA, U.S.A), 20 ng/mL epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, U.S.A), 10 ng/mL fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN), penicillin-streptomycin and L-glutamine (Life Technologies, Carlsbad, CA, U.S.A). They were cultured in 5% CO_2 incubator at 37℃ with a medium change every 3 days. To measure the inhibitory effect for colosphere formation, the number of colospheres was counted under microscope (Olympus Bx51 microscope) at day 14.

D. Drug cytotoxicity assay

The proliferation of SW620 cells treated with selected agents was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, SW620 cells were seeded at $1x10^4$ cells/well in 96-well plates with 200 μl medium. The defined doses of indomethacin or 5-FU, or vehicles were added to the wells and cultured in a 5% CO_2 incubator at 37℃. At each time point (0, 24, 48, and 72 hr), 40 μl MTT (Sigma-aldrich, St. Louis, MO, U.S.A) solution was added and cells were incubated at 37℃ for 1 hr. The media of each wells were removed, and 100 μl dimethyl sulfoxide (DMSO) was then added. The absorbance of each well at 570 nm was measured using VERSA Max (Molecular devices, Union City, CA, U.S.A). We set the control wells with medium only as zero absorbance.

E. Luciferase assay

SW620 cells were transfected with reporter plasmid containing the firefly luciferase gene driven by a PPAR-responsive element 3 (PPRE3-Luc).[24] The constructs of PPRE3-Luc have been described elsewhere.[24] PPRE3-Luc was generously donated by JW Kim (Yonsei University College of Medicine, Korea). *Renilla* vector pRL-TK was used as a transfection control. After transfection, the cells were incubated in a culture medium containing control, indomethacin 6.25 μM, 12.5 μM, 25.0 μM, or rosiglitazone 10 μM. The cells were lysated with lysis buffer (Promega, Madison, WI, U.S.A) 48 hr post transfection and incubated at room temperature for 15 min. The relative luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A) according to the manufacturer's protocol.

2. *In vivo* xenograft mouse experiments

A. Xenograft tumor models

Male Balb/c athymic nude mice, 6 wk old, were purchased from the Animal Laboratory Unit of Yonsei University College of Medicine and acclimated for 1 wk. All mouse experiments were approved by the Committee of Care and Use of Laboratory Animals of Yonsei University College of Medicine and performed in accordance with the institutional guidelines and policies.

Equal numbers (5×10^5) of 5-FU resistant SW620 cells were suspended in 150 μl matrigel diluted 1:1 in DMEM and injected subcutaneously into the left rear flank of each mouse. When tumors reached the palpable size, a total of 20 mice were allocated randomly to four treatment groups (control, 5-FU only, indomethacin only, 5-FU and indomethacin combination treatment). 5-FU (30 mg/kg body weight) was administered intraperitoneally three times a week and control animals received the vehicles with the same volume. Indomethacin crystals (Sigma-aldrich, St. Louis, MO, U.S.A) were dissolved in a 0.5% carboxy methylcellulose (CMC, Sigma-aldrich, St. Louis, MO, U.S.A) solution and this concoction was adjusted to 1.0 mg/kg body weight/0.2 ml of 0.5% CMC. Indomethcin solution at a dose of 1.0 mg/kg body weight was given by oral gavage every day for 16 days, and the same volume of 0.5% CMC solution without indomethacin was given to control mice in the same day. Tumor masses were measured every other day using calipers, and the tumor volume was calculated based on the following formula: volume= (length x width^2)/2. All mice were euthanized at 16 day after first drug treatment and the tumor masses were dissected. The excised tumors were calipered and placed in 10% buffered formalin for IHC or frozen in optical cutting temperature medium.

B. Immunohistochemistry (IHC)

All IHC studies were performed on formalin-fixed, paraffin-embedded tissue

sections using anti-CD133, anti-CD44, anti-COX2, anti-HES1, and anti-PPARγ antibody. Briefly, 5 μm-thick sections were deparaffinized in xylene, and hydrated in alcohol with the gradually decreased concentration. The antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0), using the pressure cooker in microwave for 10 min. After incubation with 3% hydrogen peroxide to block endogenous peroxidase activity, a blocking reagent was added to the sections for 10 min. Slides were then consecutively incubated with 1:100 dilution of primary antibody (overnight at 4℃) and secondary antibody (30 min at room temperature). Slides were developed with Vectastain ABC kit (Vector Laboratories, Inc, Burlingame, CA, U.S.A) and counterstained with hematoxylin. The staining was independently interpreted by 2 researchers (CM Moon and JH Kwon). In cases of the discrepant results, they were re-evaluated by the two researchers together, a third researcher was consulted. All IHC staining was evaluated by light microscopy and the immunoactivity was scored according to the proportion of immunostaining tumor cells. We counted the CD-133, CD-44, COX-2, HES1, and PPARγ−staining tumor cells among the total 100 tumor cells in the five different fields under 400x microscope and totalized the each cell counts and converted into a percentage.

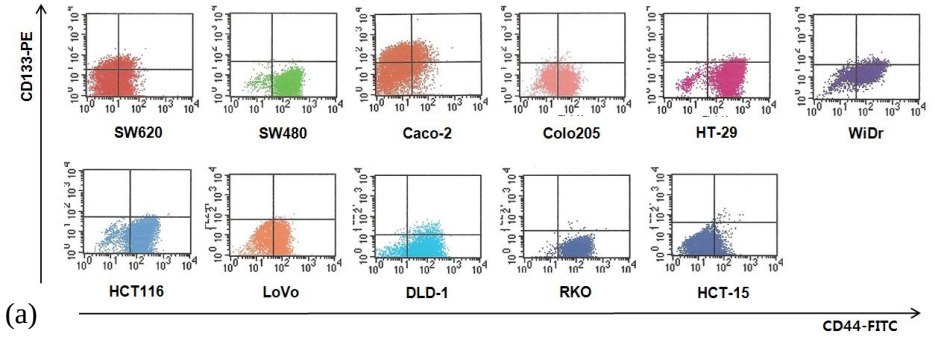
3. Statistics

All the analyses were processed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, U.S.A). In this study, Mann-Whitney's U-test was used to determine the statistical significance for the results of flow cytometric analysis, colosphere culture assay, MTT assay, luciferase assay, and interpretation of IHC. *P*-value was less than 0.05 was considered significant.

