

Induction of Apoptosis in Colon Cancer Cells by Nonsteroidal Anti-Inflammatory Drugs

Sung Pyo Hong¹, Sung Ho Ha², In Suh Park²,
and Won Ho Kim²

Epidemiological studies have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) decrease the incidence of colon cancer. In addition, NSAIDs reduce the number and size of polyps in patients with familial adenomatous polyposis. The mechanisms of the anti-neoplastic effect of NSAIDs are still far from complete understanding, but one possible mechanism is the induction of apoptosis. Several lines of evidence suggest that NSAIDs-induced apoptosis in colon cancer cells are mediated through the cyclooxygenase (COX)-independent pathway. In this study we explored the mechanism of NSAIDs-induced apoptosis in the colon cancer cell line, HT-29. We confirmed that NSAIDs induce apoptosis in HT-29 cells irrespective of their COX-selectivity. Indomethacin enhanced the expression of p21^{waf-1} in HT-29 cells. However the expression of apoptosis-related genes such as Fas, bcl-2 and bax was not affected by indomethacin. Intra- and extra-cellular calcium chelators, protein tyrosine kinase (PTK) inhibitor, protein kinase A (PKA) inhibitor and protein kinase C (PKC) inhibitors did not influence indomethacin-induced apoptosis in HT-29 cells. We concluded that NSAIDs-induced apoptosis in colon cancer cells may be independent from signals transduced through [Ca⁺⁺]_i, PTK, PKA, PKC or the expression of apoptosis-related genes. In contrast, our results demonstrating the induction of p21^{waf-1} transcription by NSAIDs suggest the possible association of NSAIDs-induced apoptosis and cell-cycle control in colon cancer cells.

Key Words: NSAIDs, colon cancer, apoptosis, cell cycle, P21^{WAF}

Epidemiological studies have indicated that non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, indomethacin and sulindac can reduce the incidence of and mortality from colon cancer (Rosenberg *et al.* 1991; Thun *et al.* 1991; Greenberg *et al.* 1993). The mechanism responsible for the anti-neoplastic effects of NSAIDs is still unknown. It is

commonly attributed to the inhibition of cyclooxygenase (COX), since eicosanoids increase the proliferation rate in colon cancer cell lines (Qiao *et al.* 1995) and the levels of prostaglandins and COX-2 gene expression are elevated in colon cancer tissue compared with normal colonic mucosal tissue (Bennett *et al.* 1987; Rigas *et al.* 1993; Eberhart *et al.* 1994). However, several lines of evidence suggest a COX-independent mechanism of NSAIDs' anti-neoplastic effect. Replacement of prostaglandins fails to reverse the anti-neoplastic effect of NSAIDs (Hanif *et al.* 1996). Sulindac is a prodrug that is converted in vivo into active metabolites, sulfide and sulfone. Both metabolites inhibit colon cancer cell growth (Ahnen *et al.* 1995) although sulindac sulfide inhibits prostaglandin synthesis but sulfone does not. Finally, NSAIDs induce apoptosis in HCT-15 cells

Received January 7, 1998

Accepted July 21, 1998

¹Department of Internal Medicine, Pundang Cha Hospital, Pochon Cha Medical University, Sunnam, and
²Department of Gastroenterology, Yonsei University College of Medicine, Seoul, Korea

This study was supported by a KOSEF for 1996 (No.: 96-0403-15-01-3).

Address reprint request to Dr. W.H. Kim, Department of Internal Medicine, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea

which lack COX transcripts (Hanif *et al.* 1996).

NSAIDs, including sulindac, alter cell cycle distribution and induce apoptosis in familial adenomatous polyposis (Pasricha *et al.* 1995) and in HT-29 colon cancer cell lines (Shiff *et al.* 1995). Apoptosis represents a physiological cell death that has evolved in multicellular organisms to remodel tissue during development, maintain tissue homeostasis, remove senescent cells, and delete cells with genetic damage. Apoptosis is defined by distinct morphological and biochemical features, such as cell shrinkage, detachment from neighboring cells, chromatin condensation and fragmentation of nuclei as well as DNA. A variety of signaling pathways can initiate apoptosis, including growth factor withdrawal, metabolic or cell cycle perturbations, alterations of extracellular matrix, DNA damage, pathogens, toxins, oxidative stress, nitric oxide, activation of specific cell death receptors, and immunologic mediated processes. Various genes have been implicated in the induction or inhibition of apoptosis. Genes upregulated in apoptosis and whose gene products are thought to cause apoptosis are death genes, while genes whose expression inhibits apoptosis and promotes cell proliferation are proto-oncogenes. Despite rapid progress in understanding the regulation and mechanisms of apoptosis, very little is known about the critical intracellular biochemical pathways that mediate apoptosis. Most studies focusing on apoptosis-related signal transduction pathways have been conducted using immune cells as a target system.

In this study, we have tried to explore the mechanisms of NSAIDs-induced apoptosis of HT-29 cells such as COX-selectivity of NSAIDs, the effect of NSAIDs on the expression of apoptosis-related genes and finally the role of signal transduction pathways including $[Ca^{++}]_i$, PTK, PKA, PKC on NSAIDs-induced apoptosis.

