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Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells

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Abstract Although the influence of selective cyclooxygenase (COX)-2 inhibitors on the proliferation of colon adenocarcinoma cells have been the subject of much investigation, relatively little research has compared the effects of different COX-2 inhibitors. Celecoxib strongly suppressed the proliferation of COX-2 expressing HT-29 cells at 10–40 μM. NS-398 and nimesulide also inhibited cell proliferation, whereas rofecoxib, meloxicam, and etodolac did not. Only celecoxib induced apoptosis of HT-29 cells, as detected on the basis of DNA fragmentation, TUNEL positivity, and caspase-3/7 activation. DNA fragmentation was also increasd in COX-2 non-expressing cell lines (SW-480 and HCT-116) by exposure to celecoxib for 6–24 h. All six COX-2 inhibitors suppressed the production of prostaglandin E_2 by HT-29 cells, suggesting that the pro-apoptotic effect of celecoxib was unrelated to inhibition of COX-2. Inactivation of Akt might explain the differential pro-apoptotic effect of these selective COX-2 inhibitors on colon adenocarcinoma cells.

Key words: Selective cyclooxygenase-2 inhibitors; Celecoxib; Proliferation; Apoptosis; Colon cancer cells; Akt

1. Introduction

Epidemiological studies have shown that chronic intake of aspirin is associated with a reduction in the incidence of colorectal cancer [1,2]. In a clinical trial, celecoxib [3] was found to reduce the number and size of polyps in patients with familial adenomatous polyposis (FAP). The effects of several non-steroidal anti-inflammatory drugs (NSAIDs) on tumor growth have also been demonstrated in animal models of FAP [4] and chemical colon carcinogenesis [5–8]. These observations suggest that NSAIDs have a potent chemopreventive effect on colon cancer.

NSAIDs have also been shown to exert anti-proliferative and pro-apoptotic effects on a variety of cell lines, particularly colon cancer cell lines [9,10], suggesting a possible mechanism for their chemopreventive action on the colon. Although cyclooxygenase (COX) is the molecular target of most NSAIDs [11], not only COX-dependent mechanisms [12,13] but also COX-independent [14–16] mechanisms have been reported for the anti-proliferative and pro-apoptotic actions of these drugs. Accordingly, the mechanisms by which NSAIDs inhibit cell proliferation and induce apoptosis remain unclear.

Classic NSAIDs not only inhibit COX-2, but also inhibit COX-1, resulting in the common side effect of gastric mucosal damage. To reduce the gastrointestinal side effects of NSAIDs, selective COX-2 inhibitors were developed [17], and the effect of these selective inhibitors on the proliferation and apoptosis of colon cancer cells has been the subject of much investigation in recent years [12,15,16,18]. However, almost all of these studies have involved the comparison of a COX-2 inhibitor with non-selective NSAID (or else no comparison at all), so relatively little is known about the comparative effects of different selective COX-2 inhibitors on cell proliferation and apoptosis. To address this issue, we investigated the effects of six major COX-2 inhibitors [17,19] (celecoxib, rofecoxib, NS-398, nimesulide, meloxicam and etodolac) on the proliferation and apoptosis of colon adenocarcinoma cells.

2. Materials and methods

2.1. Materials

NS-398 and nimesulide were purchased from Cayman Chemical (Ann Arbor, MI, USA). The caspase inhibitor *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO; Promega, Medison, WI, USA) and epidermal growth factor (EGF; Sigma, St. Louis, MO, USA) were purchased from the indicated companies. Celecoxib, meloxicam, and etodolac were obtained from Pharmacia K.K. (Tokyo, Japan), Nippon Boehringer Ingelheim (Hyogo, Japan), and Wyeth Lederle Japan (Tokyo, Japan), respectively. Rofecoxib (International Patent Publication WO95/00501) was synthesized as reported elsewhere [20]. These drugs were dissolved in dimethyl sulfoxide and the final concentration of the vehicle in all cultures was 0.1%.

