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Celecoxib inhibits the expression of survivin via the suppression of promoter activity in human colon cancer cells

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ABSTRACT

We investigated the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on human colon cancer cell lines to clarify the mechanisms underlying the chemopreventive effect of NSAIDs. Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, induced apoptosis and strongly reduced the expression of an anti-apoptotic protein, survivin, in both protein and mRNA levels in HCT-116 cells. Subsequently, we conducted luciferase reporter assay using a reporter gene driven by the human survivin promoter. A series of analyses using luciferase reporter constructs containing fragments of the survivin promoter and electrophoretic mobility shift assay indicated that the −75/−66 bp region relative to the initiating codon was involved in celecoxib action to suppress survivin promoter activity. Celecoxib also suppressed the activity of TOPflash, T-cell factor reporter plasmid, and the reporter gene driven by the human cyclin D1 promoter, suggesting that this compound inhibited the expression of Wnt/β-catenin signaling target genes. Further, we found that other NSAIDs including indomethacin, resveratrol, and SC-560 induced apoptosis and suppressed the expression of survivin and the Wnt/β-catenin signaling pathway in HCT-116 cells, indicating that these effects were likely to be common among NSAIDs. Moreover, NSAIDs (celecoxib, SC-560 and indomethacin) also suppressed the expression of cyclin D1 and survivin on other colon cancer cell lines (DLD-1 and SW-620). Our results suggested that NSAIDs could inhibit proliferation and induce apoptosis in colon cancer cells by inhibition of survivin expression and the Wnt/β-catenin signaling pathway.

1. Introduction

Apoptosis is a morphologically distinct form of genetically regulated cell death and provides a vital protective mechanism against the development of neoplasia by removing cells with DNA damage [1]. Inhibition of apoptosis confers a survival advantage on cells harboring genetic alterations and promotes the acquisition of further mutations to increase neoplastic progression [2]. The majority of cancers, including colon cancer, are known to overexpress anti-apoptotic proteins, such as survivin, to protect cells from apoptosis [3–5]. Levels of survivin expression in tumors have been reported to be correlated with aggressive phenotypes and a poor prognosis [6,7]. In human colorectal tumorigenesis,

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Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; COX-2, cyclooxygenase-2; FBS, fetal bovine serum; TCF, T-cell factor

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survivin has been thought to play an important role in the progression of cellular dysplasia from adenoma to carcinoma [8]. Survivin gene expression has been reported to be upregulated by Akt activation [9] and recent reports indicated that adenomatous polyposis coli (APC) regulates survivin expression in colonic crypts and that survivin is a target gene of the Wnt/β-catenin signaling pathway [10,11]. Most colorectal cancers have somatic mutations in APC or β-catenin, which are members of the Wnt/β-catenin/T-cell factor (TCF) signaling pathway [12–14]. Although this pathway is essential to regulate gene transcription during embryonic development, it is probably present in intestinal crypts throughout adult life, maintaining the balance between cell proliferation and differentiation [14–16].

Numerous experimental and epidemiological studies in human suggest that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) have chemopreventive activity against colon cancer [17–19]. Several randomized trials have shown the growth inhibition of polyps and decrease in number of existing polyps in patients with familial adenomatous polyposis (FAP) who received sulindac or celecoxib [20,21]. Two randomized placebo-controlled studies have shown that aspirin reduced the risk of colorectal adenomas among population with previous colorectal cancer and adenoma, excluding FAP patients [22,23]. The possible cellular mechanisms underlying these chemopreventive effects of NSAIDs have been thought to be the induction of apoptosis, cell-cycle arrest and the inhibition of angiogenesis [18,19]. Moreover, NSAIDs have been reported to inhibit the Akt [24,25] and the Wnt/β-catenin signaling pathways [26–29].

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, is the only NSAID approved by the Food and Drug Administration for the treatment of FAP patients. However, the molecular mechanism responsible for the chemopreventive effect of celecoxib is not entirely understood. In this study, we found that celecoxib induced apoptosis and suppressed the survivin expression in HCT-116 cells. Therefore, we tried to identify the mechanism by which celecoxib reduces survivin expression. Our results suggested that the −75/−66 bp region relative to the initiating codon in the survivin promoter played an important role to mediate celecoxib action. Moreover, we found that not only celecoxib but also other NSAIDs suppressed the expression of cyclin D1 and survivin in HCT-116, DLD-1 and SW-620 cells, suggesting that NSAIDs inhibit cell proliferation and induce apoptosis in colon cancer cell lines.

2. Materials and methods

2.1. Chemicals and antibodies

Wild-type cyclin D1 pGL3 basic luciferase reporter construct and its mutant were generous gifts from Drs. O. Tetsu and F. McCormick, University of California, San Francisco. TOPflash (TCF reporter plasmid), FOPflash (negative control to TOPflash) and the monoclonal anti-histone H3 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). The polyclonal anti-survivin antibody was from R&D Systems (Minneapolis, MN). The monoclonal anti-GAPDH antibody was from Abcam (Cambridge, UK). The polyclonal anti-phospho-Akt (Ser^{473}) and the polyclonal anti-Akt antibody were from Cell Signaling Technology (Danvers, MA). The polyclonal anti-cyclin D1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-β-catenin antibody was from BD Biosciences (San Jose, CA). The monoclonal anti-β-actin antibody was from Sigma (St. Louis, MO). Indomethacin was from Wako Pure Chemical Industries (Osaka, Japan). Resveratrol and SC-560 were from Cayman Chemical (Ann Arbor, MI). Celecoxib was kindly provided by Pfizer.

2.2. Cell culture

HCT-116 cells, human colon cancer cell line expressing wild-type APC and mutated β-catenin [12], and DLD-1 and SW-620 cells, human colon cancer cell lines expressing mutated APC and wild-type β-catenin [13,30], were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin G, and 0.1 μg/ml of streptomycin.

2.3. DNA electrophoresis

To detect DNA ladder formation, DNA was extracted from both detached and adherent cells, and was subjected to electrophoresis using 2.0% agarose gel as described previously [31]. An optical densitometric scan of the band in ladder was performed using Science Lab 99 Image Gauge software (Fuji Photo Film) to quantify the DNA ladders.

2.4. Caspase-3 activity assay

Caspase-3 activity was assayed using a cysteine protease protein 32/caspase-3 colorimetric protease assay kit (Medical and Biological Laboratories, Nagoya, Japan). Absorbance at 405 nm was measured to determine caspase-3 activity.

