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Prolonged activation of mitogen-activated protein kinases during NSAID-induced apoptosis in HT-29 colon cancer cells

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Abstract The mechanisms of the antineoplastic effect of nonsteroidal anti-inflammatory drugs (NSAIDs) still are unknown, but the induction of apoptosis is one of the possible mechanisms. We attempted to demonstrate the role of mitogen-activated protein (MAP) kinases, generally considered to be important mediators of proliferative and apoptotic signals, in NSAID-induced colon cancer cell apoptosis. Apoptosis was detected by demonstration of DNA fragmentation in agarose gel electrophoresis. Cell death was assessed by trypan blue dye exclusion method. MAP kinase activation was assessed by Western blot using phosphospecific antibodies to MAP kinases. Kinase assay using activating transcription factor-2 (ATF-2) fusion protein as a substrate was also performed for measuring p38 MAP kinase activity. For the inhibition of p38 MAP kinase, pyridinylimidazole compound (SB203580) was utilized. Caspase-3 activity was measured using the tetrapeptide fluorogenic substrate Ac-DEVD-AMC. Treatment of HT-29 cells with NSAIDs results in time- and dose-dependent induction of apoptosis, accompanied by sustained activation of all three MAP kinase subfamilies. The SB203580, a p38 MAP kinase inhibitor, reduced indomethacin-induced cell death by 43%, while PD098059, a MAPK/ERK kinase (MEK)1 inhibitor, did not affect cell death. p38 MAP kinase and caspase-3 activation were not significantly interlinked in indomethacin-induced apoptosis. From these results, we conclude that NSAIDs can induce prolonged activation of MAP kinases in colon cancer cells and that, of these, p38 MAP kinase may play a partial but significant role in indomethacin-induced apoptosis.

Keywords Nonsteroidal anti-inflammatory drugs (NSAIDs) · Colon cancer · Apoptosis · Mitogen-activated protein (MAP) kinases · p38 MAP kinase

Introduction

It has been demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce the occurrence of colorectal adenoma and carcinoma [1, 2, 3] and inhibit colonic carcinogenesis in epidemiological, clinical, and animal studies [4, 5, 6]. It remains unclear how NSAIDs exert their antineoplastic effects, but apoptotic induction is one of the possible mechanisms [7, 8, 9, 10]. Some reports have been published on the molecular mechanism of NSAID-induced apoptosis, e.g., caspase-3 activation in leukemia cells [11], ceramide pathway activation in colon cancer cells [12], and p38 mitogen-activated protein (MAP) kinase activation in fibroblasts [13, 14]. We have also reported on the critical role of caspase-3 activation in NSAID-induced colon cancer cell apoptosis [15].

MAP kinases are serine-threonine kinases with important functions as mediators of cellular responses to a variety of extracellular stimuli. Three major subfamilies

of structurally related MAP kinases have been identified in mammalian cells: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinases/stress-activated protein kinase (JNK/SAPK), and the p38 MAP kinase. MAP kinase subfamilies phosphorylate substrate at serine and threonine residues located adjacent to a proline residue, and members of all MAP kinases are activated as a result of the simultaneous phosphorylation of threonine and tyrosine residues by upstream dual-specificity kinases such as MAP kinase kinases and MAP kinase kinase kinases. ERK are characteristically activated by various growth factors and associated with cell proliferation, differentiation, and protection from apoptosis. In contrast, JNK/SAPK and p38 MAP kinase are strongly activated in response to a variety of cellular stresses such as ultraviolet (UV) irradiation, heat shock, hyperosmolarity, chemotherapeutic agents, and proinflammatory cytokines and are involved in growth arrest and the induction of apoptosis [16, 17, 18, 19]. Although many functions of ERK, JNK/SAPK, and p38 MAP kinase have been identified, the specificity of activating stimuli for these three subfamilies of MAP kinases is not absolute, and much of the ambiguity in their roles could be due to the different roles of each pathway in various cell types responding to different stimuli or due to the activation states of numerous other linked pathways in the cell.