2.2. Cell culture

Human colon adenocarcinoma cell lines (HT-29, SW-480 and HCT-116) were obtained from the American type culture collection (Rockville, MD, USA), and were grown in RPMI 1640 medium (Gibco,

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Abbreviations: COX, cyclooxygenase; FAP, familial adenomatous polyposis; NSAIDs, non-steroidal anti-inflammatory drugs; FBS, fetal bovine serum; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; EGF, epidermal growth factor; BrdU, 5-bromo-2′-deoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ELISA, enzyme-linked immunosorbent assay; PPARγ, peroxisome proliferator-activated receptor γ

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Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in an atmosphere of 5% CO_2.

2.3. Cell proliferation assay

Proliferation of HT-29 cells was estimated from the incorporation of 5-bromo-2′-deoxyuridine (BrdU). The cells (4×10^3 or 1×10^4/well) were exposed to test drugs during culture in 96-well plates containing RPMI 1640 medium with 1% (v/v) FBS at 37°C in an atmosphere of 5% CO_2. After 24 or 96 h, BrdU (10 μM) was added to the medium, and then the cells were incubated for another 16–18 h. Next, the cells were fixed and BrdU incorporation was determined with a cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.4. DNA fragmentation assay

Colon carcinoma cells (2×10^4/well) were treated with the test drugs during culture for 6 or 24 h in 96-well plates containing RPMI 1640 medium with 1% (v/v) FBS at 37°C in an atmosphere of 5% CO_2. Cytoplasmic DNA fragments, which are an indicator of apoptosis, were measured with a DNA cell death detection ELISA^{PLUS} kit (Roche Diagnostics) according to the manufacturer's instructions.

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

HT-29 cells (6×10^4/well) were treated with the test drugs during culture in 8-well chamber slides (IWAKI, Chiba, Japan) containing in RPMI 1640 medium with 1% (v/v) FBS at 37°C in an atmosphere of 5% CO_2. After 24 h, the cells were fixed with 4% (w/v) neutral buffered formalin for 10 min at room temperature, and then apoptotic cells were identified by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using an apoptosis in situ detection kit (Wako, Osaka, Japan) according to the manufacturer's instructions. The cells were counterstained with methyl green (Wako).

2.6. Assay of caspase activity

HT-29 cells (2×10^4/well) were treated with the test drugs during incubation in 96-well plates containing RPMI 1640 medium with 1% (v/v) FBS at 37°C in an atmosphere of 5% CO_2. After 6 h, the level of caspase activity in the cells was measured by an Apo-ONE™ homogeneous caspase-3/7 assay (Promega) according to the manufacturer's instructions.

2.7. Assay of PGE_2 production

HT-29 cells (5×10^5/well) were pretreated with the test drugs during incubation in 24-well plates containing RPMI 1640 medium with 1% (v/v) FBS at 37°C in an atmosphere of 5% CO_2. After 1 h, calcium ionophore A23187 (0.5 μM; Sigma) was added to the medium, and the cells were incubated for another 30 min. Then the PGE_2 level in the culture medium was measured using a commercial ELISA kit (Cayman) according to the manufacturer's instructions.

2.8. Western blotting

Colon carcinoma cells were lysed in solubilization buffer (10 mM Tris–HCl, pH 7.4, 1% (w/v) NP-40, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 0.15 M NaCl, 1 mM EDTA, and 10 μg/ml aprotinin), and then centrifuged at $12\,500\times g$ for 30 min to remove cell debris. Subsequently, the supernatant was concentrated by the precipitation of proteins with trichloroacetic acid at a final concentration of 3.3% (w/v). The resultant precipitate (50, 100, or 150 μg) was subjected to SDS–polyacrylamide gel electrophoresis using 10–12.5% (w/v) acrylamide slab gels under reducing conditions. The separated proteins were electrotransferred to Immobilon™ polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Then each membrane was reacted with rabbit anti-Akt antiserum (New England Biolabs, Beverly, MA, USA), rabbit anti-phospho-Akt antiserum (Ser^{473}) (Sigma), or rabbit anti-Bcl-2 polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). Immunoreactive Akt, phospho-Akt, or Bcl-2 was visualized indirectly using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as the substrates.