2.5. Purification of nucleic protein

Nucleic protein was purified from cells cultured in 100-mm dish using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) and subjected to Western blot analysis and electrophoretic mobility shift assay.

2.6. Immunoblotting

Immunoblotting analysis was performed as described previously [32]. Briefly, samples (10 μg/lane) were separated by 15% (for survivin), 12% (for cyclin D1 and Akt) or 10% (for β-catenin) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoreactive proteins on the membrane were visualized by treatment with a detection reagent (LumiGLO, Cell Signaling Technology). An optical densitometric scan was performed using Science Lab 99 Image Gauge Software.

2.7. Northern blotting

Total cellular RNA was extracted with TRIzol® Reagent (Invitrogen, Carlsbad, CA). The expression of survivin was analyzed by Northern blotting as described previously [31]. Ten micrograms of RNA was used for each lane and

hybridization was performed at 50 °C for 16 h. Survivin specific probe corresponding to positions 48–494 bp of survivin mRNA (Genbank accession NM001168) was amplified by PCR. An optical densitometric scan was performed using Science Lab 99 Image Gauge Software.

2.8. Construction of survivin reporter plasmid

The 5′-flanking region of the human survivin gene (−1594/−18 bp relative to the initiating codon) [33] was amplified by polymerase chain reaction (PCR) from human genomic DNA and subcloned into the pGL3-Basic, firefly luciferase reporter vector. Series of deletion plasmids (−418/−18, −326/−18, −211/−18, −75/−18, −65/−18, −55/−18, −45/−18, −34/−18 bp) were generated by PCR using pGL3-1594 (−1594/−18 bp) as a template. Site-directed mutagenesis was performed using a QuickChange Site-directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) to introduce the −75/−66 bp deletion and the mutations into the two consensus TCF-binding sites (TCF1: −316/−309 bp, TCTCAAAG → TATAACAT and TCF2: −262/−256 bp, CTTTGAA → ATCTAAC) [11] and the mutations were verified by sequence analysis.

2.9. Luciferase reporter assay

Cells were transfected with luciferase reporter plasmids and pRL-SV40, a *Renilla* luciferase expression plasmid for the control of transfection efficiency, using Lipofect Amine Plus reagent (Invitrogen). Cells were cultured for 18 h after transfection and stimulated with celecoxib (100 μM) for 24 h. Luciferase activity was determined with a luminometer (Lumat LB 9507, Berthold Technologies) and normalized with respect to *Renilla* luciferase activity.

2.10. Electrophoretic mobility shift assay

Complementary oligonucleotide corresponding to the −78/−50 bp in survivin promoter was synthesized (5′-TCCCGA-

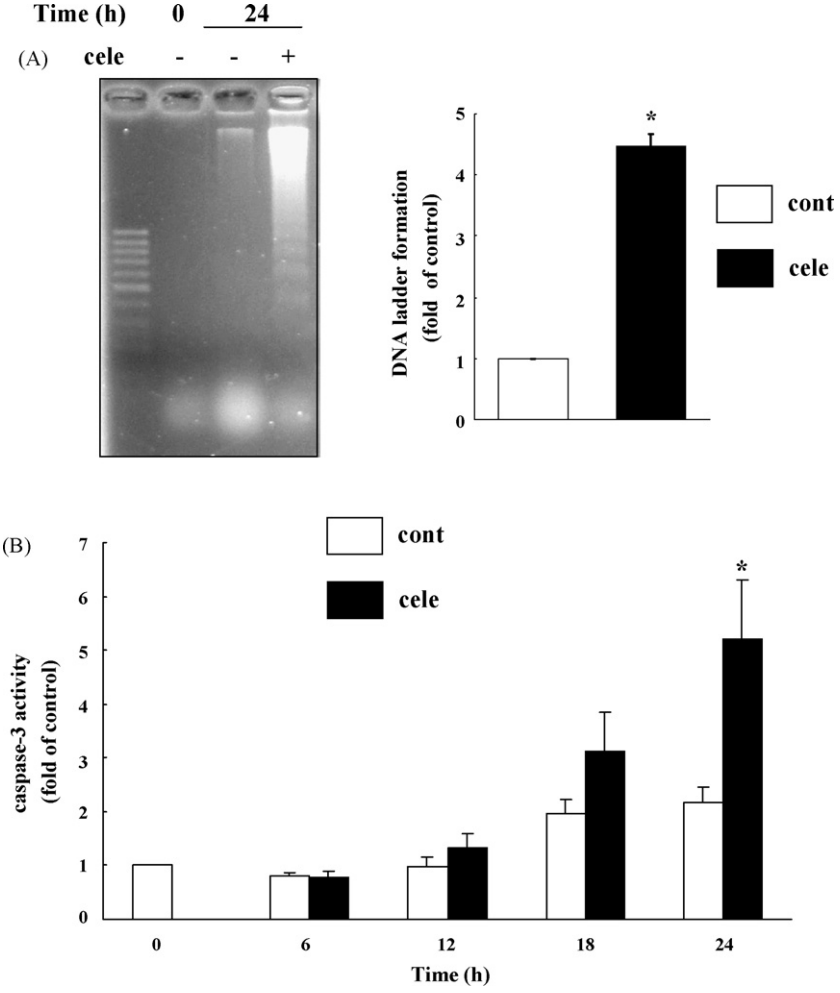

Fig. 1 – Celecoxib induced apoptosis in HCT-116 cells. (A) DNA ladder formation. HCT-116 cells were incubated with or without celecoxib (100 μM) for 24 h. Extracted DNA samples were electrophoresed to detect DNA fragmentation and the ladders were quantified with an image analyzer. Values are the means ± S.E. of three independent experiments and are shown as the fold of the control at 24 h. *$p < 0.01$ vs. control. (B) Time-course of caspase-3 activation induced by celecoxib. Cells were treated with or without celecoxib (100 μM) for the indicated periods. Samples were collected and assayed for caspase-3 activity. Values are the means ± S.E. of three independent experiments and are shown as the fold of the control at time 0. *$p < 0.05$ vs. control. cont: control; cele: celecoxib.