While many reports exist about JNK/SAPK or p38 MAP kinase activation in apoptosis induced by various stimuli, the role of MAP kinases in NSAID-induced colon cancer cell apoptosis has not been investigated. Therefore, we tried to answer the questions of whether MAP kinases are activated during NSAID-induced colon cancer cell apoptosis and, if this proved to be the case, identifying the role of such activation.

Materials and methods

Cell culture

The human colon adenocarcinoma cell line, HT-29 (ATCC HTB38), was obtained from the American Type Culture Collection (Rockville, Md., USA). HT-29 cells were grown in RPMI Medium 1640 (Gibco-BRL, Gaithersburg, Md., USA), supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Treatment of HT-29 cells with NSAIDs, specific MAP kinase inhibitors, and caspase inhibitors

HT-29 cells were treated with NSAIDs such as indomethacin (Sigma, St. Louis, Mo., USA), sulindac (Sigma), and nabumetone (Handok, Seoul, Korea). In some experiments, HT-29 cells were treated with specific p38 MAP kinase inhibitor, SB203580 (Calbiochem, La Jolla, Calif., USA), MEK1 inhibitor, PD098059 (Calbiochem), or caspase inhibitors such as acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO), and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK; Calbiochem) for 30 min before exposure to indomethacin and during the incubation period of the experiment.

Western blot analysis for the measurement of MAP kinase phosphorylation

For analysis of phosphospecific MAP kinase activation, NSAID-treated HT-29 cells were harvested and lysed in buffer containing 50 mM tris-HCl (pH 7.4), 1 mM ethylenediaminetetra-acetate (EDTA), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 µg/ml leupeptin, and 20 µg/ml pepstatin. The lysates were incubated on ice for 30 min and centrifuged at 12,000 rpm for 20 min. Extracted proteins were heated to 100°C for 5 min and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, Mass., USA). The membrane was blocked with 5% nonfat milk in TBST (10 mM tris [pH 7.4], 100 mM NaCl, 0.5% Tween 20) and then probed for 1 h with 1:1000 phosphospecific or nonphosphospecific MAP kinase antibodies and for 1 h with 1:2000 horseradish peroxidase (HRP)-conjugated antirabbit IgG (Amersham, Arlington Heights, Ill., USA). The Amersham enhanced chemiluminescence (ECL) nonradioactive method was used for detection.

All three phosphospecific MAP kinase antibodies to ERK, JNK/SAPK, and p38 MAP kinase were obtained from New England Biolabs (Beverly, Mass., USA) and nonphosphospecific antibodies to ERK, JNK/SAPK, and p38 MAP kinase were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

Detection of DNA fragmentation by agarose gel electrophoresis

Apoptosis of HT-29 cells was determined by detection of oligonucleosomal DNA fragmentation with agarose gel electrophoresis. For agarose gel electrophoresis of genomic DNA, cells were lysed at 42°C overnight in a lysis buffer containing 10 mM tris (pH 7.6), 10 mM EDTA, 50 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), and 200 µg/ml proteinase K and then centrifuged at 4°C, 16,000 *g* for 20 min. DNA was extracted from supernatant using phenol-chloroform-isoamyl alcohol (25:24:1). The extracted DNA was precipitated in ethanol containing 0.3 M NaOAc and nucleic acid precipitant was dissolved in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) containing 100 µg/ml RNase A at 37°C for 30 min. DNA samples were loaded onto 2% agarose gel for electrophoresis and then visualized with ethidium bromide. Cell survival and death were also assessed by trypan blue dye exclusion.