3. Results

3.1. Effect of COX-2 inhibitors on the proliferation of HT-29 cells

Initially, we examined the effect of six selective COX-2 inhibitors on the proliferation of HT-29 cells by measuring incorporation of BrdU to assess DNA synthesis (Fig. 1). HT-29 cells were incubated with each of the inhibitors for 24 h. Celecoxib strongly suppressed cell proliferation in a concentration-dependent manner and its IC_{50} value was 32.5 μM. The most pronounced inhibitory effect of celecoxib was observed at 40 μM, with the growth of HT-29 cells being completely suppressed. Although NS-398 and nimesulide were less effective than celecoxib, these agents also inhibited the proliferation of HT-29 cells. In contrast, rofecoxib, meloxicam, and etodolac had no effect on cell proliferation at concentrations up to 100 μM. Prolonged exposure to rofecoxib, meloxicam, or etodolac for 96 h also had no appreciable effect on cell proliferation.

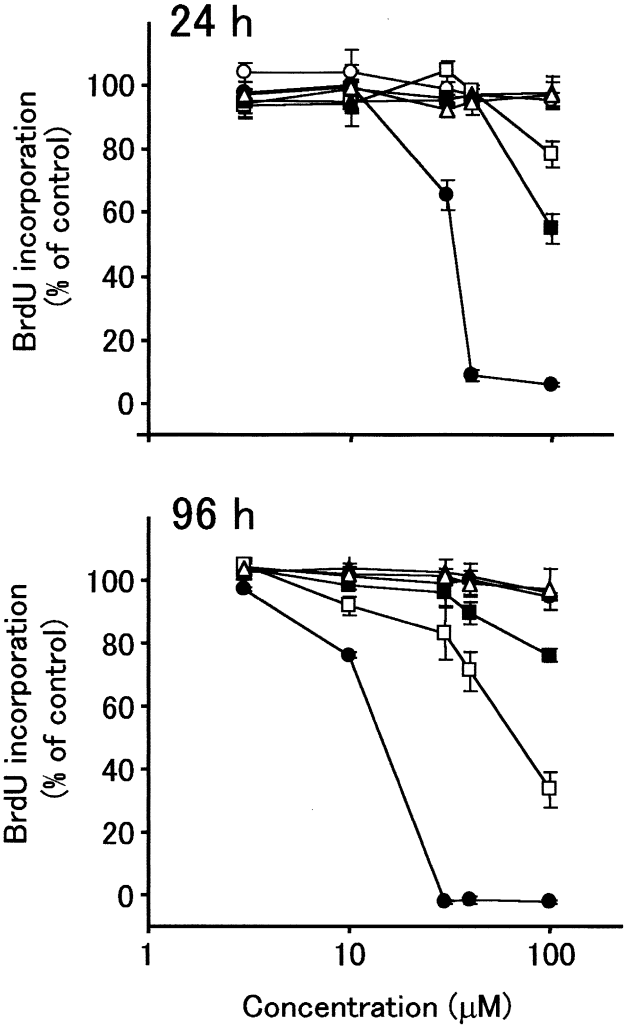

Fig. 1. Effect of various COX-2 inhibitors on the proliferation of HT-29 cells. The cells were treated with celecoxib (●), rofecoxib (○), NS-398 (■), nimesulide (□), meloxicam (▲), or etodolac (△) for 24 h or 96 h. Cell proliferation was estimated from the incorporation of BrdU and is presented as a percentage of the control value. Data are the mean ± S.D. of triplicate cultures and representative results from three independent experiments are shown.

