Non-steroidal anti-inflammatory drug-induced apoptosis in gastric cancer cells is blocked by protein kinase C activation through inhibition of c-myc

GH Zhu¹, BCY Wong¹, MC Eggo², CK Ching¹, ST Yuen³, EYT Chan³, KC Lai¹ and SK Lam¹

¹Department of Medicine, Queen Mary Hospital, University of Hong Kong, Hong Kong; ²Department of Medicine, the University of Birmingham, Birmingham, UK; ³Department of Pathology, Queen Mary Hospital, University of Hong Kong, Hong Kong

Summary Apoptosis plays a major role in gastrointestinal epithelial cell turnover, ulcerogenesis and tumorigenesis. We have examined apoptosis induction by non-steroidal anti-inflammatory drugs (NSAIDs) in human gastric (AGS) cancer cells and the role of protein kinase C (PKC) and apoptosis-related oncogenes. After treatment with aspirin or indomethacin, cell growth was quantified by MTT assay, and apoptosis was determined by acridine orange staining, DNA fragmentation and flow cytometry. The mRNA and protein of p53, p21waf1/cip1 and c-myc were detected by Northern and Western blotting respectively. The influence of PKC on indomethacin-induced apoptosis was determined by co-incubation of 12-O-tetradecanoylphorbol 13-acetate (TPA). The role of c-myc was determined using its antisense oligonucleotides. The results showed that both aspirin and indomethacin inhibited cell growth and induced apoptosis of AGS cells in a dose- and time-dependent manner, without altering the cell cycle. Indomethacin increased c-myc mRNA and protein, whereas p53 and p21waf1/cip1 were unchanged. Down-regulation of c-myc by its antisense oligonucleotides reduced apoptosis induction by indomethacin. TPA could inhibit indomethacin-induced apoptosis and accumulate cells in G₀/M. Overexpression of c-myc was inhibited by TPA and p21waf1/cip1 mRNA increased. In conclusion, NSAIDs induce apoptosis in gastric cancer cells which may be mediated by up-regulation of c-myc proto-oncogene. PKC activation can abrogate the effects of NSAIDs by decreasing c-myc expression.

Keywords: non-steroidal anti-inflammatory drugs; gastric cancer; apoptosis; antiproliferation; oncogene; protein kinase C; cell cycle

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed medications that can be obtained over the counter. Apart from their well-known analgesic, antipyretic and anti-inflammatory effects, they have also been used in the treatment of familial adenomatous polyposis (Giardiello et al, 1993). Sulindac, in particular, has been demonstrated in several epidemiological studies to significantly reduce the incidence of colorectal adenomas and adenocarcinomas (Marnett, 1992; Giovannucci et al, 1994). Aspirin use was inversely associated with fatal cancers of the oesophagus and stomach as well as colon and rectum, but not generally with fatal cancers outside the digestive tract (Thun et al, 1993). Record linkage studies in Finland and Sweden found decreased risk of stomach and colorectal cancers, but not oesophagus in patients with rheumatoid arthritis taking high doses of aspirin and other NSAIDs (Isomaki et al, 1978; Gridley et al, 1993). NSAIDs, such as indomethacin, have been shown to inhibit the growth of colon cancer cell lines and induce apoptosis in vitro, which was considered to be an important mechanism in their antineoplastic activity (Hanif et al, 1996; Shiff et al, 1996). Furthermore, apoptosis has been implicated to play a vital role in gastric epithelial cell turnover, ulcerogenesis and even carcinogenesis (Hall et al, 1994; Spyridon et al, 1994). A study of the effects of NSAIDs on apoptosis of gastric epithelial cells and its underlying intracellular events could present novel points for therapeutic intervention in gastropathy.

Protein kinase C (PKC), a serine-threonine kinase, is a family of 12 isoenzymes which mediate phosphorylation of numerous protein substrates and play a central role in signalling pathways regulating cell differentiation, tumour promotion and apoptosis (Clemens et al, 1992; Lucas and Sanchez-Margalet, 1995; Deacon et al, 1997). Phorbol esters, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), activate PKC in vitro in a manner similar to the physiological activator diacylglycerol (DAG) and have, therefore, been widely used to study the function of PKC. Both induction and inhibition of apoptosis have been reported in different cell lines treated with TPA (Lucas and Sanchez-Margalet, 1995). These contradictory findings may be because it can activate several PKC isoforms. Unlike the physiological activator DAG, TPA is a stable compound which will induce a persistent activation of PKC leading to its down-regulation. The PKC isoforms are differentially sensitive to down-regulation. So, the diverse biological effects of phorbol esters are related to the differential activation or down-regulation of one or more of the isoenzymes (Deacon et al, 1997).