Assay of p38 MAP kinase activity (in vitro kinase assay)

The p38 MAP kinase assay kit was obtained from New England Biolabs. As described in the manufacturer's instructions, the indomethacin-treated HT-29 cells were lysed and p38 MAP kinase was immunoprecipitated. Then, its catalytic activity to phosphorylate recombinant activating transcription factor-2 (ATF-2) was determined using the in vitro kinase assay, and the reaction mixture was separated by SDS polyacrylamide gel electrophoresis (-PAGE). The transferred membrane was probed with the phospho-ATF2 antibody provided by the kit, and immunoreactivity was detected by ECL.

Caspase-3-like protease activity assay

HT-29 cells were treated with indomethacin for 4 h in the presence or absence of the specific p38 MAP kinase inhibitor SB203580 or

caspase inhibitors such as Ac-DEVD-CHO, Ac-YVAD-CHO, and Z-VAD-FMK. The cells were then harvested in lysis buffer (10 mM Tris [pH 7.5], 10 mM NaH_2PO_4/Na_2HPO_4 [pH 7.5], 130 mM NaCl, 1% Triton X-100, and 10 mM NaPPi) and allowed to stay on ice for 30 min. The lysates were clarified by centrifugation, and the supernatants were used for caspase assays. Caspase activity was determined by fluorogenic assay as described previously [15]. Briefly, 300 µg of protein in lysate was incubated with 2 µg/ml fluorogenic peptide substrate, Ac-DEVD-AMC. Following incubation at 37°C for 1 h, phosphate-buffered saline (PBS) was added up to 500 µl of final volume and then the release of AMC was measured with excitation at 380 nm and emission at 450 nm using an SPF-500CTM spectofluorometer (SLM, Urbana, Ill., USA).

Statistical analysis

Data are expressed as mean±SD. The Mann-Whitney U test was used to examine the statistical difference between control and treatment groups. Statistical significance was defined as $P<0.05$.

Results

NSAIDs induced prolonged activation of MAP kinases (ERK, JNK/SAPK, and p38 MAP kinase) in HT-29 colon cancer cells. To investigate whether these MAP kinases are involved in NSAID-induced apoptosis, we first analyzed the activation of MAP kinases in NSAID-treated HT-29 colon cancer cells by Western blot analysis using phosphospecific antibodies to ERK, JNK/SAPK and p38 MAP kinase. Control cells showed low or undetectable levels of phosphorylated MAP kinases (Fig. 1). The phosphorylation of all three MAP kinases was observed at 0.1 mM of indomethacin and increased in a dose-dependent manner (Fig. 1A). Indomethacin-induced phosphorylation of all three MAP kinases became evident after only 10 min and they remained in phosphorylated form for at least 240 min (Fig. 1B).

We also confirmed the activation of p38 MAP kinase by an in vitro kinase assay using ATF-2 as a substrate. As shown in Fig. 2, the phosphorylation of ATF-2 became obvious at 0.1 mM and increased in a dose-dependent manner similar to the phosphorylation pattern of p38 MAP kinase.

To determine whether p38 MAP kinase activation is unique to indomethacin, we also investigated the effects of other NSAIDs such as nonselective cyclo-oxygenase (COX) (sulindac) and selective COX-2 (nabumetone) inhibitors on the phosphorylation of p38 MAP kinase in HT-29 cells. Both induced the phosphorylation of p38 MAP kinase in a dose-dependent manner without changes in the total amounts of p38 MAP kinase, which was similar to the effect of indomethacin (Fig. 3).