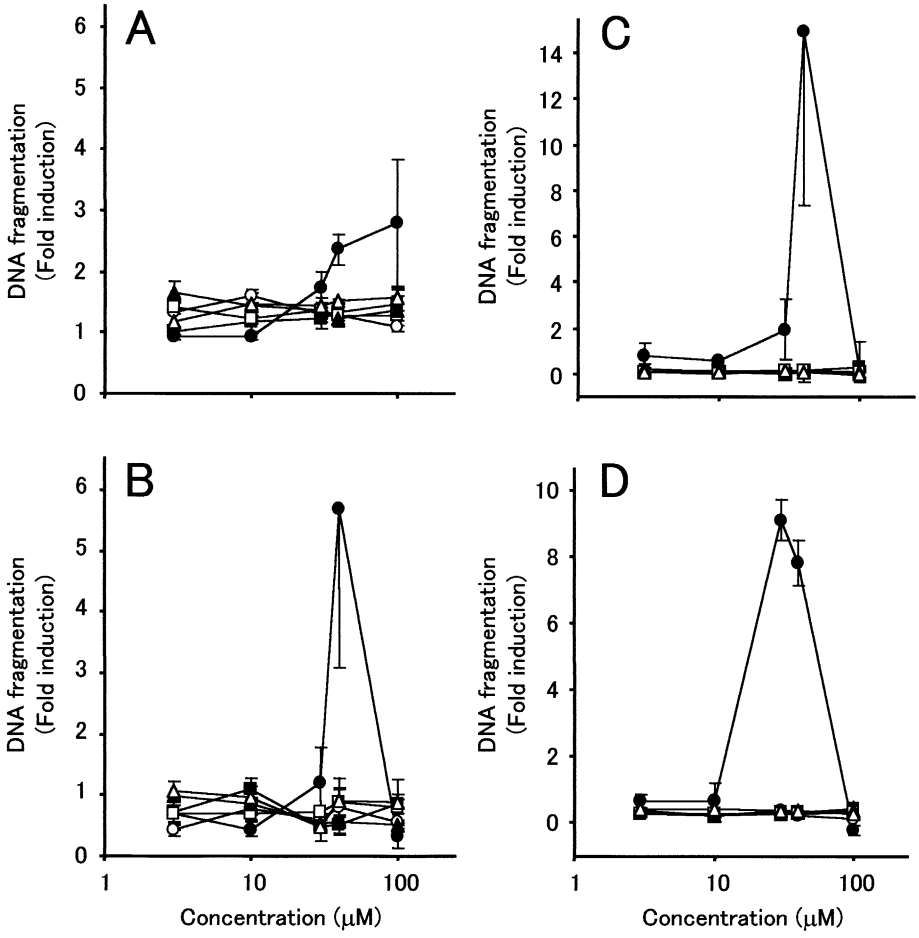

Fig. 2. Effect of various COX-2 inhibitors on DNA fragmentation in colon carcinoma cells. HT-29 cells were treated with celecoxib (●), rofecoxib (○), NS-398 (■), nimesulide (□), meloxicam (▲), or etodolac (△) for 6 h (A) or 24 h (B). SW480 cells (C) or HCT116 cells (D) were treated with celecoxib (●), rofecoxib (○), NS-398 (■), nimesulide (□), meloxicam (▲), or etodolac (△) for 24 h. Then the level of DNA fragments in the cytoplasm was measured by ELISA. The fold induction of DNA fragmentation is shown relative to the value for the control culture. Data are the mean ± S.D. of triplicate cultures and representative results from three independent experiments are shown.

3.2. *Effect of COX-2 inhibitors on apoptosis of colon carcinoma cells*

To determine whether suppression of HT-29 cell proliferation by COX-2 inhibitors was due to the induction of apoptosis, the appearance of DNA fragmentation (a hallmark of apoptosis) after incubation with each inhibitor was quantitatively analyzed by an ELISA that specifically detected cytoplasmic histone-associated DNA fragments, mononucleosomes, and oligonucleosomes. As shown in Fig. 2A, DNA fragmentation was detectable after 6 h of incubation with celecoxib at more than 40 μM. After 24 h, DNA fragmentation was increased. Following exposure to celecoxib at 100 μM, DNA fragments were released into the culture medium, indicating that the cells had undergone secondary necrosis, which usually occurs after apoptosis (Fig. 2B). In contrast, NS-398 or nimesulide (which also inhibited cell proliferation) and rofecoxib, meloxicam, or etodolac (which had no effect on proliferation) did not influence DNA fragmentation at concentrations up to 100 μM after incubation for 6 or 24 h. We also examined the effect of the selective COX-2 inhibitors on DNA fragmentation in two COX-2 non-expressing cell lines, SW-480 [16] and HCT-116 [12]. As shown in Fig. 2C,D, the effect of each COX-2 inhibitor on DNA fragmentation in these cells was very similar to the effect in HT-29 cells.