III. RESULTS

1. CSCs in various colon cancer cell lines

To select the adequate colon cancer cell lines that are prone to form CSCs, we investigated the expression of colon CSC markers and the ability to form colosphere in various colon cancer cell lines (SW620, SW480, Caco-2, Colo205, HT-29, WiDr, HCT116, LoVo, DLD-1, RKO and HCT-15). Flow cytometric analysis showed that the expression of CD133+, CD44+, and CD133+CD44+ was significantly higher in SW620 and Caco-2 than other colon cancer cell lines (Figure 1a. and Table 1). To measure the colosphere forming capacity, these colon cancer cells were incubated in the serum-free and anchorage-independent conditions. It has been known that CSC-enriched subpopulation can form tumor spheres in colon cancer cells.[22] As results, SW620 and Caco-2 cells formed more number of colspheres than other colon cancer cells (Figure 1b.). Based on these results, SW620 and Caco-2 were selected for further studies.



(a)

(b)

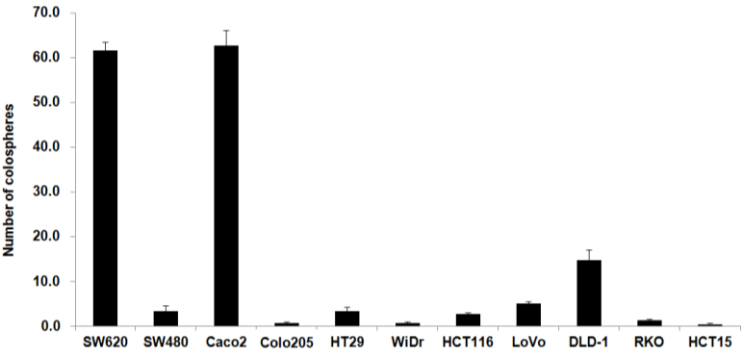


Figure 1. CSC expression in various colon cancer cell lines (SW620, SW480, Caco-2, Colo205, HT-29, WiDr, HCT116, LoVo, DLD-1, RKO and HCT-15). SW620 and Caco-2 cells showed higher expression of CSC markers (CD133+, CD44+, and CD133+CD44+) in flow cytometric analysis (a) and formed more number of colospheres compared to other colon cancer cells (b). PE, phycoerythrin; FITC, fluorescein; CSC, cancer stem cell.

Table 1. The expression of colon cancer stem cell markers in various colon cancer cell lines (flow cytometric analysis)

Cell line	*Negative (%)*	*CD133+ (%)*	*CD44+ (%)*	*CD133+ CD44- (%)*	*CD133- CD44+ (%)*	*CD133+ CD44+ (%)*
SW620	38.02	50.88	22.67	37.56	9.35	13.32
SW480	2.38	0.05	97.60	0.01	97.56	0.04
Caco-2	42.52	49.48	28.52	28.96	8.00	20.52

Colo205	29.53	0.65	70.19	0.28	69.82	0.37
HT-29	3.39	1.78	96.60	0.01	94.83	1.77
WiDr	11.64	2.19	87.63	0.08	85.53	2.11
HCT116	8.52	0.21	91.49	0.00	91.28	0.21
LoVo	48.22	4.07	50.74	1.18	47.85	2.89
DLD-1	8.95	1.94	90.97	0.08	89.11	1.87
RKO	1.85	0.25	98.15	0.01	97.90	0.25
HCT-15	73.53	0.21	26.45	0.03	26.27	0.18

2. Screening of anti-tumor agents for suppression of colon CSCs.

CSC-inhibitory effects of anti-tumor agents (indomethacin, DAPT, JLK6, butyrate, rosiglitazone, and trichostatin-A) were evaluated by flow cytometric analyses of CD133 and CD44 and colosphere forming activity. We measured the expression of CSC markers in Caco-2 cells after treatment of each agents for 96 hr (Table 2). Caco-2 cells which are treated with indomethacin (12.5 μM, 25.0 μM), DAPT (1, 5 μM), JLK6 (10, 20 μM), Butyrate (2, 4 mM), and rosiglitazone (2, 4 μM) showed the lower proportions of CD133 and CD44 compared to the controls. However, trichostatin A at 100 nM and 200 nM did not show any change of the proportion of CD133 and CD44.

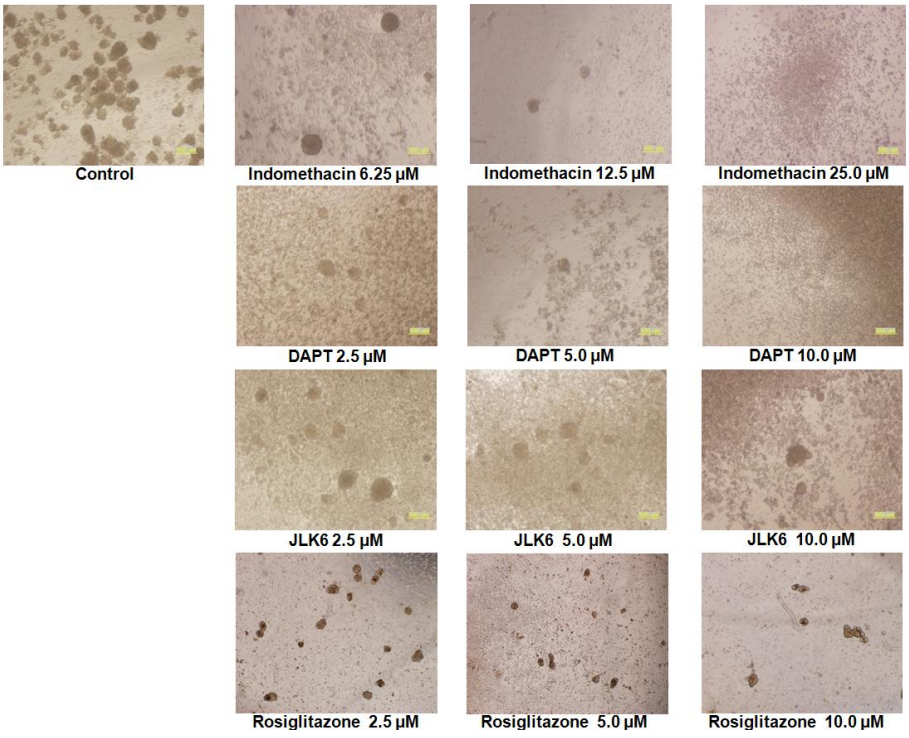
Table 2. Cancer stem cell suppressing effects of candidate agents in Caco-2 (flow cytometric analysis)

Drug	*CD133+*[1]	*CD44+*[1]	*CD133+/CD44+*[1]
Control	100	100	100
Indomethacin 12.5 μM	104.2	40.2	39.7
Indomethacin 25.0 μM	137.8	31.9	42.9
DAPT 1 μM	75.1	37.2	30.4
DAPT 5 μM	73.4	43.8	34.6
JLK6 10 μM	84.1	20.4	18.9
JLK6 20 μM	0.5	0.3	0.2
TrichostatinA 100 nM	98.6	116.8	106.7
TrichostatinA 200 nM	84.2	144.4	112.5
Butyrate 2 mM	165.9	77.1	92.3
Butyrate 4 mM	61.7	28.2	27.0
Rosiglitazone 2 μM	119.6	63.2	68.3
Rosiglitazone 4 μM	84.5	53.0	48.0