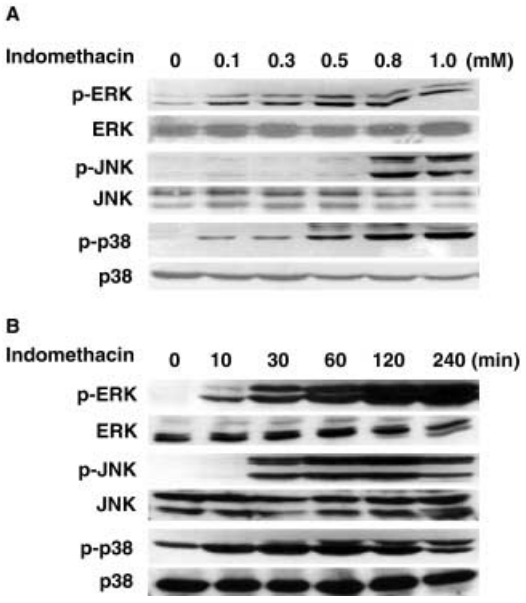

Fig. 1A,B Phosphorylation of ERK, JNK/SAPK, and p38 MAP kinase in indomethacin-treated HT-29 colon cancer cells. **A** HT-29 cells cultured alone or with varying concentrations of indomethacin (0.1–1.0 mM). After 1 h, cell lysates were prepared and subjected to Western blot analysis using phosphospecific antibodies against ERK, JNK/SAPK and p38 MAP kinase (*upper panels*) and control antibodies that recognize these kinases regardless of their phosphorylation status (*lower panels*). **B** HT-29 cells treated with indomethacin (1.0 mM). Cell lysates were prepared and subjected to Western blot analysis at varying time points

Fig. 2 Indomethacin-induced activation of p38 MAP kinase. HT-29 cells were treated with varying concentrations of indomethacin (0–1.0 mM). After 1 h, cell lysates were prepared and p38 MAP kinase activity was measured by immunoprecipitation and in vitro kinase assay using activating transcription factor-2 (ATF-2) fusion protein as a substrate

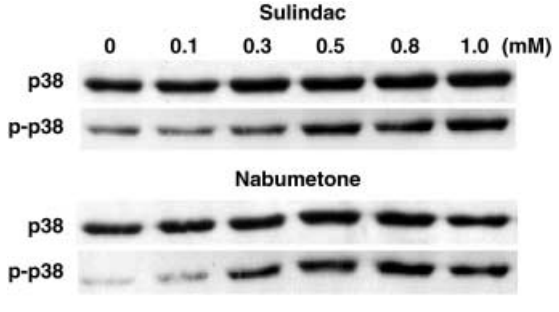

Fig. 3 Phosphorylation of p38 MAP kinase in sulindac- or nabumetone-treated HT-29 cells. After 1 h, cell lysates were prepared and subjected to Western blot analysis using a phosphospecific antibody against p38 MAP kinase (*lower panels*) and a control antibody that recognizes p38 MAP kinase regardless of their phosphorylation status (*upper panels*)

SB203580, a specific inhibitor of p38 MAP kinase, partially inhibited NSAID-induced apoptosis

We considered the possibility that p38 MAP kinase activation might be responsible for the apoptotic action of NSAIDs. To find out if the activation of p38 MAP kinase is required for NSAID-induced apoptosis, we determined whether the specific p38 MAP kinase inhibitor, SB-203580 [20], affects indomethacin-induced apoptosis. Pretreatment with SB-203580, which alone has no effect on cell viability, significantly reduced the cell death ratio in a dose-dependent manner in indomethacin-treated HT-29 cells, by 30% and 43% at 0.5 μM and 5.0 μM of SB203580, respectively (Fig. 4A). In addition, pretreatment with SB-203580 also inhibited the degree of DNA fragmentation dose-dependently (Fig. 4B), which demonstrated that the specific inhibitor of p38 MAP kinase significantly suppressed indomethacin-induced apoptosis, but not completely. A higher dose of SB-203580 (10 μM) did not increase the suppression of cell death. Thus, activation of p38 MAP kinase appears to be necessary, in part, for indomethacin-induced apoptosis.