To confirm the pro-apoptotic effect of incubation with COX-2 inhibitors, the TUNEL assay was used. As shown in Fig. 3, untreated HT-29 cells were not stained by the TUNEL method. In contrast, after incubation for 24 h in the presence of 40 μM celecoxib, 44% of HT-29 cells were TUNEL-positive, indicating the occurrence of apoptosis, whereas cells treated with the other COX-2 inhibitors (100 μM) showed no staining.

3.3. *Effect of COX-2 inhibitors on caspase-3/7 activation in HT-29 cells*

Caspases are responsible for many of the biochemical and morphological changes that occur during apoptosis, so we investigated whether selective COX-2 inhibitors induced the activation of caspase-3 and/or caspase-7 (caspase-3/7) in HT-29 cells. As shown in Fig. 4, incubation of HT-29 cells for 6 h with 40 μM celecoxib induced the activation of caspase-3/7, while activation was completely blocked by incubation with the caspase inhibitor Ac-DEVD-CHO. In contrast, the other selective COX-2 inhibitors (100 μM) did not alter the basal level of caspase-3/7 activity in HT-29 cells.

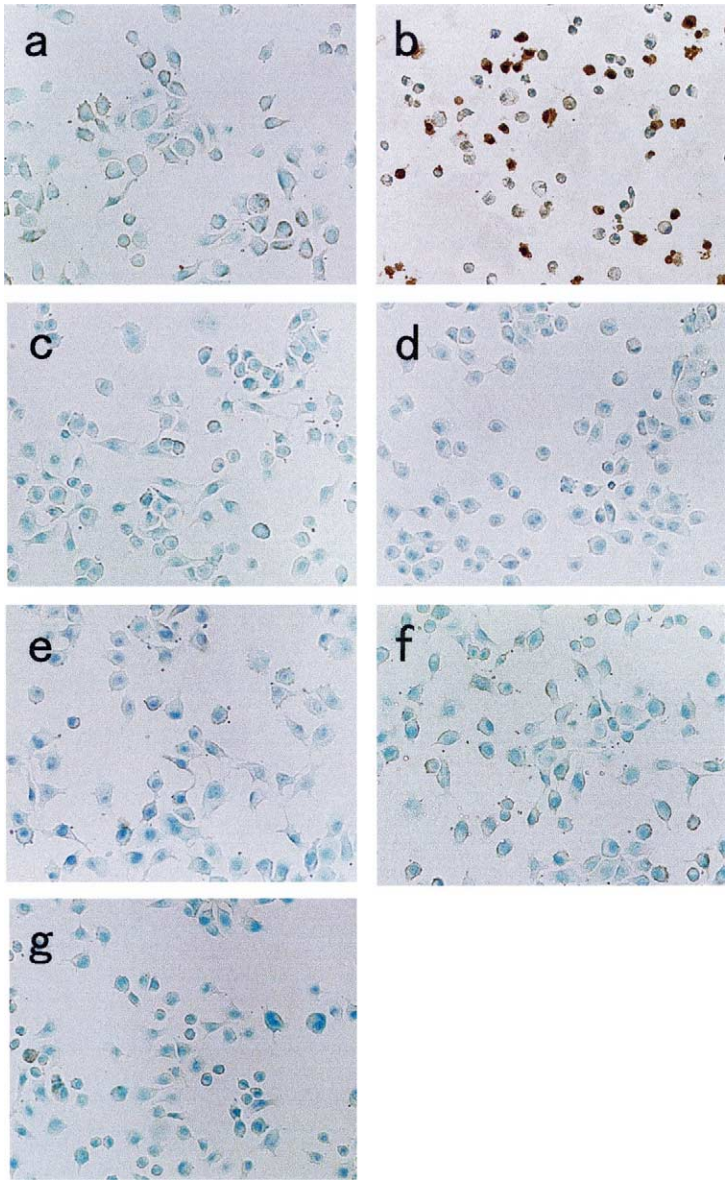

Fig. 3. Detection of apoptotic HT-29 cells by the TUNEL assay. HT-29 cells were cultured without agents (a), or else were incubated with 40 μM celecoxib (b), 100 μM rofecoxib (c), 100 μM NS-398 (d), 100 μM nimesulide (e), 100 μM meloxicam (f), or 100 μM etodolac (g) for 24 h, and then apoptotic cells were identified by the TUNEL assay. The cells were counterstained with methyl green. TUNEL-positive cells (apoptotic cells) are stained brown (magnification: ×50).