[1] : (CSC marker in Caco-2 cells treated with candidate drugs) / (CSC marker in Caco-2 cells treated with controls)

Moreover, we also investigated the efficacy of the screened agents for inhibiting colosphere forming capacity. We cultured SW620 cells in SFM with B27, EGF,

and bFGF with each compounds or vehicle and the number of spheres were counted at day 14. Compared to controls, all screened agents suppressed the colosphere formation at the low concentration. Indomethacin (6.25 μM and higher concentration), γ-secretase inhibitor (2.5 μM and higher concentration of DAPT and JLK6), and PPARγ agonist (2.5 μM and higher concentration of rosiglitazone) significantly decreased the number of colospheres compared to controls. These agents at 12.5 μM and higher concentration of indomethacin, 2.5 μM and higher that of DAPT, 10.0 μM of JLK6, and 5.0 μM and higher that of rosiglitazone, respectively induced over 50% decrease in the number of colospheres (Figure 2.). These results showed that indomethacin, γ-secretase inhibitor, and PPARγ agonist induced suppression of colospheres in dose-dependent manners. Also, we could determine the minimal concentration of each screened agents for their anti-CSC effects.



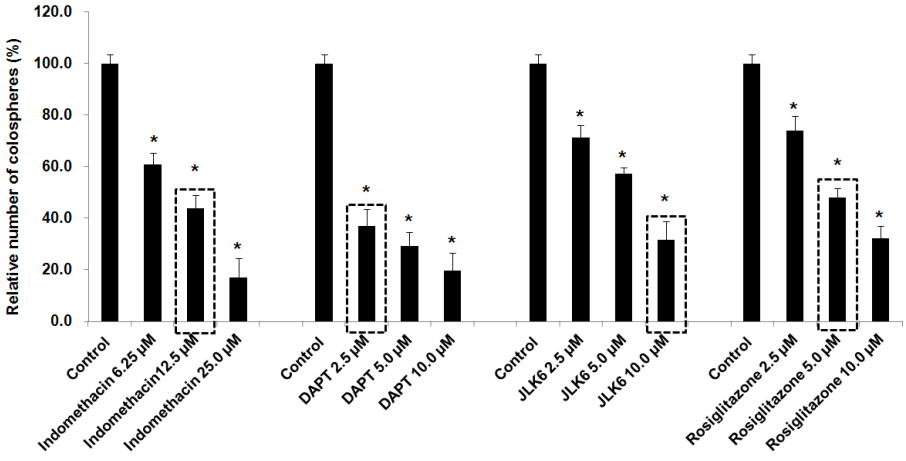


Figure 2. Colosphere formation of SW620 cells treated with the screened agents. 2,000 cells/well of SW620 cells were cultured in 24-well ultra-low adhesive plates in serum-free medium with each agents. Low concentration of indomethacin (6.25 μM and higher concentration), γ-secretase inhibitor (2.5 μM and higher concentration of DAPT and JLK6), and PPARγ agonist (2.5 μM and higher concentration of rosiglitazone) significantly decreased the number of colospheres compared to controls. The dotted rectangle means the minimal dose of each agents that induced over 50% decrease in the number of colospheres. Data are expressed as mean±standard error, $^{*}P < 0.05$. PPARγ, peroxisome proliferator-activated receptor γ.

3. Inhibitory effect of indomethacin on chemotherapy induced increase of CSC.

A. Indomethacin selectively inhibited CSCs and 5-FU induced increase of CSC.

After the selection of the agents, flow cytometric analysis of CD133 and CD44 in SW620 cells were evaluated after treatment with indomethacin with/without 5-FU for 4 days to evaluate the inhibitory effects of indomethacin on 5-FU

induced CSC population. Figure 3. showed the relative proportion of CSC population at each conditions compared to the control. The treatment of 2.0 μM of 5-FU for 4 days led to the significant increases of CD133+CD44+ cells (228.2%, *P* = 0.014). In contrast, the treatment of 12.5 μM indomethacin significantly decreased the CSCs population (72.1%, *P* = 0.014). Treatment of indomethacin combined with 5-FU (133.1%) for the same period significantly reduced CD133+CD44+ cells compared to 5-FU alone (*P* = 0.021). These results could suggest that indomethacin can selectively suppress CSCs and also decreased 5-FU-inducued increase of CSC population in CRC.

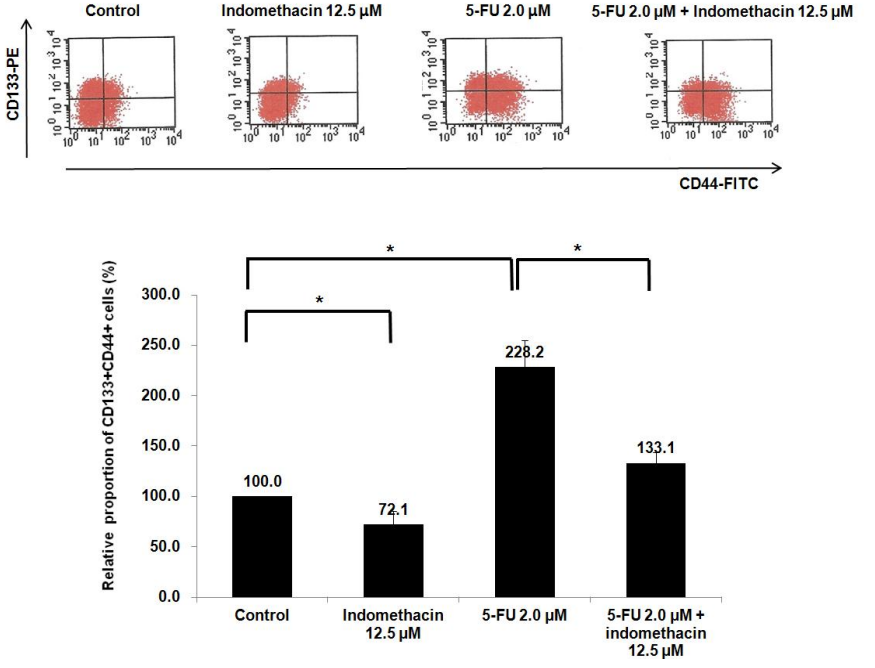


Figure 3. CSC suppressing effect of indomethacin (flow cytometric analysis). Compared to the control (100%), the treatment of low dose indomethacin (12.5 μM) for 4 days significantly decreased CD133+CD44+ cells (72.1%, P = 0.014), treatment of low dose 5-FU (2.0 μM) for 4 days led to the significant increases of CD133+CD44+ cells (228.2%, P = 0.014), and this 5-FU induced increase of

CD133+CD44+ cells was inhibited by combination with indomethacin for the same period (133.1%, P = 0.021). Data are expressed as mean±standard error. $^{*}P < 0.05$. PE, phycoerythrin; FITC, fluorescein; CSC, cancer stem cell; 5-FU, 5-fluorouracil.