MATERIALS AND METHODS

Materials

Cell lines: The human colon adenocarcinoma cell line HT-29 (ATCC HTB38) was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). HT-29 cells were incubated at

37°C, in 5% CO₂ with a medium consisting of Dulbecco's modified eagle medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 2 μ mol/L glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 10% heat-inactivated fetal calf serum.

Reagents: Indomethacin (Sigma, St. Louis, MO, USA), sulindac (Sigma), and nabumetone (Smith-Kline Beecham, Handok remedial industrial Co. Seoul) were used as NSAIDs. Propidium iodide (PI), DAPI (4',6'-diamidine-2'-phenylindole dihydrochloride), EGTA [ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid], staurosporine, and MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue] solution were obtained from Sigma and BAPTA/AM [1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid], herbimycin A, H89, H7 [1-(5-isoquinoline sulfonyl)-2-methyl-piperazine dihydrochloride] were purchased from Calbiochem (San Diego, CA, USA).

Methods

Detection of apoptosis: Apoptosis of HT-29 cells were evaluated by four different methods; (a) morphologic evaluation with phase contrast microscopy and confocal microscopy after PI and DAPI staining; (b) agarose gel electrophoresis of genomic DNA to detect the DNA fragmentation; (c) TUNEL (TdT-mediated dUTP-biotin nick-end labelling) assay to measure fragmented DNA content; (d) measurement of the DNA content of cells by FACS analysis after PI staining.

(a) Morphology of cells; Cellular morphology and detachment were examined with phase contrast microscopy. Nuclear morphology was examined with confocal microscopy (TCS NT, Leica Lasertechnik GmbH, Heidelberg, Germany) after staining with PI and DAPI. For PI or DAPI staining, harvested cells were attached on glass plates treated with 3-aminopropyltriethoxysilane by cytospin. Attached cells were fixed with 80% methanol for 30 min. After rinsing with PBS (phosphate buffered saline), nuclei were stained with PI (50 μ g/mL) or DAPI solution (1 μ g/mL).

(b) Electrophoresis; 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% SDS,

and 200 $\mu\text{g/mL}$ proteinase K and then centrifuged at 4°C , $16,000\times g$ for 20 min. DNA was extracted from supernatant using phenol-chloroform-isoamyl alcohol (25 : 24 : 1, Sigma) and chloroform. The extracted DNA was precipitated in ethanol containing 0.3 M NaOAc and nucleic acid precipitant was lysed in solution containing 10 mM Tris, 1 mM EDTA (pH 8.5) supplemented with 0.2 U RNase A for 30 min to eliminate RNA. DNA was loaded onto 1.2% agarose gels and stained with ethidium bromide.

(c) **TUNEL assay;** Cells were harvested by trypsinization and centrifuged at 1500 rpm for 5 min after washing with PBS. Cells were suspended in PBS containing 1% formaldehyde and stored on ice for 15 min. Cell membranes were permeabilized by resuspension in 70% ethanol at -20°C . After washing with PBS at room temperature, cells were incubated in a reaction mixture containing TdT, biotin-16-dUTP, and other dNTP for 30 min at 37°C . After resuspension with PBS, cells were incubated in a staining buffer containing FITC-labelled avidin in the dark for 30 min at room temperature. Fluorescence of cells was measured by flow cytometry.

(d) **Measurement of DNA content;** To quantify the DNA content, cells were fixed in methanol and incubated in PBS containing 5 $\mu\text{g/mL}$ PI, 0.05% Triton X-100, 1.8 $\mu\text{g/mL}$ EDTA, and 100 U/mL RNase for 30 min. Fluorescence of cells was then analyzed by flow cytometry.

Cell viability assay: Cell viability was determined using MTT assay. In brief, 2×10^4 cells per

well were cultured in a 96-well microtiter plate (Costar, Cambridge, MA, USA) and supernatant was discarded. Fifty μL of MTT solution (2 $\mu\text{g/mL}$) was added to each well and the plate was incubated at 37°C for 4 hours. The plate was centrifuged at $450\times g$ for 5 min and supernatant was removed. In each well, 150 μL DMSO was added and incubated for 10 min. The optical density (OD) of wells was measured at 570 nm with a multiwell spectrophotometer (ELISA processor II; Behringerwerke, Marburg, Germany). The measured OD of wells treated with Triton X-100 for cell lysis was regarded as total OD. Cell viability was calculated by the following equation;

$$\% \text{ Cell Viability} = \frac{\text{sample OD} - \text{total OD}}{\text{spontaneous OD} - \text{total OD}} \times 100$$

RT-PCR: Cells were lysed in 500 μL solution containing 4 M guanidine thiocyanate, 25 mM sodium acetate (pH 7.0), 0.5% sodium sarcosine, 100 mM 2-mercaptoethanol and total RNA was extracted using guanidine thiocyanate-phenol-chloroform. cDNA was synthesized using reverse transcriptase based on 1 μg of RNA denatured at 94°C for 10 min. PCR buffer solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl_2 , 0.01% gelatin was added with 100 pM primer, 300 μM dNTP, 0.5 U Taq polymerase and 5 μL of cDNA. PCR was performed for 30 cycles, using a thermal cycler (Perkin-Elmer) at 94°C for 1 min, 63°C for 1 min,