3.4. *Effect of selective COX-2 inhibitors on PGE_2 production by HT-29 cells*

To examine the effect of COX-2 inhibitors on PGE_2 production by HT-29 cells, the cells were treated with COX-2 inhibitors for 1 h and then stimulated with A23187. As shown in Fig. 5, all of the COX-2 inhibitors suppressed PGE_2 production in a concentration-dependent manner over the range from 0.001 to 0.1 μM.

3.5. *Effect of COX-2 inhibitors on Akt activation in colon carcinoma cells*

Since serine/threonine kinase Akt (protein kinase B) is a regulator of cell survival and apoptosis and its activation has been shown to protect a variety of cells against apoptosis [21], we examined the effect of the six selective COX-2 inhibitors on Akt activation in two colon carcinoma cell lines. The total amount of phosphorylated Akt was measured after 24 h of incubation with COX-2 inhibitors using an anti-phospho-Akt (Ser^{473}) antibody, because activation of Akt occurs after phosphorylation. As shown in Fig. 6A, 40 μM celecoxib decreased the level of phospho-Akt in HT-29 cells, while rofecoxib did not. When HT-29 cells (Fig. 6B) or SW-480 cells (Fig. 6C) were incubated with 40 μM celecoxib and then stimulated with EGF, suppression of Akt phosphorylation in response to EGF was observed. In contrast, these effects were not seen with other selective COX-2 inhibitors.

In addition, we examined the effect of the six selective COX-2 inhibitors on the expression of Bcl-2, which is linked to inhibition of apoptosis, in HT-29 cells. Western blotting (Fig. 7) showed that Bcl-2 was expressed by HT-29 cells, and little change was seen after treatment with any of the COX-2 inhibitors.

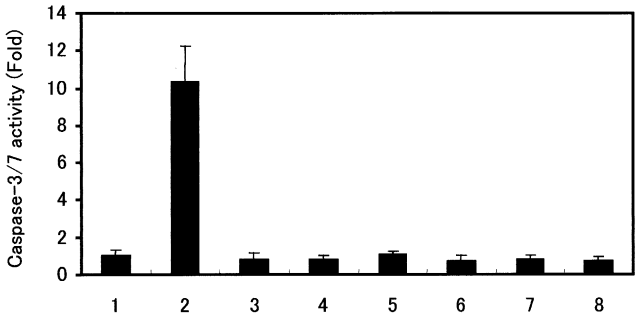

Fig. 4. Effect of various COX-2 inhibitors on caspase-3/7 activation in HT-29 cells. The cells were cultured without agents (bar 1), or else were incubated with 40 μM celecoxib (2), 40 μM celecoxib+10 μM Ac-DEVD-CHO (3), 100 μM rofecoxib (4), 100 μM NS-398 (5), 100 μM nimesulide (6), 100 μM meloxicam (7), or 100 μM etodolac (8) for 6 h, and then caspase activity was measured by the Apo-ONE™ homogeneous caspase-3/7 assay (Promega). The fold changes of caspase activity are shown relative to the value for the control culture. Data are the mean ± S.D. of triplicate cultures and representative results from two independent experiments are shown.

4. Discussion

In this study, we evaluated the effects of six major selective COX-2 inhibitors (celecoxib, rofecoxib, NS-398, nimesulide, meloxicam and etodolac) on the proliferation and apoptosis of colon adenocarcinoma cells. We clearly showed that celecoxib could strongly suppress cell proliferation. Although NS-398 and nimesulide also inhibited the proliferation of HT-29 cells at higher concentrations, rofecoxib, meloxicam and etodolac had no effect. Among these six COX-2 inhibitors, only celecoxib induced the characteristic features of apoptosis, including DNA fragmentation, TUNEL-positivity and caspase-3/7 activation, in colon carcinoma cells (HT-29, SW-480 and HCT-116 cells). It has been reported that NS-398 also induces apoptosis of colon carcinoma cells in vitro [15,18]. In the present study, however, no such pro-apoptotic effect was observed, although NS-398 did inhibit cell proliferation. This discrepancy may be explained by differences of the culture conditions. In previous studies, apoptosis was determined after a relatively long culture period (4 days) when compared with our study (6–24 h). The pro-apoptotic effect of NS-398 on colon carcinoma cells may have been more apparent after 4 days of treatment. In contrast, we found that celecoxib induced apoptosis after only 6 h. Taken together, these results indicate that the pro-apoptotic mechanism of celecoxib may be different from that of other selective COX-2 inhibitors.