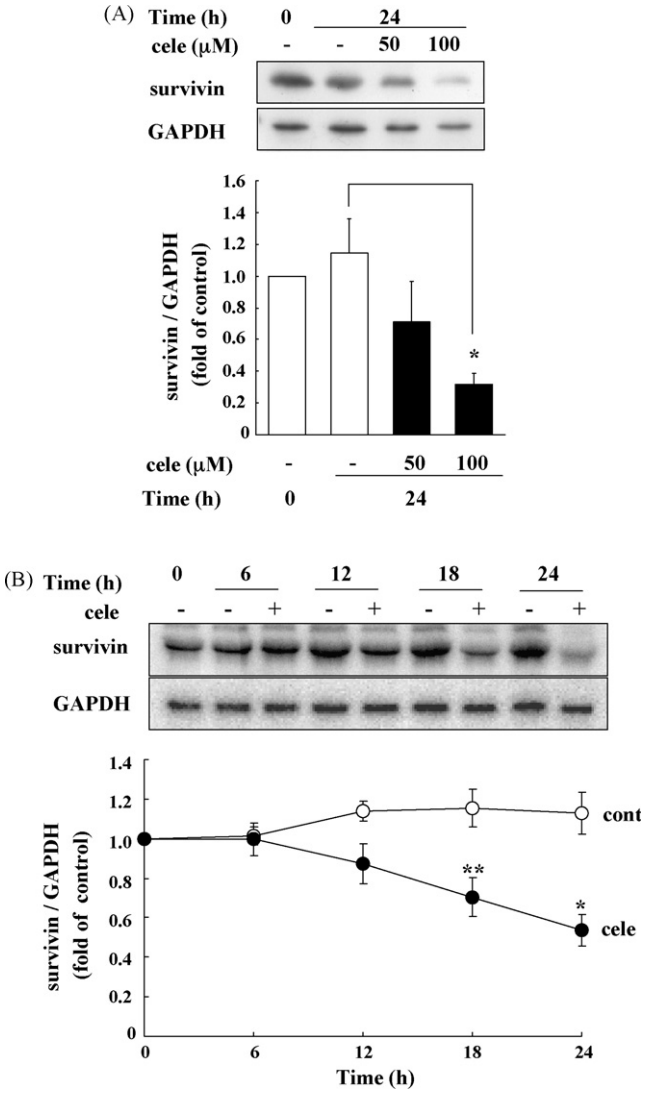

Fig. 2 – The effect of celecoxib on survivin protein and mRNA. (A) Western blot analysis. HCT-116 cells were incubated with or without celecoxib (50, 100 μM) for 24 h. Protein samples were collected and separated by 15% SDS-PAGE and immunoblotted with the anti-survivin antibody. The membrane was reprobed with the anti-GAPDH antibody. The levels of protein bands were quantified and are shown as the fold of the control level at time 0. Values are the means ± S.E. of three independent experiments. $^{*}p < 0.01$ vs. control at 24 h. (B) Northern blot analysis. HCT-116 cells were incubated with or without celecoxib (100 μM) for the period indicated. Total cellular RNA were extracted and subjected to Northern blot analysis. The membrane was reprobed with a GAPDH probe. The levels of mRNA bands were quantified and are shown as the fold of the control level at time 0. Values are the means ± S.E. of three independent experiments. $^{*}p < 0.01$ vs. control; $^{**}p < 0.05$ vs. control.

CATGCCCCGCGGCGCGCCATTAA-3′). Nuclear extracts (10 μg) were incubated with ^{32}P-end-labelled oligonucleotide in the absence or presence of the 100-fold molar excess amount of unlabelled oligonucleotide as a competitor. Electrophoretic mobility shift assay was performed as described previously [34].

3. Results

3.1. Celecoxib induced apoptosis in HCT-116

We examined whether celecoxib induces apoptosis in a human colon cancer cell line, HCT-116. Cells were incubated with or without celecoxib for 24 h and the level of apoptosis was determined by examining the formation of DNA fragmentation and caspase-3 activity. As shown in Fig. 1A, celecoxib strongly induced DNA fragmentation after 24 h incubation. The effect of celecoxib on caspase-3 activity was examined and we found that celecoxib stimulated caspase-3 activity in a time-dependent manner (Fig. 1B). These results indicated that celecoxib induced apoptosis in HCT-116 cells.

3.2. Celecoxib suppressed the protein and mRNA expression levels of survivin

Subsequently, we examined the effect of celecoxib on survivin protein and mRNA expression. As shown in Fig. 2A, celecoxib decreased the protein level of survivin in a dose-dependent manner. Fig. 2B shows that celecoxib significantly decreased the mRNA level of survivin after 18 h incubation. These results indicated that celecoxib decreased the survivin protein amount due to the suppression of mRNA expression in HCT-116 cells.

3.3. Celecoxib reduced survivin promoter activity

The 5′-flanking region of the human survivin gene was obtained by PCR and constructed with a luciferase reporter plasmid (pGL3-Basic). Reporter plasmids containing different lengths of survivin promoter were generated and analyzed for promoter activity. As shown in Fig. 3A, although the luciferase activity of survivin promoter-constructed plasmids were increased up to two- to three-fold of the control at time 0 after 24 h incubation, celecoxib almost completely inhibited this increase when constructs longer than 58 bp (−1594/−18, −418/−18, −326/−18, −211/−18, and −75/−18 bp) were transfected. Next, we examined the effect of celecoxib on survivin promoter activity using further deletion of the 58 bp (−75/−18 bp) promoter region to indicate the minimal responsive region in the survivin promoter. As shown in Fig. 3B, although the basal activity of these constructs was quite small compared with the −75/−18 bp construct, celecoxib showed no significant effect when constructs shorter than 48 bp (−65/−18, −55/−18, −45/−18 and −34/−18 bp) were transfected. This result might indicate that the responsive element to celecoxib treatment exists between −75 and −66 bp. However, luciferase activities of the −75/−18 and −65/−18 bp constructs were quite small compared with the −418/−18 bp construct. Therefore, a deletion reporter construct which is based on −418/−18 bp construct but lacks between −75 and −66 bp was generated to clarify if this region played an important role in celecoxib action. As shown in Fig. 3C, although celecoxib still had a significant effect, the effect was weakened when

deletion construct was transfected (Fig. 3C). Celecoxib decreased luciferase activity to 26.9 ± 2.6% (mean ± S.E., $n = 7$) of control in wild-type-transfected cells and to 44.4 ± 1.9% (mean ± S.E., $n = 6$) in deletion mutant-transfected cells (Fig. 3C, right panel). These results indicated that celecoxib strongly inhibited survivin promoter activity and the −75/−66 bp region relative to the initiating codon in survivin promoter might partially mediate the effect of celecoxib in HCT-116 cells. Subsequently, an electrophoretic mobility shift assay was performed to examine the effect of celecoxib on the binding of this promoter region (−75/−66 bp) to nuclear extracts from HCT-116 cells. As shown in Fig. 3D, a DNA oligonucleotide containing this region bound to nuclear protein (lane 2) and the addition of 100-fold molar excess of the unlabelled nucleotide resulted in a marked reduction of the band (lane 4). The binding of the oligonucleotide was remarkably increased when extract from the celecoxib-treated cells was used (lane 3). This result might suggest that celecoxib stimulate the binding of unknown protein which is involved in the suppression of the survivin mRNA transcription.