Table 1. Alignments of nucleotide sequences of primers

Gene	Nucleotide sequences	
β -actin (250 bp)	5'-sense	5'-CGTGGGCCGCCCTAGGCACCA-3'
	3'-antisense	5'-TTGGCCTTAGGGTTCAGGGGGG-3'
bcl-2 (385 bp)	5'-sense	5'-ACTTGTGGCCCAGATAGGCACCCAG-3'
	3'-antisense	5'-CGACTTCGCCGAGATGTCCAGCCAG-3'
bax (540 bp)	5'-sense	5'-CAGCTCTGAGCAGATCATGAAGACA-3'
	3'-antisense	5'-GCCCCATCTTCTCCAGATGGTGAGC-3'
Fas (400 bp)	5'-sense	5'-CGGAGGATTGCTCAACAAC-3'
	3'-antisense	5'-TTGGTATTCTGGGTCCG-3'
c-myc (292 bp)	5'-sense	5'-TCGGAAGGACTATCCTGTCTG-3'
	3'-antisense	5'-GCTTTTGCTCCTCTGCTTGG-3'
p21 ^{waf-1} (500 bp)	5'-sense	5'-CGGGATCCGGCGCCATGTCAGAACC-3'
	3'-antisense	5'-CGGAATTCTTAGGGCTTCTCTTGA-3'

and 72°C for 1 min. Following the amplification, an aliquot of reaction mixture was fractionated by electrophoresis in a 2% agarose gel. Nucleotide sequences of primers are illustrated in Table 1.

RESULTS

NSAIDs decreased viability of HT-29 cells

The effect of NSAIDs on HT-29 cell viability was assessed quantitatively by MTT assay. Both non-selective COX inhibitors such as indomethacin and sulindac as well as the selective COX-2 inhibitor nabumetone, dose-dependently decreased the viability of HT-29 cells (Fig. 1).

Decreased survival was caused by NSAIDs-induced apoptosis in HT-29 cells

To evaluate whether NSAIDs-induced apoptosis is the cause of decreased viability of HT-29 cells, apoptosis was assessed quantitatively as well as qualitatively.

Phase contrast microscopy demonstrated shrinkage and floating of cells treated with NSAIDs. PI staining illustrated nuclear fragmentation of apop-

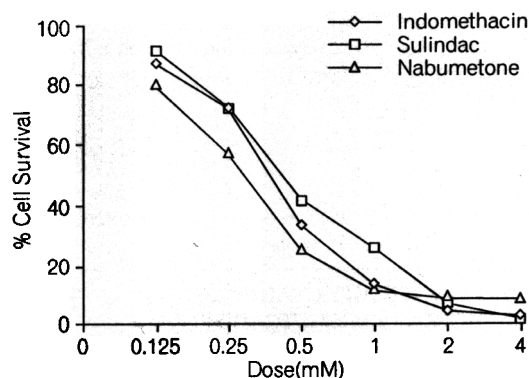


Fig. 1. Effect of various NSAIDs on the survival of HT-29 cells. HT-29 cells were treated with the indicated dose of indomethacin, sulindac or nabumetone, respectively for 24 hours and then cell viability was measured by MTT assay. Both non-selective COX inhibitors (indomethacin and sulindac) and selective COX-2 inhibitor (nabumetone) decreased the viability of HT-29 cells dose-dependently.

totic cells. DAPI staining identified clumped chromatin which was arrayed along the nuclear membrane (Fig. 2). The degree of change was directly proportional to the dosage of NSAIDs but not different according to the COX-selectivity of NSAIDs.

Agarose gel electrophoresis of DNA isolated from HT-29 cells treated with indomethacin demonstrated the characteristic ladder pattern indicative of internucleosomal DNA cleavage into 180 to 200 bp fragments (Fig. 3). DNA cleavage of NSAIDs-treated HT-29 cells was also identified by TUNEL assay. FITC-labelled cells were observed by fluorescent microscopy and cellular fluorescence was quantitatively measured using flow cytometry. Mean fluorescence intensity was higher in indomethacin-treated cells compared with control HT-29 cells. In addition, mean fluorescence intensity in indomethacin-treated cells was directly proportional to the dosage