Oncogenes and tumour-suppressor genes are well documented to be involved in mediating apoptosis (Liebermann et al, 1995; Hale et al, 1996). p53 is a transcription factor which stimulates the synthesis of inhibitors of cyclin-dependent kinases (CDKs), such as p21waf1/cip1 which binds to CDKs and inhibits their action, thereby blocking cell proliferation (Xiong et al, 1993; El Deiry et al, 1994). When mammalian cells are exposed to ionizing radiation or other DNA-damaging agents, the cells are arrested at G₁.
phase until the genomic lesions are fully repaired. If the damage to DNA is irreparable, p53 triggers apoptosis. Cells without p53 or with mutant p53 genes lack this checkpoint and cells enter S-phase without appropriate DNA repair, leading to fixation and propagation of genetic alteration (Hale et al, 1996). The c-myc proto-oncogene plays a role in the control of cell proliferation, differentiation as well as apoptosis (Askew et al, 1991; Evans et al, 1992). Deregulated expression of c-myc can induce apoptosis in conjunction with growth-regulating signals, such as growth factor deprivation, high cell density and chemotherapeutic treatment (Evans et al, 1992; Lotem and Sachs, 1993). c-myc antisense oligodeoxynucleotides can inhibit the intracellular expression of the targeted gene specifically because of the exquisite specificity of the Watson–Crick base-pair interactions that result in the formation of mRNA–DNA hybrid structure. Antisense techniques have been widely used in studying the role of c-myc in apoptosis.

In this study, we have tested the effects of aspirin and indomethacin on a gastric (AGS) cancer cell line and investigated the roles of both drugs on cell growth and apoptosis. The mechanisms associated with these changes were also studied, including the PKC signalling pathways and apoptosis-related genes including p53, p21\textsuperscript{waf1/cip1} and c-myc.

MATERIALS AND METHODS

Cell culture

AGS, a poorly differentiated gastric adenocarcinoma cell line, was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and used in the present study. Cells were passaged into RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U ml\textsuperscript{−1} penicillin, 100 \textmu g ml\textsuperscript{−1} streptomycin (Gibco BRL, Life Technologies, NY, USA). Cells were cultured in 25 cm\textsuperscript{2} culture flasks (Corning, New York, NY, USA) as a monolayer in a 95% air/5% carbon dioxide humidified atmosphere at 37°C.

Both indomethacin and aspirin (Sigma, St Louis, MO, USA) were freshly prepared in absolute ethanol before use. TPA (CalBiochem, La Jolla, CA, USA) was prepared in DMSO. Stock preparations of the reagents were stored at −20°C. Vehicle controls of absolute ethanol and DMSO (less than 0.1%) were included in the studies. Cells were incubated for 24 h with different concentrations of indomethacin, aspirin, and TPA alone or in combination. After treatment, adherent cells were removed by trypsinization and combined with the floating cells in the medium (Hanif et al, 1996). Cells were collected by centrifugation for further analysis. Antisense and sense oligonucleotides for c-myc were purchased from Biognostik (Germany). For oligonucleotide experiments, cells growing in log phase were seeded in 24-well plates at a density of 1 \times 10\textsuperscript{5} cells per well. Specified concentrations of oligonucleotides were added to the cell culture for 14 h, then indomethacin (400 \textmu M) was added to the culture for a fixed time period for further analysis.

MTT assay

Antiproliferation effects were measured by a modified MTT assay (Carmichael et al, 1988). The assay detects living but not dead cells, and the signal generated is dependent on the degree of activation of the cells. About 5000 cells per well were plated in 96-well microtitre plates and incubated overnight in 100 \textmu l of culture media. Then cells were treated with various concentrations of indomethacin or aspirin for fixed time intervals. Ten microlitres of stock MTT (2.5 mg ml\textsuperscript{−1}) was then added to each well, and the cells were further incubated at 37°C for 4 h. The supernatant was removed and 100 \textmu l of 0.04 M hydrochloric acid in isopropanol was added to each well. The absorbance at a wavelength of 595 nm was measured by a microELISA reader (Biorad, USA). The negative control well has no cells, but the culture medium only.

Flow cytometry

Cells from both treatment and control groups were collected and washed with ice-cold phosphate buffered saline (PBS, pH 7.4). They were fixed in ice-cold 70% ethanol in PBS and stored at −20°C. Before analysis, the cells were washed and resuspended in PBS. One hundred microlitres of RNase I (1 mg ml\textsuperscript{−1}) and 100 \textmu l of propidium iodide (PI, 400 \textmu g ml\textsuperscript{−1}, Sigma) were added and incubated at 37°C for 30 min. The analysis of samples was performed by a flow cytometry (Coulter Epics XL, UK). The cell cycle phase distribution was calculated from the resultant DNA histogram using Multicycle AV software (Phoenix Flow System, San Diego, CA, USA) and expressed as a percentage of cells in G\textsubscript{0}/G\textsubscript{1}, S- and G\textsubscript{2}/M phases. The apoptotic cells can be observed on a DNA histogram as a subdiploid or ‘pre-G\textsubscript{1}’ peak (Darzynkiewicz et al, 1992).
NSAID-induced apoptosis in gastric cancer cells