B. Indomethacin does not affect the cell survival at low concentrations.

We then evaluated whether the CSC-inhibitory effect of indomethacin was related to the cellular death or not. We performed a MTT assay on SW620 cells treated with the same dose of indomethacin combined with/without 5-FU. As shown in Figure 4., there was no significant difference in viability of the cells among 5-FU, indomethacin and combination of these two compounds. These results could suggest that the low concentration of indomethacin in this study does not affect the cell viability and can suppress the CSC formation independent to the cell viability.

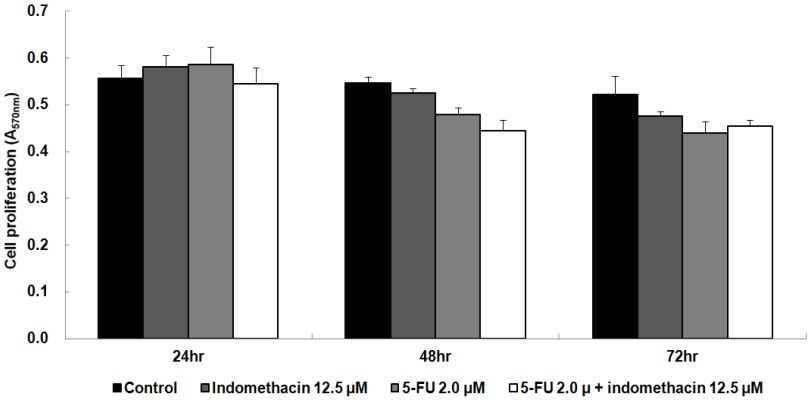
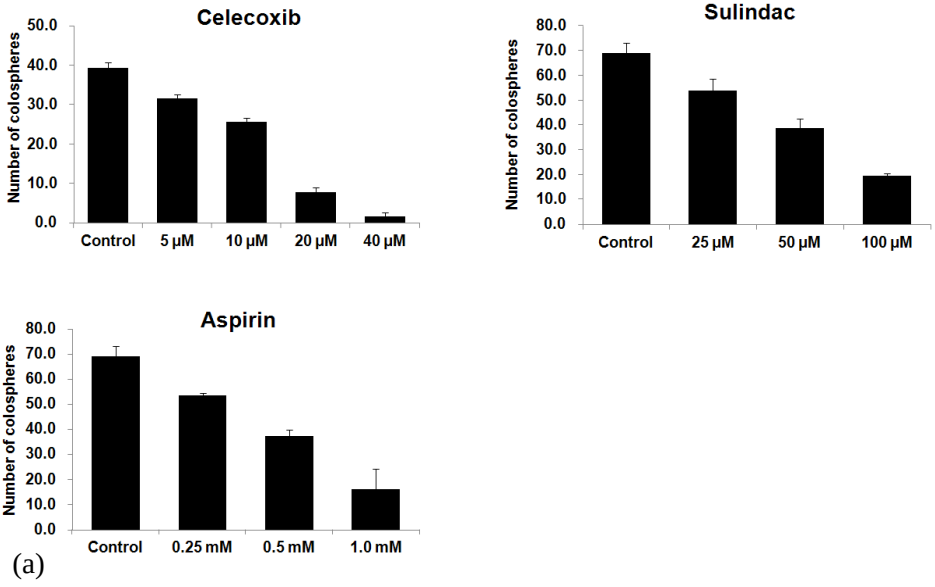


Figure 4. Cell viability with the treatment of indomethacin, 5-FU, and combination of indomethacin and 5-FU (MTT assay). There was no significant differences in cell survival between each treated groups (control, indomethacin 12.5 μM, 5-FU 2.0 μM, 5-FU 2.0 μM + indomethacin 12.5 μM). Data are

expressed as mean±standard error. A_{570nm}: absorbance at 570 nm; 5-FU, 5-fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide.

4. CSC-inhibitory effect of NSAIDs through COX-2-dependent pathway

We investigated whether COX-2-dependent pathway is the relevant mechanism in suppression of CSCs. In colosphere forming assay, a low concentration of COX-2 selective inhibitor (celecoxib), as well as other NSAIDs (aspirin, sulindac), significantly decreased the number of colospheres (Figure 5a.). In addition, CSC-inhibitory effect of indomethacin was reversed by PGE2. The proportion of CD133+CD44+ cells was significantly decreased after 4 day treatment of indomethacin (50 μM). However, the treatment of PGE2 (5, 10 μM) for the same period increased CD133+CD44+ cells in a dose-dependent manner (Figure 5b.).



(a)

(b)

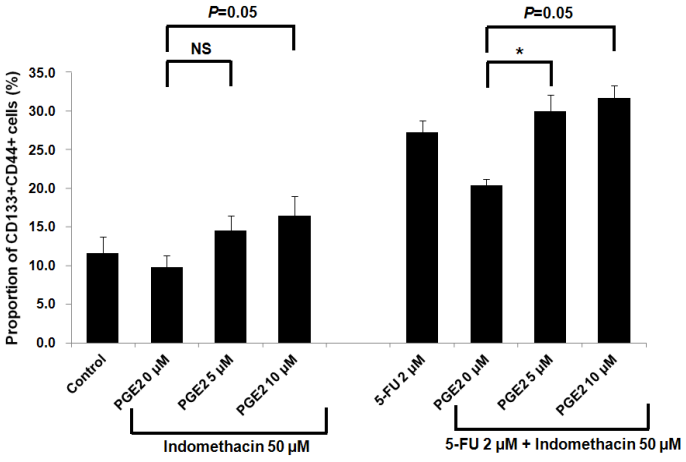


Figure 5. CSC-inhibitory effect of NSAIDs via COX-2-dependent pathway. COX-2 selective inhibitor (celecoxib), as well as other NSAIDs, significantly suppressed the colosphere formation (a). In flow cytometric analysis, CSC-inhibitory effect of indomethacin was reversed by PGE2 in a dose-dependent manner (b). Data are expressed as mean±standard error, $^{*}P <$ 0.05, NS presented no statistical significance. CSC, cancer stem cell; NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; PGE2, prostaglandin E2.

5. Indomethacin up-regulated the PPARγ expression in CRC.

Since indomethacin was considered to activate the PPARγ,[25, 26] Luciferase assay was performed using PPRE-Luc in SW620 cells treated with indomethacin and PPARγ agonist. Treatment of 6.25, 12.5, and 25.0 μM of indomethacin as well as 10 μM of rosiglitazone significantly increased PPRE transcriptional activity in SW620 cells in a dose-dependent manner (Figure 6.). These results suggest that indomethacin can increase the expression of PPARγ in colon cancer cells.