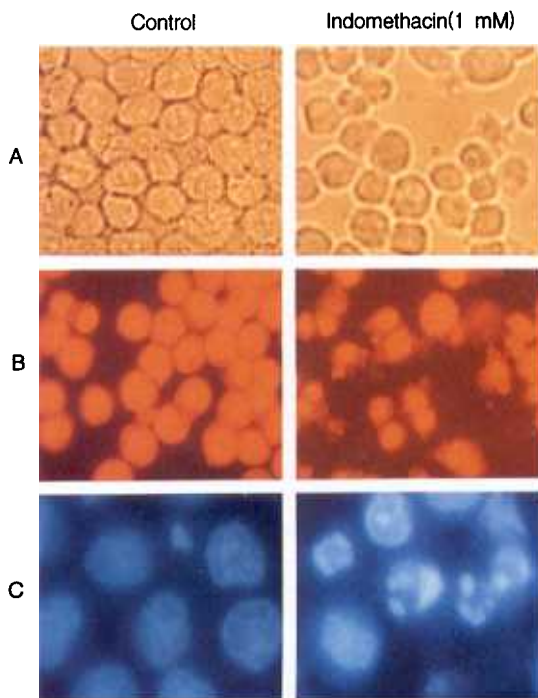


Fig. 2. Photomicrographs of HT-29 cells treated with 1mM indomethacin for 24 hours. (A) Cellular morphology observed by phase contrast microscopy demonstrating shrinkage and detachment of HT-29 cells after indomethacin treatment ($\times 100$). (B) PI staining illustrated nuclear fragmentation ($\times 200$). (C) Confocal microscopy after DAPI stain showed clumped chromatin which was arrayed along the nuclear membrane ($\times 400$).

of indomethacin (Fig. 4). The DNA content of cells measured by flow cytometry after PI staining demonstrated the dose-dependent increase of the sub-G1 peak (Fig. 5). These findings, including morphological changes and DNA cleavage, suggested that

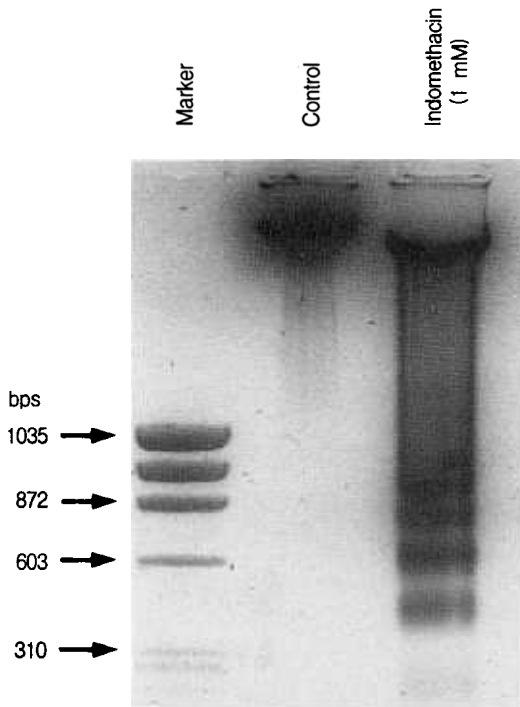


Fig. 3. Agarose gel electrophoresis of DNA isolated from HT-29 cells treated with 1 mM indomethacin for 24 hours. Indomethacin induced the characteristic 'ladder' pattern of internucleosomal DNA cleavage into 180 to 200 bp fragments.

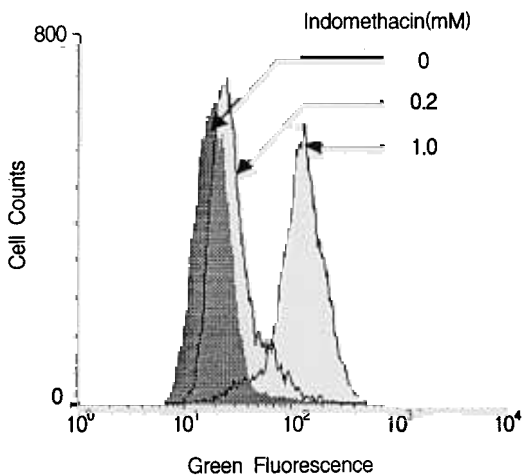


Fig. 4. Flow cytometric illustrations of TUNEL assay. The intensity of green fluorescence representing the degree of DNA fragmentation in HT-29 cells was increased by indomethacin treatment dose-dependently.