3.4. *Effect of celecoxib on the Wnt/β-catenin signaling pathway and the Akt pathway*

To clarify the effect of celecoxib on the Wnt/β-catenin signaling pathway, we next examined the effect of celecoxib on T-cell factor (TCF) transcriptional activity using luciferase reporter plasmid, TOPflash. We transfected TOPflash to HCT-116 cells and stimulated with celecoxib for 24 h. As shown in Fig. 4A, celecoxib reduced the luciferase activity of TOPflash, whereas it had no significant effect on luciferase activity when FOPflash, a reporter plasmid containing a mutated TCF binding site (a negative control to TOPflash), was transfected to the cells. As it has been reported that TCF-binding sites in the survivin promoter play an important role in the regulation of promoter activity, we introduced mutations in these TCF-binding sites and the effect of celecoxib on these reporter plasmids were also analyzed. Unexpectedly, mutations in TCF-binding sites did not influence the effect of celecoxib (Fig. 4B). These results indicated that celecoxib strongly inhibited survivin promoter activity but TCF-binding sites in the survivin promoter did not play a significant role in the action of celecoxib. To examine the effect of celecoxib on the Akt pathway, phosphorylation status of Akt was examined. Surprisingly, as shown in Fig. 4C, celecoxib elevated the phosphorylation level of Akt, indicating that this pathway is not involved in celecoxib action to suppress survivin mRNA expression in HCT-116 cells.

3.5. *Celecoxib reduced the protein level of cyclin D1 and cyclin D1 promoter activity through the TCF binding site*

As we found that celecoxib affected the Wnt/β-catenin signaling pathway and suppressed TCF transcriptional activity, we subsequently examined the effect of celecoxib on cyclin D1 protein expression and promoter activity to clarify the effect of celecoxib on the Wnt/β-catenin signaling target genes. As shown in Fig. 5A, celecoxib significantly reduced the protein level of cyclin D1 after 24 h incubation. Next, we

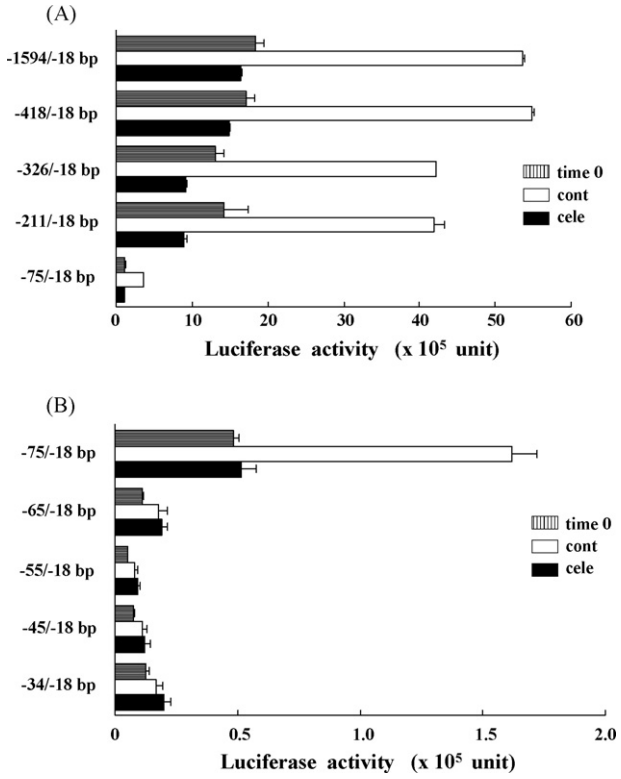

Fig. 3 – The effect of celecoxib on survivin promoter activity. Celecoxib inhibited survivin promoter activity. (A) Effect of celecoxib on the longer constructs (from −1594/−18 to −75/−18 bp). (B) Effect of celecoxib on the shorter constructs (from −75/−18 to −34/−18 bp). The luciferase reporter vector pGL3-Basic, containing the indicated region of the 5′-flanking region of the survivin gene, and pRL-SV40 were co-transfected into HCT-116 cells. After 18 h incubation (time 0), cells were stimulated with or without celecoxib (100 μM) for 24 h. Values are the means ± S.E. of three independent experiments performed in duplicate. (C) Comparison of the effect of celecoxib on wild-type and deletion mutant constructs. Wild-type and deletion mutant, which lacks −75/−66 bp, constructs based on −418/−18 bp were co-transfected with pRL-SV40. After 18 h incubation, cells were stimulated with or without celecoxib (100 μM) for 24 h. Values are the means ± S.E. of three independent experiments performed in duplicate. In right panel, luciferase activity of celecoxib-treated cells was shown as the percentages of the control cells. $^{*}p < 0.05$ vs. wild-type. (D) Electrophoretic mobility shift assay. Nuclear protein was extracted from HCT-116 cells with or without celecoxib-treatment for 24 h and electrophoretic mobility shift assay was performed. The bands were quantified with an image analyzer. Values are the means ± S.E. of three independent experiments and are shown as the fold of the control at 24 h. $^{*}p < 0.05$ vs. control.

examined the effect of celecoxib on cyclin D1 promoter activity. For this experiment, we employed two different types of cyclin D1 reporter plasmids, wild-type and mutant, which lacks the TCF consensus site located at −81/−75 bp [35]. Fig. 5B