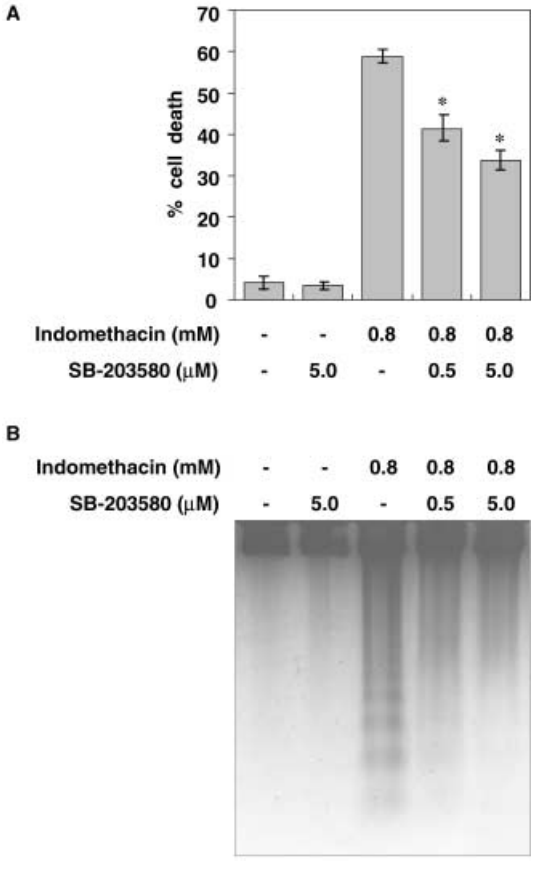

Fig. 4A,B Partial inhibition of indomethacin-induced apoptosis by p38 MAP kinase inhibitor, SB203580. **A** HT-29 cells were exposed to 0.8 mM indomethacin for 24 h with or without pretreatment of 0.5 μM or 5.0 μM SB203580 for 30 min, and cell viability was assessed by the trypan blue dye exclusion method. Data shown represent the mean±SD of three independent experiments. *Statistical significance (P<0.05) compared to indomethacin-treated HT-29 cells without pretreatment. **B** HT-29 cells were pretreated with 0.5 μM and 5.0 μM SB203580 for 30 min and then exposed to 0.8 mM indomethacin for 24 h. DNA fragmentation was detected by agarose gel electrophoresis

p38 MAP kinase and caspase-3 activation were not significantly interlinked in NSAID-induced apoptosis

We reported previously that caspase-3 plays an important role for NSAID-induced apoptosis in the colon cancer cell line, HT-29 [15] and that p38 MAP kinase activation is partially responsible for the apoptotic action of indomethacin. In view of these findings, we tried to determine whether p38 MAP kinase activation was part of the upstream or downstream signaling pathway associated with capase-3 activation. HT-29 cells were pretreated for 30 min with the specific p38 MAP kinase inhibitor, SB203580 (10 μM), or three kinds of caspase inhibitors (10 μM) with different specificities (Ac-DEVD-CHO, Ac-YVAD-CHO, and Z-VAD-FMK) and then treated with indomethacin (0.8 mM) for 4 h. Caspase-3-like protease activity was measured by fluorogenic assay. As shown in Fig. 5, caspase inhibitors significantly blocked caspase-3 activation. However, p38 MAP kinase inhibitor, SB203580, failed to inhibit caspase-3 activation.

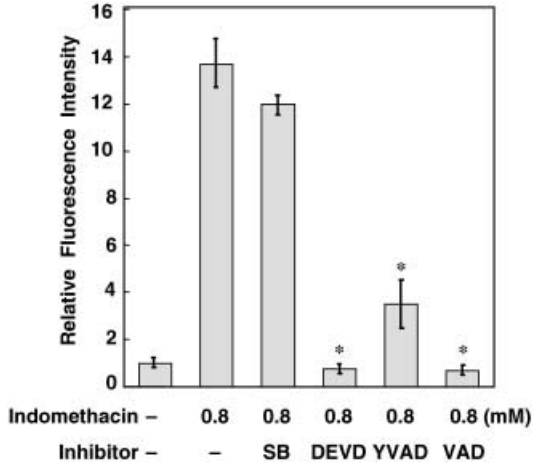

Fig. 5 Effect of p38 MAP kinase inhibitor, SB203580, on caspase-3 activity. HT-29 cells were treated with indomethacin (0.8 mM) for 4 h in the presence or absence of specific p38 MAP kinase inhibitor, SB203580 (10 μM), or caspase inhibitors (10 μM), and caspase-3-like protease activity was determined by fluorogenic assay. Relative fluorescence intensities were calculated with respect to the fluorescence intensity of control HT-29 cells. Data shown represent the mean±SD of three independent experiments. *Statistical significance (P<0.05) compared to indomethacin-treated HT-29 cells without inhibitor pretreatment