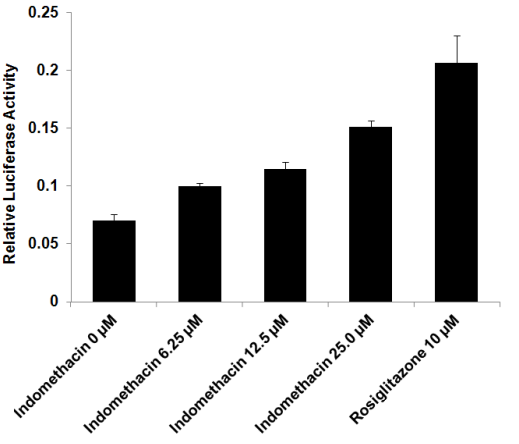


Figure 6. Indomethacin activates the PPARγ expression in CRC. SW620 cells were transiently transfected with PPRE-Luc reporter and renilla (control) for 48 hr. After transfection, cells were treated with vehicle, indomethacin (6.25, 12.5, 25.0 μM), and rosiglitazone (10 μM). Relative activity of firefly luciferase was normalized using renilla luciferase activity. Indomethacin, as well as rosiglitazone, increased PPRE transcriptional activity in a dose-dependent manner. Data are expressed as mean±standard error. PPARγ, peroxisome proliferator-activated receptor γ; CRC, colorectal cancer; PPRE, PPAR-responsive element.

6. Inhibitory effect of indomethacin on CSC of xenograft tumor

A. Combination of indomethacin and 5-FU inhibited tumor growth compared to 5-FU alone.

We performed *in vivo* tumor growth in the xenograft mice model by treatment with vehicle, 5-FU (30 mg/kg, 3 times/week), indomethacin (1.0 mg/kg, daily), and the same dose and frequencies of 5-FU and indomethacin combination. 5-FU resistant SW620 cells were used instead of parent SW620 cells. The

effects of indomethacin and 5-FU on CSCs were expected to be more distinct because 5-FU resistant cells had much higher proportion of CSCs than parent SW620 cells (CD133+CD44+ cells : 47.1% vs. 15.4%) (Figure 7.). Xenograft tumor nodules were formed in all nude mice and were harvested at 16 day except one. One mouse was expired at 10 day and excluded at the final analysis. As shown in Figure 8., the size of tumors treated with the combination of 5-FU and indomethacin was significantly smaller than those treated with 5-FU alone at the day 16 (p = 0.009). The combination of 5-FU and indomethacin decreased 41.3% in the tumor size compared 5-FU alone treatment. However, the difference of tumor size between mice treated with controls and indomethacin alone did not reach statistical significance.

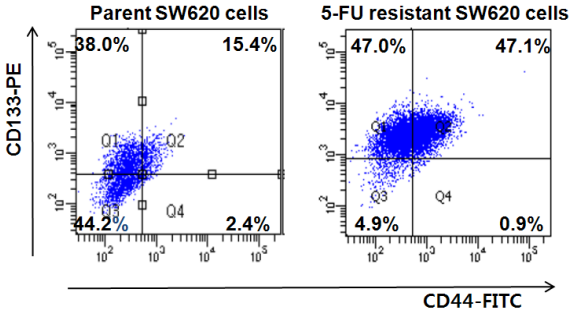


Figure 7. Comparison of the expression of CD133 and CD44 between 5-FU resistant SW620 cells and parent SW620 cells. 5-FU resistant cells were more enriched for CD133+CD44+ cells than parent SW620 cells (47.1% vs. 15.4%). PE, phycoerythrin; FITC, fluorescein; 5-FU, 5-fluorouracil.

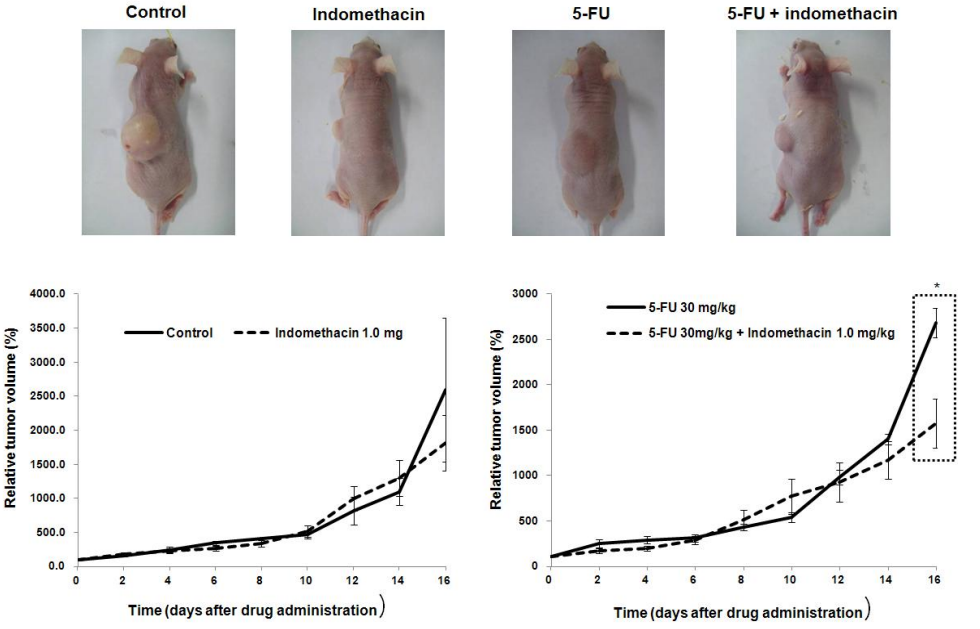
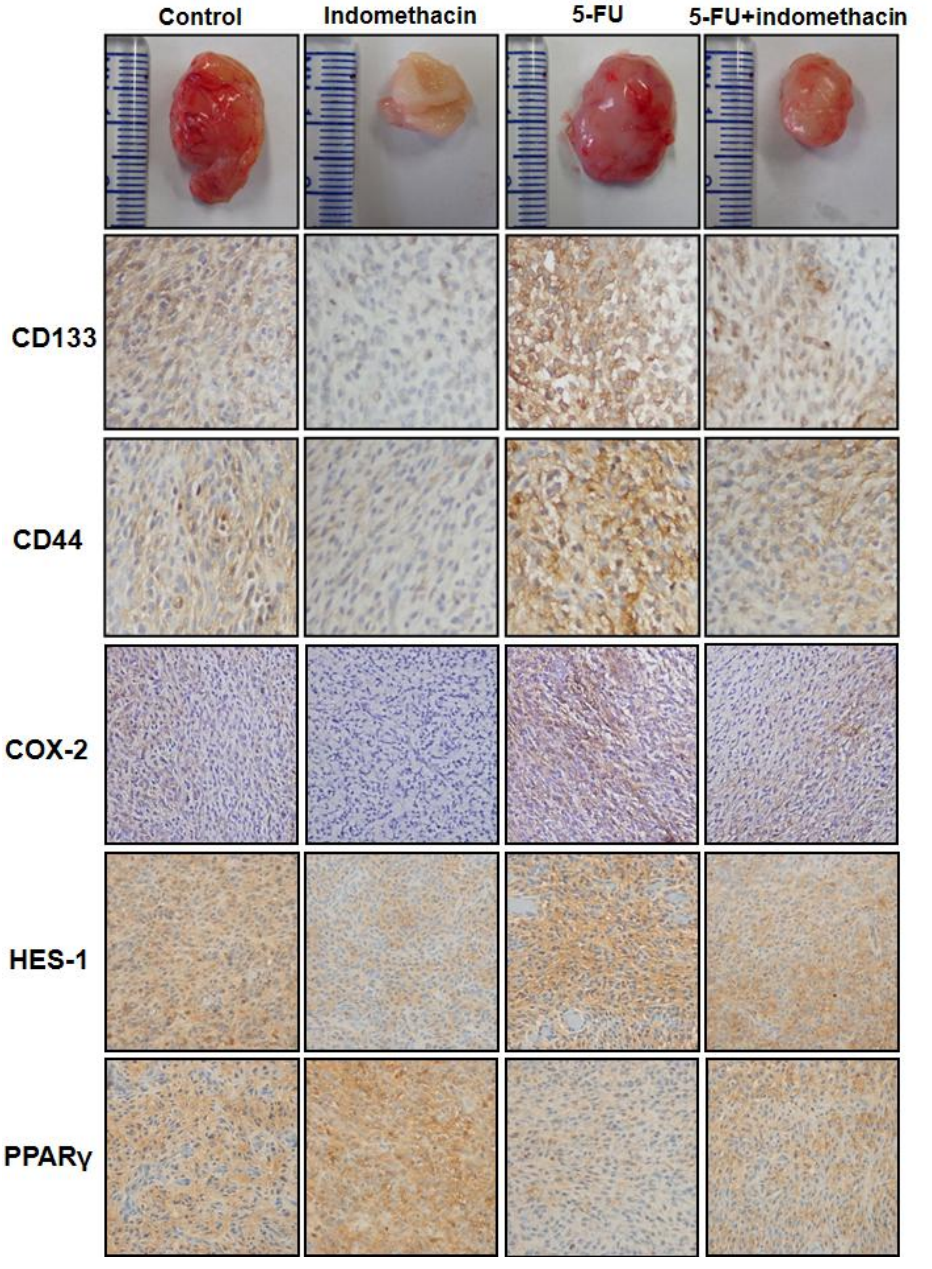