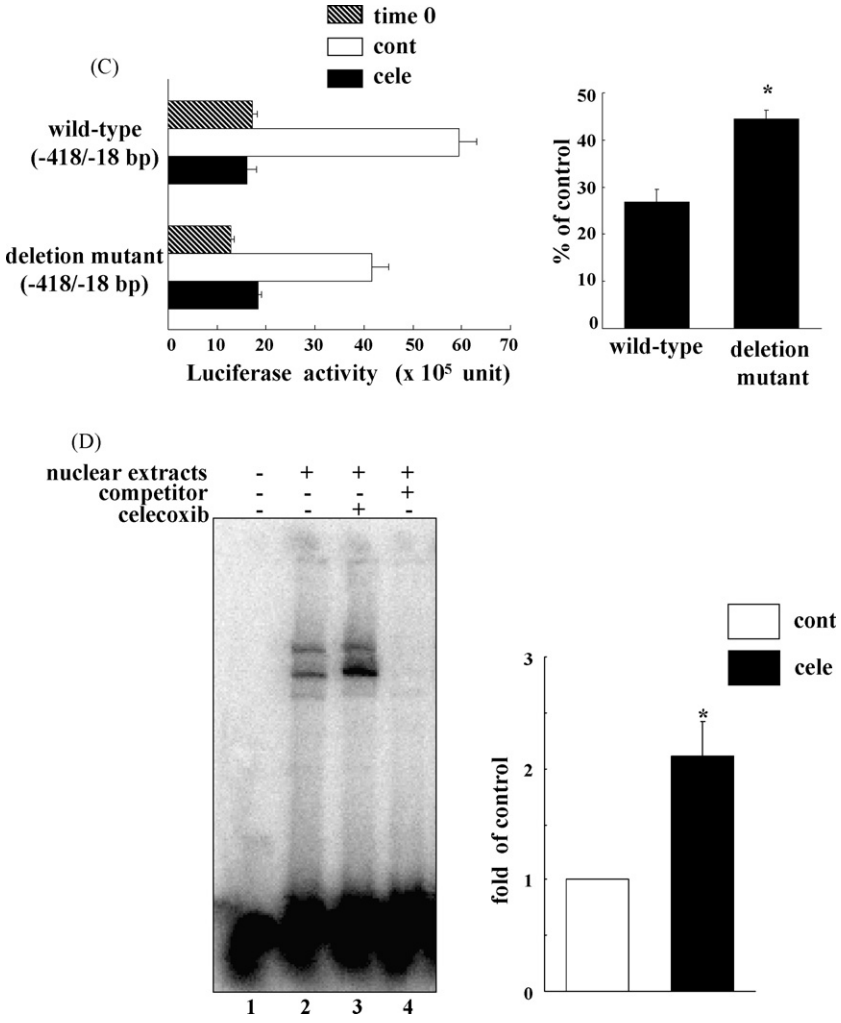

Fig. 3. (Continued).

shows that celecoxib reduced wild-type promoter activity after 24 h stimulation, whereas it exhibited no significant effect on mutant promoter activity. These results indicated that celecoxib inhibited cyclin D1 promoter activity through the TCF-binding site and reduced cyclin D1 protein expression, suggesting that celecoxib suppressed the expression of Wnt/β-catenin signaling target genes. As TCF transcriptional activity can be modulated by the level of β-catenin protein amount, we analyzed whether celecoxib affects the cellular β-catenin protein amount. However, as shown in Fig. 5C, celecoxib had no significant effect on the total and nuclear β-catenin protein amount. These results suggested that celecoxib affected the Wnt/β-catenin signaling pathway and suppressed TCF transcriptional activity without affecting the β-catenin protein amount.

3.6. *Other NSAIDs also induced apoptosis and suppressed survivin expression and TCF transcriptional activity in HCT-116 cells*

Subsequently, we examined whether other NSAIDs also have the same effect as celecoxib on HCT-116. We employed different types of NSAIDs including indomethacin (nonselective COX inhibitor), resveratrol and SC-560 (selective COX-1 inhibitors). As shown in Fig. 6A, these three NSAIDs induced DNA fragmentation after 24 h incubation. In these drugs, indomethacin had the strongest effect to induce apoptosis. Subsequently, we examined the effect of these drugs on survivin expression. All drugs significantly decreased the protein and mRNA level of survivin after 24 h incubation (Fig. 6B and C). As shown in Fig. 6D, NSAIDs significantly reduced TOPflash activity after 24 h stimulation. Similar to celecoxib, NSAIDs had no significant effect on luciferase activity when FOPflash was transfected to the cells (data not shown). These results indicated that not only celecoxib but also other NSAIDs induced apoptosis and suppressed the expression of survivin and Wnt/β-catenin signaling in HCT-116 cells.

3.7. *Effect of NSAIDs on the expression of survivin, cyclin D1, and β-catenin in other colon cancer cell lines*

The effect of celecoxib, SC-560, and indomethacin on the expression of β-catenin, cyclin D1 and survivin was examined using human colon cancer cell lines (DLD-1 and SW-620). Since the same concentrations of NSAIDs used for HCT-116 cells (celecoxib, 100 μM; SC-560, 100 μM; indomethacin, 400 μM) did not have significant effects on these