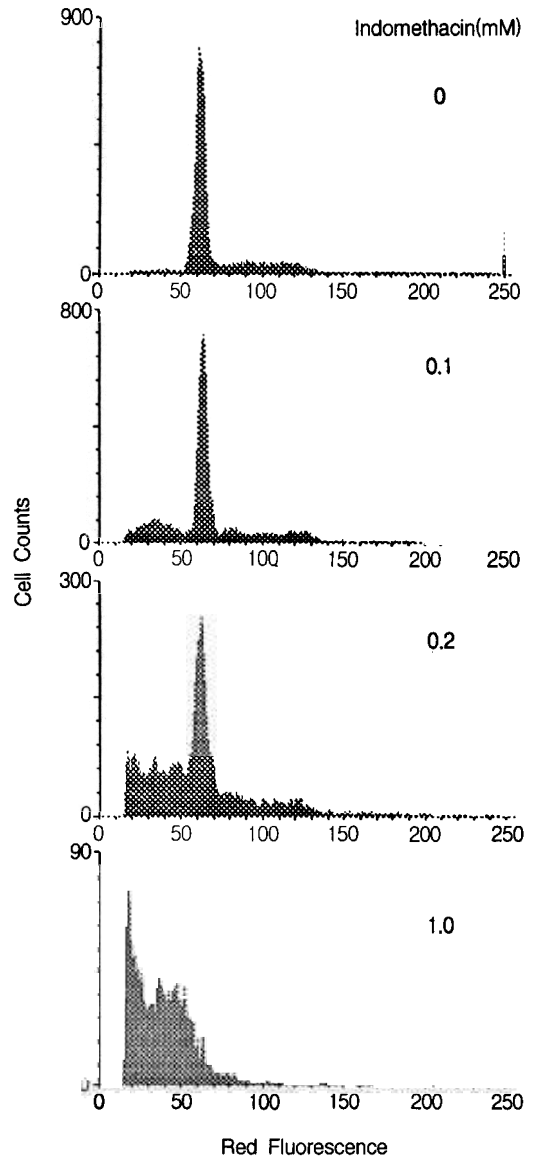


Fig. 5. Flow cytometric illustrations of DNA contents in HT-29 cells treated with the indicated dose of indomethacin. Red fluorescence represents the PI-stained DNA content of cells. Fraction of characteristic sub-G1 peak representing apoptotic cells increased as indomethacin dosage was increased.

NSAIDs-induced apoptosis can be attributed to the decreased viability of HT-29 cells.

NSAIDs enhanced $p21^{waf-1}$ mRNA expression but not other apoptosis-related genes

RNA was extracted from HT-29 cells treated with indomethacin doses of 0, 0.2, 0.5, and 1 mM for 4 hours. Using RT-PCR, the expression of apoptosis-related genes such as $p21^{waf-1}$, Fas, bcl-2, bax, and c-myc was examined. Indomethacin enhanced $p21^{waf-1}$ mRNA expression dose-dependently (Fig. 6). However, the expression of other apoptosis-related genes such as Fas, bcl-2, bax, and c-myc was unaffected by indomethacin (Fig. 7).

NSAIDs-induced apoptosis was not affected by intra- and extracellular calcium chelators and PTK, PKA, or PKC inhibitors

To evaluate whether signals were transduced

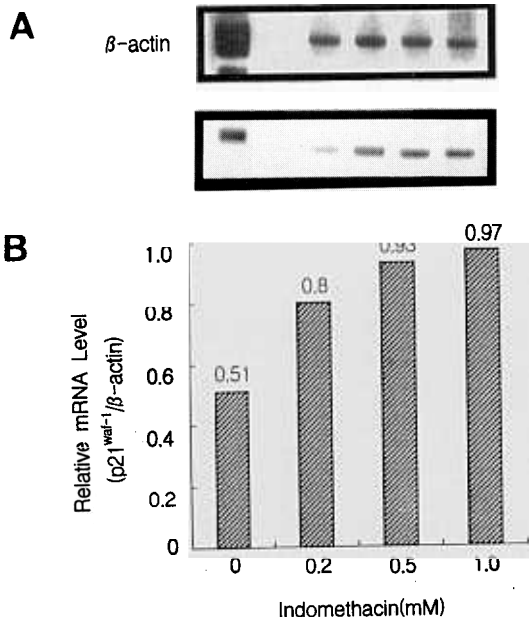


Fig. 6. Effect of indomethacin on the expression of $p21^{waf-1}$ mRNA in HT-29 cells. HT-29 cells were treated with the indicated dose of indomethacin for 4 hours and the total RNAs were extracted. They were then reverse transcribed and amplified by RT-PCR. (A) Indomethacin enhanced the expression of $p21^{waf-1}$ mRNA. (B) The levels of $p21^{waf-1}$ mRNA expression are depicted as the relative ratio to those of β -actin.

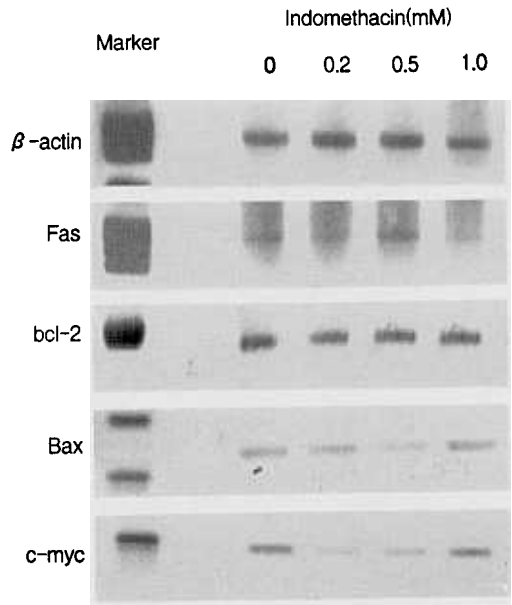


Fig. 7. Effect of indomethacin on the expression of apoptosis-related genes in HT-29 cells. HT-29 cells were treated with the indicated dose of indomethacin for 4 hours and the total RNAs were extracted. They were then reverse transcribed and amplified by RT-PCR. Indomethacin did not alter the expressions of apoptosis-related genes such as Fas, bcl-2, Bax and c-myc.