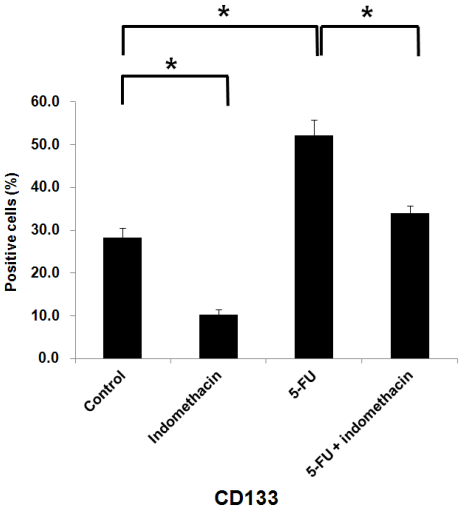
Figure 8. The effects of indomethacin on *in vivo* tumor growth in xenograft mice model. We evaluated the xenograft tumor growth in nude mice by treatment with vehicle, 5-FU (30 mg/kg, 3 times/week), indomethacin (1.0 mg/kg, daily), and the same dose and frequencies of 5-FU and indomethacin combination and sacrificed mice at 16 day. 5-FU treatment combined with indomethacin significantly reduced tumor growth compared to 5-FU alone treated group. Data are expressed as mean±standard error, $^{*}P < 0.05$. 5-FU, 5-fluorouracil.

B. Indomethacin decreased the expression of CSC markers, COX-2, and HES-1 and increased PPARγ expression in xenograft tumors.

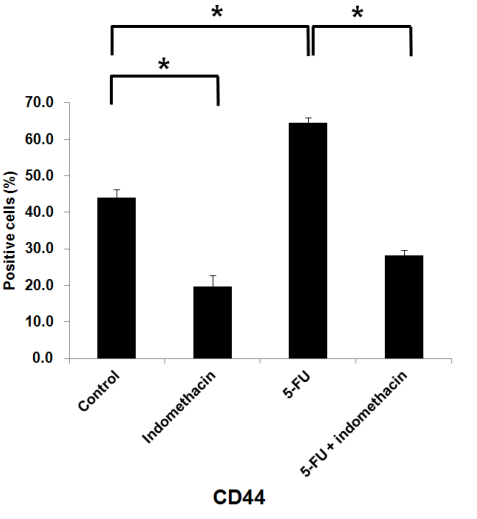
To investigate CSC-suppressing effect of indomethacin *in vivo*, the expression of surviving CSCs were compared in the xenograft tumors treated with vehicle, 5FU, indomethacin, and 5-FU combined with indomethacin. IHC staining on xenograft tumors was performed for CSC markers (CD133 and CD44), COX-2,

Notch signaling pathway (HES-1), and PPARγ (Figure 9.). When the stained cells were analyzed quantitatively, indomethacin significantly decreased CD133+ and CD44+ cells ($P < 0.05$), whereas 5-FU increased CD133+ and CD44+ cells compared to controls ($P < 0.05$). Furthermore, the combination treatment of 5-FU and indomethacin significantly decreased CD133 and CD44 compared to 5-FU alone ($P = 0.001$, and $P = 0.002$, respectively). Indomethacin treatment also showed significant down-regulation of COX-2 ($P = 0.010$) and HES-1 ($P = 0.001$) and up-regulation of PPARγ expression ($P = 0.009$) in xenograft tumors compared to the control group. In addition, the treatment of 5-FU combined with indomethacin reduced COX-2 ($P = 0.037$) and HES-1 ($P = 0.002$) and induced PPARγ expression ($P = 0.001$) compared to 5-FU alone treated mice.