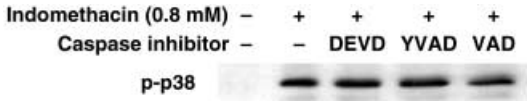

Fig. 6 The effect of caspase inhibitors on indomethacin-induced p38 MAP kinase activation. HT-29 cells were treated with indomethacin (0.8 mM) for 1 h in the presence or absence of caspase inhibitors (10 μM), and p38 MAP kinase activity was determined by Western blot using phosphospecific antibody against p38 MAP kinase

To examine the effect of caspase activation on p38 MAP kinase phosphorylation, HT-29 cells were exposed to indomethacin (0.8 mM) for 1 h with or without the pretreatment of three kinds of caspase inhibitors (10 μM), Ac-DEVD-CHO, Ac-YVAD-CHO, and Z-VAD-FMK, for 30 min, and the p38 MAP kinase phosphorylation status was determined by Western blot. Pretreatment with caspase inhibitors did not affect p38 MAP kinase phosphorylation in indomethacin-treated HT-29 cells (Fig. 6). These results suggest that while both caspase-3 and p38 MAP kinase activation have a clear role in the indomethacin-induced apoptosis of HT-29 cells individually, they are not significantly linked to each other.

Discussion

Epidemiological, clinical, and animal studies have shown that NSAIDs have an antineoplastic effect. The mechanisms responsible for these remarkable antineoplastic effects are not completely understood, but emerging data indicate that NSAIDs are likely to target several steps along the carcinogenesis pathway. These include the NSAID-induced inhibition of cell turnover (such as the induction of apoptosis and inhibition of cell proliferation), the stimulation of immune surveillance, and the antiangiogenic effect [7, 8, 9, 21, 22, 23].

These effects are commonly attributed to the inhibition of COX-2 [24], since eicosanoids increase proliferation rates in colon cancer cell lines [25] and the levels of prostaglandins and COX-2 gene expression are higher in colon cancer tissue than in normal colonic mucosal tissue [26, 27]. In addition, the overexpression of COX-2 in epithelial cells inhibits apoptosis [28, 29]. However, some NSAIDs were shown to induce apoptosis in colon cancer cells by a mechanism that may be unrelated to the ability of these drugs to inhibit prostaglandin synthesis [8, 30, 31, 32], as the addition of exogenous prostaglandins to cultures failed to offset the antineoplastic and antiangiogenic effects of NSAIDs [13, 23, 33], and sulindac sulfone, the oxidized metabolite of sulindac that inhibits neither COX isoform, also inhibits colon cancer cell growth and angiogenesis [31, 34]. Therefore, their chemopreventive effects are also believed to involve COX-independent pathways.

NSAIDs alter cell cycle distribution and induce apoptosis in colonic polyps and colon cancer cell lines [7, 8, 9, 12]. Salicylate and sulindac prevent cell cycle progression from G0/G1 into the S phase [30, 32], which is associated with an alteration in the levels of major components of the molecular cell cycle machinery and several tumor cell suppressor proteins [9, 32, 35]. The induction of apoptosis was recently considered to be its main antineoplastic action mechanism. However, very little is known about the precise molecular mechanism that regulates NSAID-induced apoptosis.