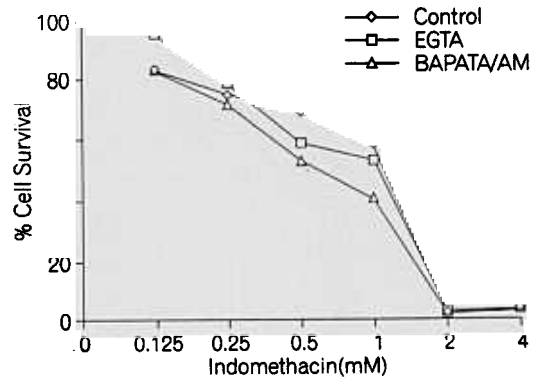


Fig. 8. Effect of Ca^{++} chelators on indomethacin-induced apoptosis in HT-29 cells. HT-29 cells were pretreated with or without Ca^{++} chelators such as 10 g/mL BAPTA/AM or 10 mM EGTA for 30 minutes and then treated with the indicated dose of indomethacin for 24 hours. Cell viability was measured by MTT assay. Both intracellular Ca^{++} chelator (BAPTA/AM) and extracellular Ca^{++} chelator (EGTA) did not influence the survival of HT-29 cells.

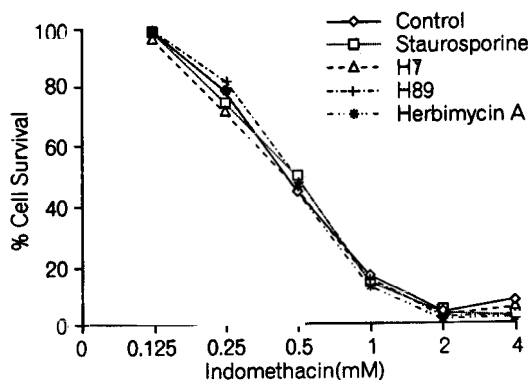


Fig. 9. Effect of signal inhibitors on indomethacin-induced apoptosis in HT-29 cells. HT-29 cells were pretreated with or without signal inhibitors such as 100 nM staurosporine, 50 nM H7, 4 μ M H89 or 40 nM herbimycin A for 30 minutes and then treated with the indicated dose of indomethacin for 24 hours. Cell viability was measured by MTT assay. Percent survival of HT-29 cells was not influenced by pretreatment of protein kinase C inhibitor (staurosporine, H7), protein kinase A inhibitor (H89) or protein tyrosine kinase inhibitor (herbimycin A).

through $[Ca^{++}]_i$, PTK, PKA, and PKC mediate NSAIDs-induced HT-29 apoptosis, intra- and extracellular calcium chelators (BAPTA/AM, 10 μ g/mL; EGTA, 10 mM), PTK inhibitor (herbimycin A, 40 nM), PKA inhibitor (H89, 4 μ M), and PKC inhibitors (staurosporine, 100 nM; H7 50 nM) were added respectively 30 min before treatment with indomethacin, then cell viability was measured by MTT assay. Neither calcium chelators nor signal inhibitors affected NSAIDs-induced apoptosis in HT-29 cells (Fig. 8, 9).

DISCUSSION

One possible alternative mode of NSAIDs' anti-neoplastic action is an induction of apoptosis. However, the molecular mechanism responsible for an apoptosis-inducing effect of NSAIDs is still far from complete understanding. Anti-proliferative and apoptosis-inducing effects of NSAIDs seem to be mediated by a prostaglandin-independent pathway (Hanif *et al.* 1996). In this study, we also confirmed that NSAIDs induce apoptosis in HT-29 cells irrespec-

tive of their COX-selectivity.

Apoptosis is considered as a coordinately regulated process with a cell cycle. Apoptotic cells are similar in shape to mitotic cells (King and Cidlo-wski, 1995). In addition, apoptosis is almost exclusively found in proliferating tissues and artificial manipulation of the cell cycle can either prevent or potentiate apoptosis (Meikrantz and Schlegel, 1995). Several genes that are involved in the cell cycle and cellular proliferation are also involved in apoptosis. This is the case in NSAIDs-induced apoptosis of colon cancer cells (Ahnen *et al.* 1995; Koutsos *et al.* 1995; Shiff *et al.* 1995; Goldberg *et al.* 1996). NSAIDs block the cell cycle progression by reducing the level and activity of cyclin-dependent kinases (cdks) such as $p34^{cdc2}$, $p34^{cdk4}$, and $p33^{cdk2}$ (Koutsos *et al.* 1995; Shiff *et al.* 1995) and by increasing the levels of the cdk inhibitor $p21^{waf-1}$ (Goldberg *et al.* 1996). $p21^{waf-1}$ regulates the cell cycle by inhibiting cdks or by inhibiting DNA synthesis through interaction with PCNA (Goldberg *et al.* 1996). We have demonstrated that NSAIDs enhance $p21^{waf-1}$ expression at the transcription level.