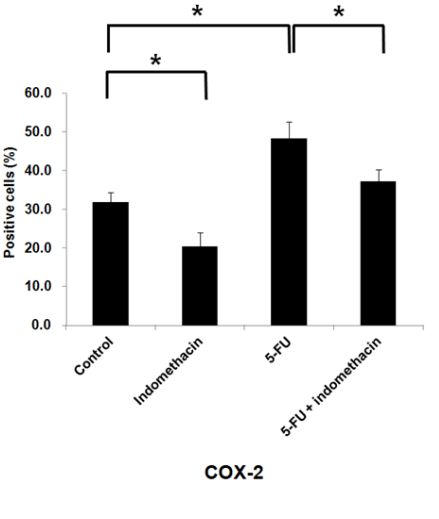

Control
Indomethacin
5-FU
5-FU+indomethacin
CD133
CD44
COX-2
HES-1
PPARγ



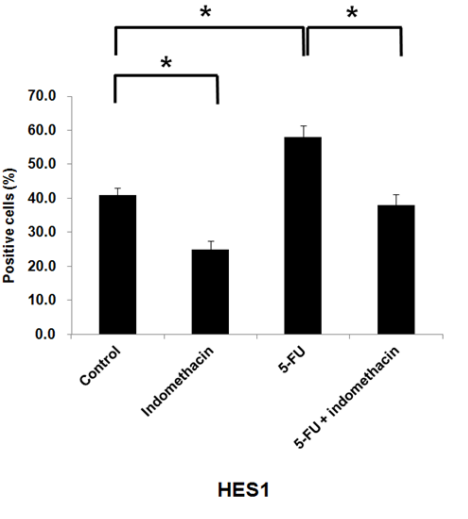
CD133



CD44



COX-2



HES1

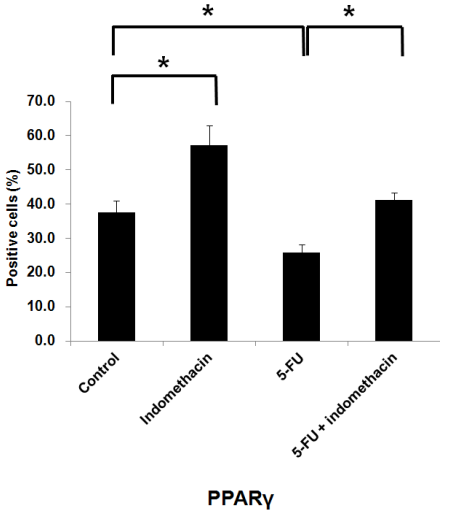


Figure 9. IHC for CD133, CD44, COX-2, HES1, and PPARγ of xenograft tumors from mice. 5-FU resistant SW620 cells were injected subcutaneously into the left flank of nude mice. Mice were allocated randomly to four groups (vehicle, indomethacin alone, 5-FU alone, and the combination of 5-FU and indomethacin). The treatment of indomethacin alone and combination of 5-FU and indomethacin decreased the expression of CD133, CD44, COX-2 and HES-1, and increased PPARγ expression, compared to control and 5-FU alone treated group, respectively. Data are expressed as mean±standard error, $^{*}P <$ 0.05. IHC, immunohistochemistry; COX-2, cyclooxygenase-2; PPARγ, peroxisome proliferator-activated receptor γ; 5-FU, 5-fluorouracil.

IV. DISCUSSION

In this study, we have identified the effective agents for suppressing CSCs to increase the anti-cancer therapeutic effect of current chemotherapy in human CRC. To explore the CSC-inhibitory effect of the agents, we performed colosphere forming assay and flow cytometric analysis of CSC markers. As a result, a low concentration of indomethacin showed CSC-suppressing effect in colon cancer cell line. Indomethacin significantly reduced colosphere formation and the expression of CD133 and CD44 compared to controls. Likewise, anti-CSC effect of indomethacin was also ascertained in xenograft mouse model.

NSAIDs have been identified as the chemopreventive agent in CRC and many experimental and clinical studies have consistently reported that NSAIDs may reduce the risk of colorectal adenoma or cancer.[27-32] As for the premalignant lesion, colorectal adenoma, four randomized controlled trials demonstrated that regular aspirin use had a significant reduction of adenoma risk in both average and high-risk populations.[27, 33-35] Also, the relative risk of CRC was 40-50% reduced in the individuals with chronic intake of aspirin or other NSAIDs for over 10-15 years.[36-38] The most convincing evidence is that NSAIDs substantially reduce the number and size of polyps in familial adenomatous polyposis (FAP) patients with existing adenomas.[39-41] In three different animal models (*Apc*^{Min} mouse,[42-44] azoxymethane (AOM)-treated rat,[45-47] and xenograft nude mouse[48, 49]), either non-selective or COX-2 selective NSAIDs have been shown to suppress the CRC growth. Because CSC was identified to be involved in tumor initiation and growth,[8-11] our results suggested that preventive and therapeutic effect of NSAIDs might be related to its CSC-suppressing ability in CRC. Moreover, our study also found that treatment of indomethacin reduced 5-FU-induced increase of CSCs. In the Cancer and Leukemia Group B (CALGB) 89803 trial, which was a postoperative adjuvant chemotherapy trial

with stage III colon cancer, aspirin intake was significantly associated with a lower risk of cancer recurrence or death.[50] Based on these results, we would hypothesize that indomethacin could prevent CRC recurrence through inhibitory effect of CSCs. However, further studies are needed to establish this issue more definitely.

Until now, the CSC-inhibitory effect of NSAIDs and the relevant mechanisms in CRC has not been entirely elucidated. First of all, we have investigated whether anti-CSC effect of NSAIDs were related to COX-2-dependent pathway. The anticarcinogenic activity of NSAIDs in CRC may mostly depend on the inhibition of COX-2 activity. COX-2 would be a very plausible target for anti-cancer effect of NSAIDs because prostaglandins (PGs) play an important role in tumorigenesis in CRC.[51, 52] Oshima et al.[53] reported that a COX-2 gene (Ptgs2) null mutation significantly reduced the number and size of polyps on $Apc^{\Delta 716}$ mice and COX-2 inhibitor decreased the polyp number more significantly than sulindac on $Apc^{\Delta 716}$/*Cox2* wild-type mice. In addition, COX-2 overexpression was documented in 85% of human CRC and about 50% of colorectal adenomas[52] and this phenomenon was also identified in animal models.[54, 55] In this study, COX-2 selective inhibitor (celecoxib) significantly suppressed the colosphere formation as well as other non-selective NSAIDs and anti-CSC effect of indomethacin was reversed by PGE2. These results suggested that COX-2 pathway may be one of the relevant mechanisms for suppressing colon CSC by NSAIDs.