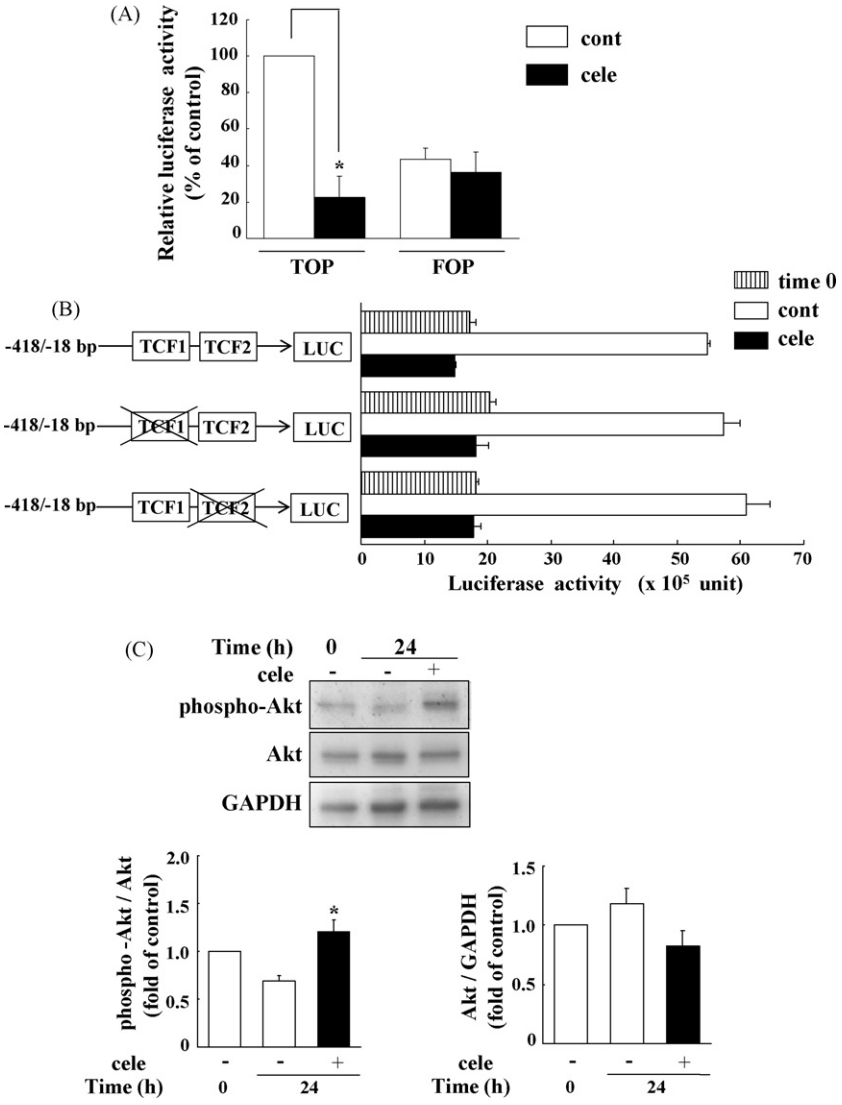

Fig. 4 – Celecoxib inhibited TCF transcriptional activity. (A) Effect of celecoxib on TCF transcriptional activity. TOPflash or FOPflash was co-transfected with pRL-SV40 into HCT-116 cells. After 18 h incubation, cells were stimulated with or without celecoxib for 24 h. Luciferase activity is shown as the percentages of the TOPflash activity of control. Values are the means ± S.E. of three independent experiments done in duplicate. $^{*}p < 0.01$ vs. control. (B) Effect of celecoxib on luciferase activity of TCF-binding site-mutated survivin promoter constructs. TCF-binding sites in −418/−18 bp constructs were mutated as described in Section 2. Wild-type and mutated −418/−18 bp constructs were co-transfected with pRL-SV40. After 18 h incubation (time 0), cells were stimulated with or without celecoxib (100 μM) for 24 h. Values are the means ± S.E. of three independent experiments performed in duplicate. (C) Western blot analysis. HCT-116 cells were incubated with or without celecoxib (100 μM) for 24 h. Immunoblot was performed with the anti-phospho-Akt (Ser^{473}), anti-Akt and anti-GAPDH antibodies. The levels of protein bands were quantified and are shown as the fold of the control level at time 0. Values are the means ± S.E. of three independent experiments. $^{*}p < 0.01$ vs. control at 24 h.

cells, we used higher concentrations of NSAIDs (celecoxib, 200 μM; SC-560, 200 μM; indomethacin, 800 μM) for this experiment. As shown in Fig. 7, all these NSAIDs greatly reduced the expression of survivin and cyclin D1 after 24 h incubation and did not have significant effect on the expression of β-catenin in these cell lines. These results indicated that NSAIDs reduced the expression of both survivin and cyclin D1 in colon cancer cell lines, while the sensitivity to NSAIDs are different among cell lines.

4. Discussion

In this study, we showed that celecoxib induced apoptosis in human cancer cells and also reduced survivin expression at both protein and mRNA levels. Overexpression of survivin in cancer overcomes the apoptotic checkpoint and favors the aberrant progression of transformed cells through mitosis [36,37]. The ability of celecoxib to increase apoptosis by decreasing survivin may be responsible for the chemopreventive effects of this drug. Indeed, it has been reported that

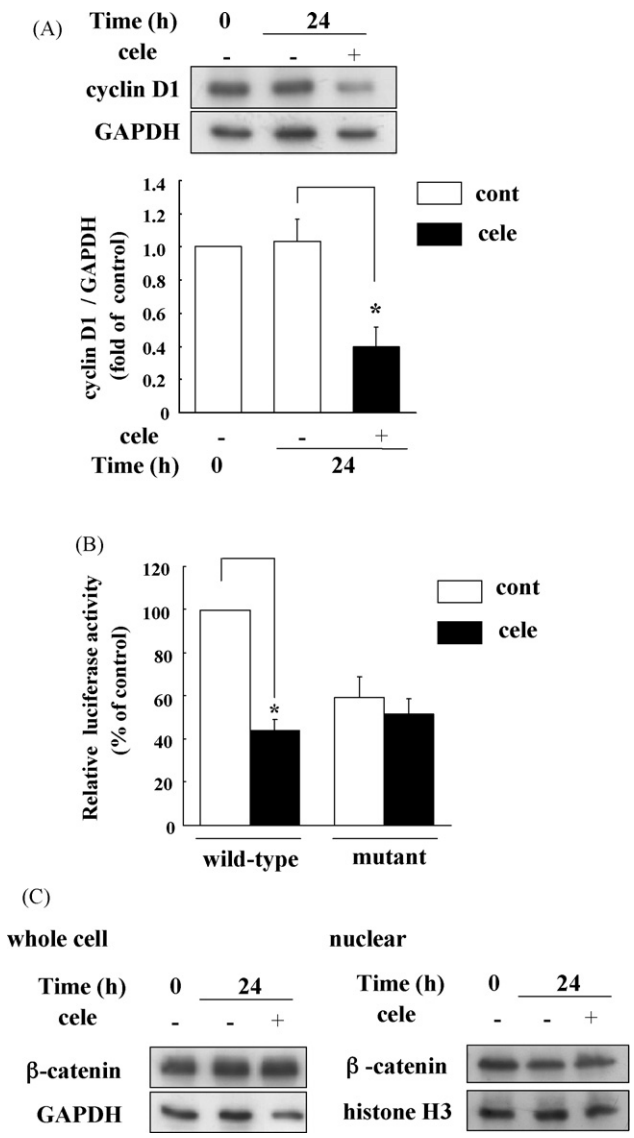

Fig. 5 – The effect of celecoxib on cyclin D1 protein and promoter activity. (A) Western blot analysis for cyclin D1. HCT-116 cells were incubated with or without celecoxib for 24 h. Protein samples were collected and separated by 12% SDS-PAGE and immunoblotted with the anti-cyclin D1 antibody. The membrane was reprobed with the anti-GAPDH antibody. The levels of protein bands were quantified and are shown as the fold of the control level at time 0. Values are the means ± S.E. of three independent experiments. *$p < 0.01$ vs. control at 24 h. (B) Reporter gene assay with wild-type and mutated cyclin D1 promoter. HCT-116 cells were co-transfected with wild-type or mutated cyclin D1 promoter and pRL-SV40. After 18 h incubation, cells were stimulated with or without celecoxib (100 μM) for 24 h. Luciferase activity is shown as the percentages of luciferase activity in wild-type-transfected cells. Values are the means ± S.E. of three independent experiments performed in duplicate. *$p < 0.01$ vs. control. (C) Effect of celecoxib on β-catenin amount in whole cell and nuclear extract. HCT-116 cells were incubated with or without celecoxib (100 μM) for 24 h. Protein samples were collected and separated by 10% SDS-PAGE and immunoblotted with the anti-β-catenin antibody. The membrane was reprobed with the anti-GAPDH and anti-histone H3 antibody. The results are representative of three other experiments.