HT-29 cells have served as a useful model for examining the mechanisms by which NSAIDs reduce the proliferation of colon cancer cells [9,10,14,15]. HT-29 cells express both COX-1 and COX-2 proteins and mRNAs [14]. Although all six selective COX-2 inhibitors that we tested shared the ability to inhibit PGE_2 production by HT-29 cells, these compounds showed differential suppression of cell proliferation and induction of apoptosis. In addition, much higher doses of the three active agents (celecoxib, NS-398 and nimesulide) were required to achieve inhibition of cell growth than to inhibit PGE_2 production. Moreover, the differential effects of the COX-2 inhibitors on DNA fragmentation in COX-2 non-expressing cell lines (SW-480 [16] cells and HCT-116 [12] cells) were very similar to the effects of these agents on HT-29 cells. These results indicate that inhibition of COX-2 was not associated with the anti-proliferative and pro-apoptotic effects of these COX-2 inhibitors on colon carcinoma cells. However, there seems to be little doubt that COX-2 contributes to tumorigenesis in the colon, because all six COX-2 inhibitors that we tested (celecoxib [6], rofecoxib [4], NS-398 [7], nimesulide [5], meloxicam [22] and etodolac [8]) show an anti-tumor effect in animal models such as chemical colon carcinogenesis. These findings, combined with the present observations, suggest that inhibition of cell proliferation and induction of apoptosis do not fully explain the mechanism by which selective COX-2 inhibitors exert anti-tumor activity in vivo. It is possible that indirect mechanisms, such as an anti-angiogenetic action [23,24], may be more important than the direct effects of such agents on colon cancer cells in vivo.

It is also possible that some selective COX-2 inhibitors induce apoptosis of colon carcinoma cells via targets that are not necessarily related to COX-2 inhibitory activity. In this respect, the observation that some NSAIDs act as a direct ligand for peroxisome proliferator-activated receptor γ (PPARγ) is of interest [25]. We recently found that various NSAIDs, such as indomethacin, diclofenac, oxaprozin and zaltoprofen, induced the apoptosis of rheumatoid synovial fibroblasts in association with PPARγ activation [26]. Furthermore, recent studies have indicated that PPARγ is expressed by colon cancer cells, including HT-29 cells [27,28], and a selective PPARγ ligand (troglitazone) causes growth inhibition and induces apoptosis in HT-29 cells [28]. However, we found that celecoxib and NS-398 did not induce transcriptional activation of PPARγ

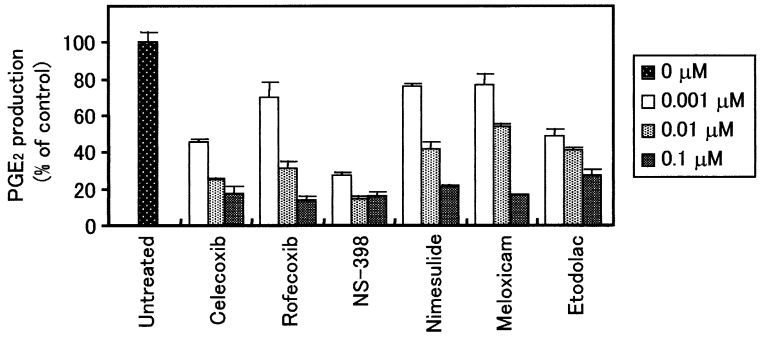

Fig. 5. Effect of various COX-2 inhibitors on PGE_2 production by HT-29 cells. The cells were preincubated with the test drugs for 1 h at 37°C, and then 0.5 μM calcium ionophore A23187 was added. After incubation for another 30 min, the PGE_2 level in the culture medium was measured by enzyme immunoassay. Data are the mean ± S.D. of triplicate cultures. The PGE_2 level in the control culture was 1.40 ± 0.08 ng/ml. Representative results of three independent experiments are shown.