We have also investigated COX-2-independent pathways (Notch pathway and PPARγ) of NSAIDs as a mechanism of the anti-CSC activity. Previous studies have reported that traditional NSAIDs presented anti-cancer effects via COX-2-independent mechanisms.[56, 57] Moreover, recent one study has just revealed that NSAIDs eliminates oncogenic intestinal stem cells through induction of second mitochondria-derived activator of caspase (SMAC)-mediated apoptosis.[58] In the results of this study, Notch signaling and

PPARγ might be the relevant mechanisms how NSAIDs can inhibit CSCs in CRC. Luciferase study showed that indomethacin significantly increased PPRE transcriptional activity like PPARγ agonist. In addition, the treatment of indomethacin alone and combination treatment of 5-FU and indomethacin significantly decreased the expression of HES-1 and increased PPARγ expression, compared to control and 5-FU alone treated mice, respectively. In many previous reports, NSAIDs have shown to inhibit Notch signaling pathway[59-61] and activate the PPARγ expression.[25, 26] Notch signaling has been shown to be oncogenic in CRC through inhibiting the terminal differentiation of secretory cells.[62-64] Recently, it was reported that dysregulation of the Notch signaling was implicated in the self-renewal and maintenance of CSCs in CRC.[20] On the contrary, PPARγ activation resulted in growth arrest and induced differentiation of colon cancer cells.[65] CSC-inhibitory effect of PPARγ agonist was demonstrated in brain CSCs through Jak-Stat pathway.[66] On the basis of these evidence and our results, Notch pathway and PPARγ may be related to CSCs in CRC and they were down- and upregulated by NSAIDs, respectively. Thus, we suggested that NSAIDs could inhibit the colon CSCs via COX-2-independent pathways, such as Notch pathway and PPARγ, as well as COX-2-dependent pathway.

V. CONCLUSION

Some of the chemopreventive agents, such as NSAIDs, PPARγ agonist, and γ-secretase inhibitor, presented CSC-suppressing effects in CRC. NSAIDs can biologically inhibit Notch signaling pathway and activate the PPARγ. CSC-inhibitory effect of indomethacin was prevented by PGE2, and other NSAIDs (sulindac and aspirin) and COX-2 selective inhibitor also showed anti-CSC effect. NSAIDs could selectively reduce the chemotherapy-induced increase of CSCs independent with cell viability. In xenograft mouse model,

indomethacin significantly decreased tumor growth and the CSC population, and down-regulated the Notch signaling and COX-2 and up-regulated PPARγ expression.

In conclusion, our studies provide that NSAIDs could selectively reduce the CSCs and suppress chemotherapy induced increase of CSCs through COX-2-dependent and -independent pathways, such as PPARγ and Notch pathway. These suggest that NSAIDs could play an important role of adjunctive treatment with conventional chemotherapy in CRC.

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ABSTRACT(IN KOREAN)

대장암에서 cyclooxygenase-2 의존 및 비의존 경로를 통한 비스테로이드성 항염증제의 암줄기세포 억제효과

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문 창 모

암줄기세포는 종양의 기원 및 성장에 관여하는 종양 내 소수 세포들로 많은 기존 항암화학제에 대한 내성의 원인으로 설명되어지고 있다. 본 연구의 목적은 대장암에서 항암치료제의 항암효과를 증가시키기 위해 암줄기세포를 억제하는 효과적인 약제를 선정하는 것이었다.

Cyclooxygenase (COX) 억제제 뿐만 아니라, peroxisome proliferator-activated receptor γ (PPARγ) agonist와 γ-secretase 억제제의 효과가 알려진 비스테로이드성 항염증제(nonsteroidal anti-inflammatory drugs; NSAIDs)의 암줄기세포 억제 효과를 확인하기 위해 colosphere 형성능 및 암줄기세포 표지자(CD133, CD44)의 유세포 분석을 시행하였다. SW620 세포주를 이용한 실험에서, 대조군, indomethacin, 5-fluorouracil (5-FU), 5-FU와 indomethacin 병합 처치 후 암줄기세포 표지자와 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT) 분석을 시행하였다. 또한,

연구자들은 세포주 실험에서와 동일한 약물 조합에서 5-FU 내성 SW620 세포주를 이용한 이종 이식 누드 마우스실험을 진행하였다. 기저 메커니즘을 밝히기 위해서, COX-2 선택적 억제제 (celecoxib)과 다른 NSAIDs (sulindac, aspirin), indomethacin과 prostaglandin E2 (PGE2)의 병합 처리 후, 암줄기세포 분획의 변화를 측정하였으며, PPAR-responsive element (PPRE)-Luc을 이용한 reporter assay를 시행하였다. 이종 이식 실험에서, 면역염색을 통해 CD133과 CD44와 함께 HES1 (Notch 신호 체계 표지자), PPARγ, COX-2의 발현을 평가하였다.

그 결과, indomethacin, sulindac, aspirin, celecoxib 등 NSAIDs, γ-secretase inhibitor (DAPT, JLK6), PPARγ agonist (rosiglitazone)는 SW620 세포주에서 대조군과 비교하여 유의하게 CD133+CD44+ 세포를 감소시키고, 50% 이상 colosphere 형성능을 억제하였다. 저농도의 indomethacin (12.5 μM)을 4일간 처리하였을 때, 대조군(100%)와 비교하여 유의하게 CD133+CD44+세포들(72.1%, P = 0.014)을 감소시켰으며, 저농도의 5-FU(2.0 μM)는 CD133+CD44+ 세포들(228.2%, P = 0.014)을 유의하게 증가시켰고, 이렇게 5-FU에 의해 증가된 CD133+CD44+세포들은 동일한 기간 indomethacin 병합처리에 의해 억제되었다(133.1%, P = 0.021). MTT 분석시 각 군별로 세포 생존에 통계적인 차이가 없었으며, 이러한 indomethacin의 암줄기세포 억제효과는 PGE2에 의해 농도의존적으로 억제되었다. Indomethacin은 rosiglitazone과 같이 PPRE 전사 활동을 유의하게 증가시켰다. 이종이식 마우스실험 결과, 5-FU와 indomethacin 병합 처치는 5-FU 단독 처치군과 비교하여 종양 용적을 유의하게 감소시켰다. 또한, indomethacin 단독 처치와 5-FU와 indomethacin 병합 처치군은 각각 대조군, 5-FU 단독 처치군과

비교하여 CD133, CD44, HES1과 COX-2의 발현을 감소시켰고, PPARγ 발현을 증가시켰다.

결론적으로, NSAIDs는 COX-2 의존 및 PPARγ와 Notch 신호와 같은 COX-2 비의존 경로를 통해서 선택적으로 대장암줄기세포를 감소시키고, 5-FU에 의해 증가된 암줄기세포를 억제하였다. 이러한 결과로 NSAIDs는 대장암에서 기존의 항암화학요법에 보조적 치료로 중요한 역할을 할 수 있을 것으로 기대된다.

핵심되는 말 : 암줄기세포, 대장암, 비스테로이드성 항염증제

PUBLICATION LIST