forced overexpression of survivin rescues various cells from apoptosis induced by anticancer drugs including taxol, doxycyclin and hedamycin [38–40]. Therefore, we attempted to clarify the mechanism by which celecoxib reduced the expression of survivin and found that celecoxib strongly inhibited the promoter activity of survivin. To identify the responsive element to celecoxib treatment, we generated a series of reporter plasmids containing different lengths of the 5′-flanking region of survivin gene. Our results suggested that the possible responsive element to celecoxib treatment exists between −75 and −66 bp. The deletion of this region from the survivin promoter attenuated the effect of celecoxib whereas this chemical still had a significant effect on the deletion promoter activity after 24 h stimulation. Moreover, the result from the electrophoretic mobility shift assay suggested that celecoxib might stimulate the binding of unknown protein to this region and suppress the transcription of survivin mRNA. Further studies are required to clarify the mechanisms how celecoxib reduces survivin promoter activity and what kind of protein is involved in the celecoxib action.

Sp-1 and TCF are the two major transcriptional factors involved in the regulation of survivin promoter activity [33]. In these transcriptional factors, we paid special attention to TCF, since we found that celecoxib reduced the activity of TCF reporter plasmid, TOPflash (Fig. 4A). However, mutations in the TCF binding site in the survivin promoter did not prevent the effect of celecoxib (Fig. 4B), indicating that the TCF binding site was not responsive to celecoxib treatment for that gene. Different lengths of survivin promoter were constructed in the luciferase reporter plasmid and the activity of these constructs was analyzed. As shown in Fig. 3A, there was a large difference in basal luciferase activity between −211/−18 bp construct and −75/−18 bp construct. According to Li and Altieri [33], numerous SP1 sites exist between −231 and −84 bp regions in the survivin promoter. On the other hand, when comparing −326/−18 bp construct and −211/−18 bp construct, there was no significant effect when two TCF-binding sites (TCF1: −316/−309 bp, TCF2: −262/−256 bp) were eliminated. Therefore, these results might indicate that SP1 sites and not TCF sites are important basal transcriptional requirements of survivin gene expression. Moreover, as shown in Figs. 4B and 5B, the deletion of TCF site from cyclin D1 promoter greatly decreased the basal luciferase activity whereas the deletion of TCF site from survivin promoter did not have a significant effect. These results suggested that TCF site plays an important role in cyclin D1 gene expression but not in survivin gene expression.

β-Catenin is a key component of the Wnt/β-catenin signaling pathway. The accumulated β-catenin promotes its translocation to the nucleus and interacts with TCF transcription factors to modulate the transcription of downstream target genes. Celecoxib reduced TOPflash activity and cyclin D1 promoter activity, which is one of the Wnt/β-catenin signaling target genes, without affecting the protein level of β-catenin. As several Wnt/β-catenin signaling target genes are thought to play important roles in the progression of colon