Apoptosis is tightly regulated by counterbalanced effects of death genes which were upregulated in apoptosis. Death gene products are thought to cause apoptosis. Proto-oncogenes inhibit apoptosis and promote cell survival. Of these apoptosis-related genes, the *bcl-2* gene family has emerged a critical negative regulator of apoptosis. *Bcl-2* was originally identified at the chromosomal breakpoint of B-cell lymphomas bearing the t (14 : 18) translocation. Over-expression of *bcl-2* can prolong cell survival by blocking apoptosis (Patel and Gores, 1995). The *bcl-2* gene does not contain any identifiable sequences for signal transduction pathways and the mechanism of action of *bcl-2* remains undetermined. Potential mechanisms include a role in intracellular calcium regulation, nuclear transport, and control of signal transduction pathways as well as its anti-oxidant effect (Reed, 1994). However, the anti-apoptotic effect of *bcl-2* is not universal because *bcl-2* expression does not protect cells from apoptosis in some circumstances, such as CTL-mediated apoptosis (Vaux, 1993). The *bcl-2* family consists of several *bcl-2* related genes that share sequence and functional homology with *bcl-2*. One such homolog, Bax, forms heterodimers with *bcl-2* and inactivates

it. Therefore overexpression of Bax promotes apoptosis in contrast with bcl-2. In this study, we observed that NSAIDs affect neither bcl-2 nor Bax expression. Recently, c-myc, whose expression has been implicated in cell growth and proliferation, has also been linked to cell death by apoptosis (Evan *et al.* 1992). c-myc induces both cell proliferation and apoptosis depending on the presence of survival factors. We could not find any significant changes in the c-myc mRNA expression of HT-29 cells treated with NSAIDs. p53, whose mutations are the most common genetic alterations observed in human cancers, also plays an important role during apoptosis. p53 can act as a transcription factor and has a sequence-specific DNA binding protein. DNA damage dramatically induces p53 expression and its transcriptional activity. This accumulation of p53 leads to cell cycle arrest at the G1-S checkpoint, allowing the cell to repair its DNA before replication. The cell undergoes apoptosis if the damage is irreversible as a defence mechanism to protect it from the propagation of cells that have sustained mutations. NSAIDs-induced apoptosis in HT-29 cells is a p53-independent process because this cell line carries p53 mutation, which results in loss of the gene's function (Goldberg *et al.* 1996). p21^{waf-1}, an important link between apoptosis and the cell cycle in response to exogenous stimuli, is mainly induced by activation of the p53-mediated DNA damage-response checkpoint. Induction of p21^{waf-1} by NSAIDs is p53-independent, because HT-29 carries p53 mutation and several lines of evidence suggest that p21^{waf-1} can also be regulated by p53-independent mechanisms.

Studies on cellular signaling during the execution of apoptosis have revealed a rise in cytoplasmic calcium concentration ($[Ca^{++}]_i$) associated with the induction of apoptosis in several systems (Jiang, 1996). However, there are many instances where a rise in $[Ca^{++}]_i$ does not appear to be involved in the apoptotic process and in some cases it can even inhibit apoptosis (Alnemri and Litwack, 1990; McConkey *et al.* 1990; Lennon *et al.* 1992; McConkey *et al.* 1994). NSAIDs such as indomethacin and sodium diclofenac promote mitogen-activated T-cell proliferation which is related with a rise in $[Ca^{++}]_i$ as a secondary messenger of signal transduction (Flescher *et al.* 1991). A rise in $[Ca^{++}]_i$, which is

very shortly maintained, is mediated by an influx of extracellular calcium in this circumstance because the extracellular calcium chelator, EGTA, blocks this process. We have tried to explore whether NSAIDs-induced apoptosis in HT-29 cells is associated with a rise in $[Ca^{++}]_i$ using intracellular calcium chelator, BAPTA/AM and the extracellular calcium chelator, EGTA. Neither BAPTA/AM nor EGTA affected the dose-response of NSAIDs-induced decrease of cell survival, suggesting that a rise in $[Ca^{++}]_i$ is not an important secondary messenger in NSAIDs-induced apoptosis. NSAIDs are known to transduce signals associated with GTP-binding protein and finally induce transcription factors in human immune cells (Abramson *et al.* 1991; Flescher *et al.* 1995). Therefore, we have also examined the role of signals transduced through pathways including PTK, PKA, and PKC, all of which are known to be associated with apoptosis induced by various stimuli (McConkey *et al.* 1994; Migita *et al.* 1994; Yousefi *et al.* 1994; Lucas and Sanchez-Margalet, 1995). A NSAIDs-induced decrease of cell survival was not influenced by any of the signal transduction pathway blockers including herbimycin A, a PTK inhibitor, H89, a PKA inhibitor, or staurosporine and H7, both of which are known as PKA inhibitors, suggesting segregation of these signal transduction pathways from NSAIDs-induced apoptosis in HT-29 cells.