In earlier studies, we demonstrated that indomethacin induces apoptosis and caspase-3 activation in HT-29 colon cancer cells and that indomethacin-induced apoptosis is attenuated by caspase-3 inhibitor (Ac-DEVD-CHO), suggesting that caspase-3 plays an important role in indomethacin-induced colon cancer cell apoptosis [9, 15]. Recently, MAP kinase subfamilies were considered as important mediators of apoptotic signals in many systems. Therefore, we studied the involvement of MAP kinases to elucidate the signal transduction pathway leading to apoptosis in NSAID-treated colon cancer cells. Schwenger et al. showed that sodium salicylate induces apoptosis via p38 kinase activation but inhibits TNF-induced ERK and JNK/SAPK activation in normal human FS-4 fibroblasts [13]. In addition, Jones et al. demonstrated that indomethacin and NS-398 inhibit ERK2 activity in endothelial cells, which is related to the inhibition of angiogenesis [23]. However, the role of MAP kinase activation in NSAID-induced colon cancer cell apoptosis has not been investigated.

Our present results show that indomethacin induces the prolonged activation of all three MAP kinase subfamilies (ERK, JNK/SAPK, and p38 MAP kinase) in a time- and dose-dependent manner. It has been recognized in many systems that the activations of the JNK/SAPK or the p38 MAP kinase pathway are involved in stress stimuli-induced apoptosis, while in some studies, ERK were shown to protect cells from apoptosis [17, 19, 36]. Although many functions of ERK, JNK/SAPK, and p38 MAP kinase have been identified, the specificity of activating stimuli for the specific subfamilies of MAP kinase is not absolute, and it is difficult to generalize about their functions. Therefore, the discrepancy between ERK activation in our present study and other studies could be due to different roles of the ERK pathway in various cell types in response to different stimuli or due to the activation states of numerous other linked pathways in the cell [10, 37]. This discrepancy in ERK activation should be resolved by further study. Although ERK was activated in our present study, the MEK1 inhibitor PD098059 (10 μM, 30 min pretreatment) had no significant effect on indomethacin-induced colon cancer cell apoptosis (data not shown), which suggests that ERK activation does not play an important role in indomethacin-induced apoptosis.

While it is known that JNK/SAPK is related to apoptotic signals in many systems, it was also found to induce cell proliferation and differentiation [38, 39]. This ambiguity could be due to differences between activated up- or downstream pathways or activated isoforms of JNK/SAPK. Unfortunately, its role in indomethacin-induced apoptosis could not be evaluated because a suitable JNK inhibitor is not available.

In our present study, indomethacin treatment in HT-29 colon cancer cells induced apoptosis that was partially prevented by the selective p38 MAP kinase inhibitor, SB-203580, suggesting a partial but significant role for p38 MAP kinase activation in indomethacin-induced apoptosis. This is in agreement with a report which demonstrated an essential role of p38 MAP kinase in sodium salicylate-induced FS-4 fibroblast apoptosis [13].

We next investigated whether p38 MAP kinase activation is linked with caspase-3, a common executor of apoptosis in many systems. However, our experiment using p38 MAP kinase inhibitor and caspase inhibitors demonstrated that while both caspase-3 and p38 MAP kinase by themselves have a clear role in indomethacin-induced HT-29 cell apoptosis, their actions are substantially independent and they are not significantly linked.

In conclusion, our results suggest that various NSAIDs, irrespective of their COX selectivity, can induce prolonged activation of MAP kinases including ERK, JNK/SAPK, and p38 MAP kinase, and the activation of p38 MAP kinase has a partial but significant role in indomethacin-induced colon cancer cell apoptosis. As another possible mechanism of NSAID-induced colon cancer cell apoptosis, NSAID-induced ceramide generation from sphingomyelin has been reported [12], and many apoptotic stimuli have been shown to induce ceramide generation as a general secondary mediator of apoptosis, which is related to MAP kinase activation, caspase activation, or other signal pathways involved in apoptosis [12, 40]. Therefore, it will be interesting to determine how the activation of MAP kinases is related to the ceramide pathway and how the inhibition of JNK/SAPK activation affects NSAID-induced colon cancer cell apoptosis.

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