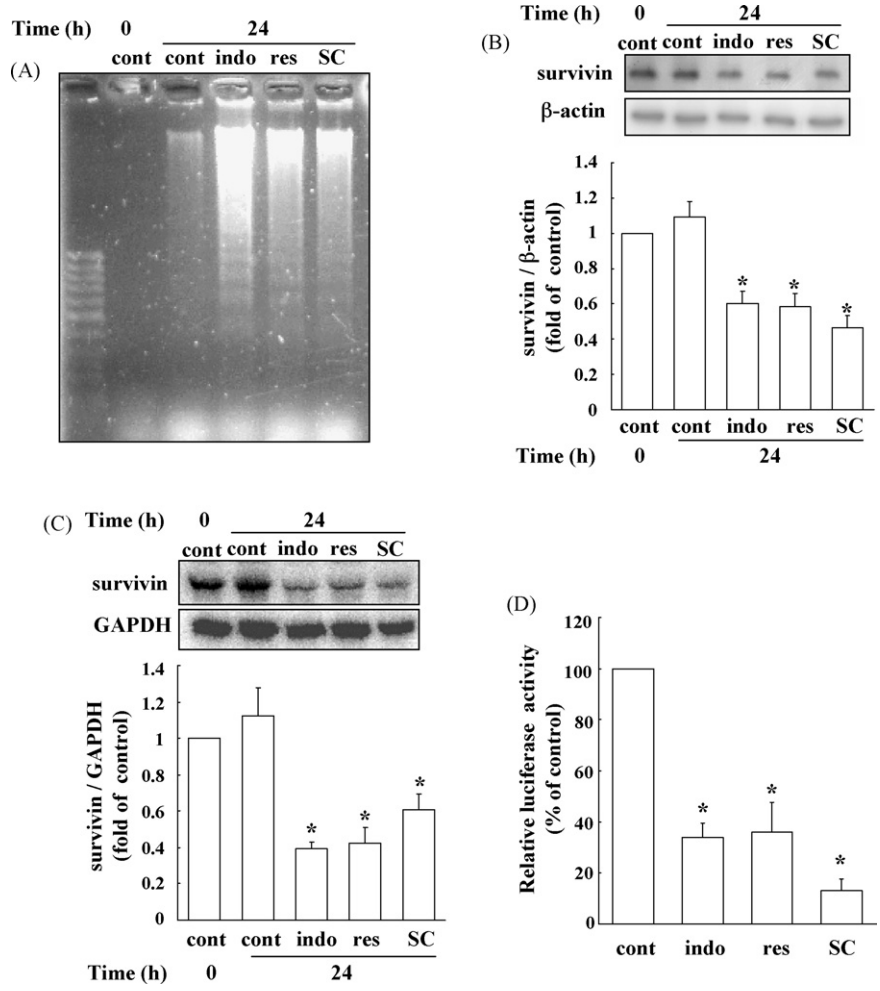

Fig. 6 – Effect of various NSAIDs on HCT-116 cells. HCT-116 cells were incubated with or without the indicated NSAIDs for 24 h. (A) DNA ladder formation. Extracted DNA samples were electrophoresed to detect DNA fragmentation and the ladders were quantified with an image analyzer. (B) Western blot analysis for survivin. The levels of protein bands were quantified and are shown as the fold of the control level at time 0. Values are the means ± S.E. of three independent experiments. *$p < 0.01$ vs. control at 24 h. (C) Northern blot analysis for survivin. The levels of mRNA bands were quantified and are shown as fold of the control level at time 0. Values are means ± S.E. of three independent experiments. *$p < 0.01$ vs. control at 24 h. (D) Luciferase activity. TOPflash was co-transfected with pRL-SV40 into HCT-116 cells. After 18 h incubation, cells were stimulated with or without the indicated drugs for 24 h. Luciferase activity is shown as the percentages of the control. Values are the means ± S.E. of three independent experiments performed in duplicate. *$p < 0.01$ vs. control. cont: control; indo: indomethacin (400 μM); res: resveratrol (100 μM); SC: SC-560 (100 μM).

cancer [35,41,42], suppression of this signaling pathway could be an important effect of celecoxib as an anticancer drug. We were not able to determine the mechanism by which celecoxib reduced the promoter activity of the Wnt/β-catenin signaling target genes without affecting the protein level of β-catenin. However, recently it has been reported that the suppression of β-catenin-dependent transcription by NSAIDs depends on the coexpression of other nuclear factors such as peroxisome proliferator-activated receptor γ (PPAR γ) and retinoid-X-receptor α (RXR α) [43]. Further, it has been reported that transcriptional activity of the β-catenin/TCF pathway is regulated by binding the transcriptional cofactor cyclic AMP-response element binding protein (CREB)-binding protein to β-catenin [44,45]. Therefore, not only β-catenin and TCF but also other nuclear factors might play important roles in the action of celecoxib.

COX-2 is the key enzyme in the conversion of arachidonic acid to prostaglandins which promote cell proliferation, angiogenesis and inhibit the induction of apoptosis; therefore, the chemopreventive effect of NSAIDs is thought to be attributed to their COX-2 inhibitory activity. However, some studies have suggested that celecoxib has anticarcinogenic effects in cells which do not express COX-2 and COX-2-deficient tumors in a nude mice model [46,47]. In fact, the HCT-116 cells used in this experiment do not express COX-2, and not only COX-2 inhibitor but also COX-1 inhibitor (SC-560) suppressed survivin expression and induced apoptosis. Thus anticarcinogenic effects of

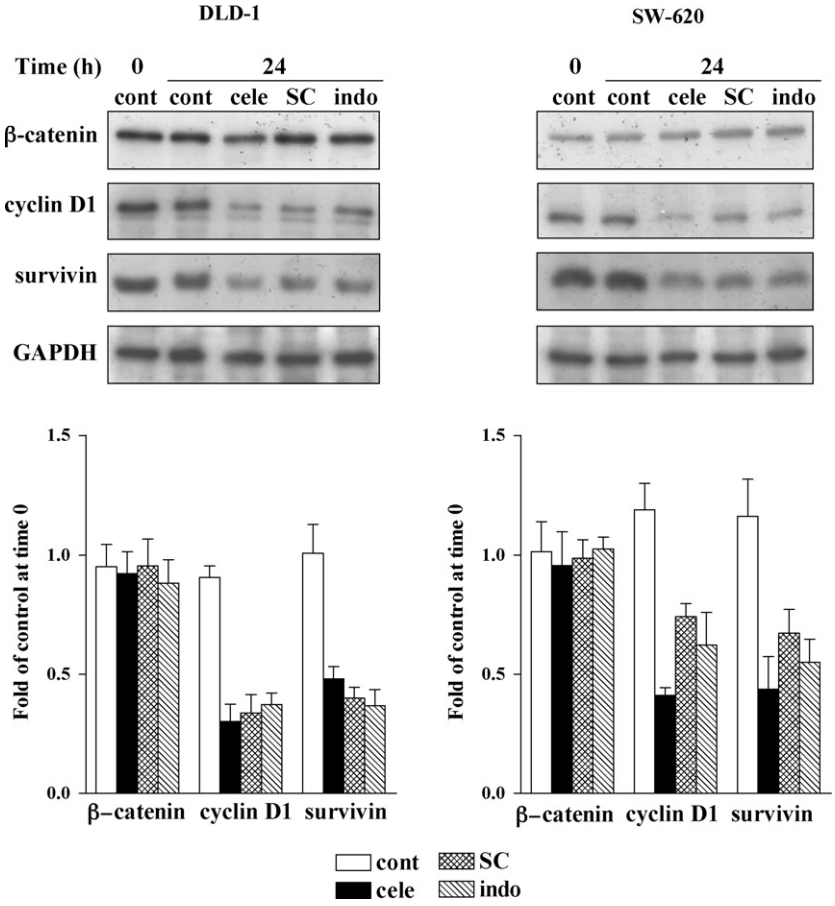

Fig. 7 – Effect of various NSAIDs on other colon cancer cell lines. DLD-1 and SW-620 cells were incubated with or without the indicated NSAIDs (celecoxib, 200 μM; SC-560, 200 μM; indomethacin, 800 μM) for 24 h. Protein samples were collected and separated by 12% SDS-PAGE and immunoblotted with the anti-β-catenin, anti-cyclin D1, anti-survivin, and anti-GAPDH antibodies. The levels of protein bands were quantified and normalized to those of GAPDH. The results are shown as the fold of the control level at time 0. Values are the means ± S.E. of three independent experiments. cont: control; cele: celecoxib; SC: SC-560; indo: indomethacin.

celecoxib can be explained by both COX-2-dependent and -independent mechanisms.

In summary, our results indicated that celecoxib induced apoptosis with decreased survivin expression by the suppression of transcriptional activity partially through the −75/−66 bp region relative to the initiating codon in survivin promoter. Moreover, not only celecoxib but also other NSAIDs induced apoptosis and suppressed the expression of survivin and cyclin D1 in colon cancer cell lines.

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