REFERENCES

- Abramson SB, Leszczynska-Piziak J, Haines K, Reibman J: Non-steroidal anti-inflammatory drugs: effects on a GTP binding protein within the neutrophil plasma membrane. *Biochem Pharmacol* 41: 1567-1573, 1991
- Ahnen D, Piazza G, Alberts D, Paranka N, Burt R, Pamukcu R: Sulindac sulfide(SS) and sulfone(FGN-1) both inhibit the growth of colon cancer cell lines by inducing apoptosis(Abstract). *Gastroenterology* 108(suppl): A443, 1995
- Alnemri ES, Litwack G: Activation of internucleosomal DNA cleavage in human CEM lymphocytes by glucocorticoid and novobiocin. *J Biol Chem* 265: 17323-17333, 1990
- Bennett A, Civier A, Hensby CN, Melhuish PB, Stamford IF: Measurement of arachidonate and its metabolites extracted from human normal and malignant gastrointestinal tissues. *Gut* 28: 315-318, 1987

- Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, Dubois RN: Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107: 1183-1188, 1994
- Evan G, Wyllie A, Gilbert C, Littlewood T, Land H, Brooks M, Waters C, Penn L, Hancock D: Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69: 119-128, 1992
- Flescher E, Fossum D, Gray PJ, Fernandes G, Harper MJK, Talal N: Aspirin-like drugs prime human T cells; modulation of intracellular calcium concentrations. *J Immunol* 146: 2553-2559, 1991
- Flescher E, Ledbetter JA, Ogawa N, Vela-Roch N, Fossum D, Dang H, Talal N: Induction of transcription factors in human T lymphocytes by aspirin-like drugs. *Cell Immunol* 160: 232-239, 1995
- Goldberg Y, Nassif II, Pittas A, Tsai L, Dynlacht BD, Rigas B, Shiff SJ: Sulindac and sulindac sulfide alter the levels of pRb and p53 and the cyclin dependent kinase inhibitor p21 in HT-29 colon cancer cells. *Oncogene* 12: 893-901, 1996
- Greenberg ER, Baron JA, Freeman DH Jr, Mandel JS, Haile R: Reduced risk of large-bowel adenomas among aspirin users. The Polyp Prevention Study Group. *J Natl Cancer Inst* 85: 912-916, 1993
- Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SJ, Rigas B: Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 52: 237-245, 1996
- Jiang S: Regulation of T cell apoptosis by calcium and zinc ions. Stockholm, Karolinska Institute, 1996, 30-33
- King KL, Cidlowski JA: Cell cycle and apoptosis: common pathways to life and death. *J Cell Biochem* 58: 175-180, 1995
- Koutsos MI, Shiff SJ, Qiao L, Rigas B: Aspirin and other NSAIDs inhibit the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis(Abstract). *Gastroenterology* 108(suppl): A492, 1995
- Lennon SV, Kilfeather SA, Hallett MB, Campbell AK, Cotter TG: Elevations in cytosolic free Ca^{2+} are not required to trigger apoptosis in human leukaemia cells. *Clin Exp Immunol* 87: 465-471, 1992
- Lucas M, Sanchez-Margalet V: Protein kinase C involvement in apoptosis. *Gen Pharmacol* 26: 881-887, 1995
- McConkey DJ, Nicotera P, Orrenius S: Signalling and chromatin fragmentation in thymocyte apoptosis. *Immunol Rev* 142: 343-363, 1994
- McConkey DJ, Orrenius S, Jondal M: Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. *J Immunol* 145: 1227-1230, 1990
- Meikrantz W, Schlegel R: Apoptosis and the cell cycle. *J Cell Biochem* 58: 160-174, 1995
- Migita K, Eguchi K, Kawabe Y, Misokami A, Tsukada T, Nagataki S: Prevention of anti-CD3 monoclonal antibody-induced thymic apoptosis by protein tyrosine kinase inhibitors. *J Immunol* 153: 3457-3465, 1994
- Pasricha PJ, Bedi A, O'Connor K, Rashid A, Akhtar AJ, Zahurak ML, Piantadosi S, Hamilton SR, Giardiello FM: The effects of sulindac on colorectal proliferation and apoptosis in familial adenomatous polyposis. *Gastroenterology* 109: 994-998, 1995
- Patel T, Gores GJ: Apoptosis and hepatobiliary disease. *Hepatology* 21: 1725-1741, 1995
- Qiao L, Kozoni V, Koutsos MI, Tsioukas GJ, Hanif R, Shiff SJ, Rigas B: Eicosanoids increase the proliferation rate in human colon carcinoma cell lines and mouse colon cells(Abstract). *Gastroenterology* 108(suppl): A527, 1995
- Reed J: Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 124: 1-6, 1994
- Rigas B, Goldman IS, Levine L: Altered eicosanoid levels in human colon cancer. *J Lab Clin Med* 122: 518-523, 1993
- Rosenberg L, Palmer JR, Zauber AG, Warshauer ME, Stolley PD, Shapiro S: A hypothesis: nonsteroidal anti-inflammatory drugs reduce the incidence of large-bowel cancer. *J Natl Cancer Inst* 83: 355-358, 1991
- Shiff SJ, Qiao L, Tsai LL, Rigas B: Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells. *J Clin Invest* 96: 491-503, 1995
- Thun MJ, Namboodiri MM, Heath CW Jr: Aspirin use and reduced risk of fetal colon cancer. *N Engl J Med* 325: 1593-1596, 1991
- Vaux D: Toward an understanding of the molecular mechanisms of physiological cell death. *Proc Natl Acad Sci USA* 90: 786-789, 1993
- Yousefi S, Green DR, Blaser K, Simon H: Protein-tyrosine phosphorylation regulates apoptosis in human eosinophils and neutrophils. *Proc Natl Acad Sci USA* 91: 10868-10872, 1994