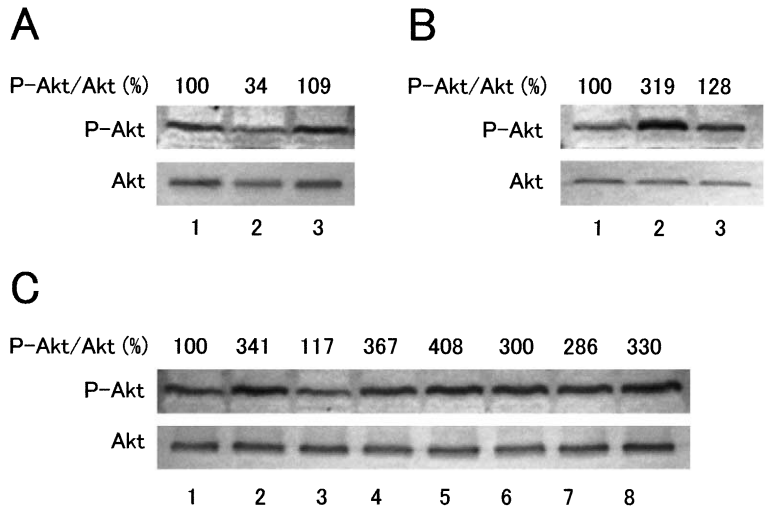

Fig. 6. Effects of COX-2 inhibitors on Akt activation in colon carcinoma cells. A: HT-29 cells were cultured without agents (lane 1), or else were incubated with 40 μM celecoxib (2) or 100 μM rofecoxib (3) for 24 h. HT-29 cells (B) or SW480 cells (C) were incubated in serum-free medium for 18 h. The cells were cultured without agents (lanes 1 and 2), or else were incubated with 40 μM celecoxib (3), 100 μM rofecoxib (4), 100 μM NS-398 (5), 100 μM nimesulide (6), 100 μM meloxicam (7), or 100 μM etodolac (8) for 3 h, and then co-incubated with the drug and 100 ng/ml EGF (2–8) for 10 min. Protein extracts prepared from the cells were subjected to Western blotting using antiserum that detected Akt or phospho-Akt, as described in Materials and methods. The amount of phospho-Akt (P-Akt) was semi-quantified by densitometric analysis and was calculated relative to that in control cells after normalizing for the amount of Akt.

in a luciferase reporter assay using a peroxisome proliferator response element-driven luciferase reporter plasmid (data not shown).

The serine/threonine kinase Akt (protein kinase B) is a regulator of cell survival and apoptosis, and its activation has been shown to protect a variety of cells against apoptosis [21]. Recently, celecoxib was reported to induce apoptosis by blocking the activation of anti-apoptotic Akt in prostate cancer cells via an action that was independent of Bcl-2 [29]. Since the optimum concentration of celecoxib and the incubation time for induction of apoptosis in colon carcinoma cells in the present study were similar to those reported for prostate cancer cells, the effect of celecoxib on colon carcinoma cells may also involve inhibition of Akt activation. In our study, only celecoxib among six selective COX-2 inhibitors decreased the level of phospho-Akt in HT-29 cells and SW-480 cells by suppressing its phosphorylation. This mechanism may explain the unique pro-apoptotic activity of celecoxib and the differential pro-apoptotic effects of various selective COX-2 inhibitors on colon carcinoma cells.

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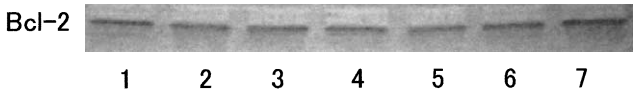

Fig. 7. Effect of selective COX-2 inhibitors on Bcl-2 expression by HT-29 cells. HT-29 cells were cultured without agents (lane 1), or else were incubated with 40 μM celecoxib (2), 100 μM rofecoxib (3), 100 μM NS-398 (4), 100 μM nimesulide (5), 100 μM meloxicam (6), or 100 μM etodolac (7) for 6 h. Protein extracts prepared from the cells were subjected to Western blotting using antiserum that detected Bcl-2, as described in Materials and methods.

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