DNA fragmentation analysis

DNA fragmentation was analysed as described previously (Grant et al, 1992) with some modification. Briefly, after treatment with various agents as described above, cells were harvested and washed twice in ice-cold PBS. The final pellet was lysed in 10 mM Tris–HCl (pH 7.4) buffer containing 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K (Sigma) and incubated at 50°C for 12–18 h. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol and sodium acetate. Total cellular RNA was isolated following the methods as described previously (Rhoads, 1975) after some modification. Briefly, cells were washed with ice-cold PBS, lysed with 4 M lithium chloride/8 M urea/6 mM EDTA and then precipitated overnight at 4°C. After centrifugation for 15 min at 13,000 r.p.m. at 4°C, the RNA pellet was dissolved in DEPC-treated water, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and then precipitated with ethanol and sodium acetate. Northern blot analysis was performed according to the method previously described (Ausubel et al, 1995). Equal amounts (20 μg) of total RNA per lane were electrophoresed on 1.0% denaturing formaldehyde agarose gel and transferred onto nylon membranes (Amersham, UK). The cDNA probes of p53, c-myc (Oncogene Research Products, Cambridge, MA, USA) and waf1 probe from pSXV-WAF1 (ATCC) excised by EcoRI and XhoI (New England Biolabs, Beverly, MA, USA) were radiolabelled with [α-32P]dCTP (Amersham) by the Megaprime random prime labelling system (Amersham). After hybridization with a different probe overnight at 42°C, the membrane was washed with 2.0 × sodium chloride, sodium phosphate EDTA buffer (SSPE)/0.1% SDS, 1.0 × SSPE/0.1% SDS and 0.2 × SSPE/0.1% SDS at 42°C, and exposed to radiographic film (Kodak, Rochester, NY, USA) at –70°C. The membrane was reprobed for use in another hybridization in which a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as an internal control. GAPDH oligonucleotide probe (Oncogene Research Products) was radio-labelled with [γ-32P] ATP by 5′-end labelling kit (Amersham).

Western blot analysis

After the drug treatment, the cells were extracted with lysis buffer containing 1% Triton X-100, 50 mM sodium chloride, 50 mM sodium fluoride, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.2 mM PMSF and 0.5% NP-40. The protein concentration was determined by using Bicinchoninic acid assay (BCA) (Smith et al, 1985) with bovine serum albumin (Sigma) as standard. Western blotting was carried out as described previously (Li et al, 1996). In brief, equal amount of cell lysates (60 μg) were solubilized in sample buffer by boiling for 5 min and subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto a nitrocellulose membrane (Sigma). The membrane was incubated first with an appropriate primary antibody and then with peroxidase-conjugated anti-mouse IgG antibody in the second reaction. The membranes were washed and developed by chemiluminescence (ECL) Western blot detection system (Transduction Laboratories, Lexington, KY, USA) and exposed to film. Mouse monoclonal antibodies p53 (Ab-6), p21waf1/kip1 (Ab-1), c-Myc (Ab-1) and peroxidase-conjugated anti-mouse IgG were purchased from Oncogene Research Products.

Acridine orange staining

After 24 h of drug treatment, the cells were washed with ice-cold PBS, harvested and fixed in 1% formalin/PBS and stained with acridine orange (AO, 10 μg/ml, Sigma). A drop of the stained cell suspension was placed on a microscope slide. Cells were visualized under UV fluorescence microscope with blue–green filter, as described previously (Elder et al, 1996). Apoptotic cells were defined as cells showing cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation morphologically. At least 300 cells were counted and the percentage of apoptotic cells was determined.
Statistical analysis

The data shown were mean values of at least three different experiments and expressed as means ± s.d. Student’s t-test was used to compare the results. A P-value of less than 0.05 is considered statistically significant.

RESULTS

Effects of aspirin and indomethacin on cell growth

To evaluate the effects of aspirin and indomethacin on the growth of gastric cancer cells in vitro, cells were seeded in 96-well culture plates at a density of 5000 cells per well. Various concentrations of aspirin (0.1–10 mM) and indomethacin (50–800 μM) were added in the culture medium for 1, 2 or 3 days. Cell growth was determined by MTT assay. As Figure 1 shows, both indomethacin and aspirin could inhibit gastric cancer cell growth in a dose- and time-dependent manner. Indomethacin showed a more potent effect on reducing AGS cell growth compared with aspirin.

Effect of aspirin and indomethacin on the cell cycle phase distribution

To investigate the antiproliferation mechanisms of aspirin and indomethacin, we studied the effect of these compounds on cell cycle phase distribution of gastric adenocarcinoma cells using FACS analysis. The results showed that after the indicated treatments for 24 h, neither aspirin nor indomethacin interfered with cell cycle phase distribution of AGS cells when compared with the control (Figure 2).

Apoptosis of gastric cancer cells induced by aspirin and indomethacin

Apoptosis of gastric cancer cells after treatment was evaluated by three different methods as described above: (1) measurement of DNA content of cells by PI staining and FACS analysis to detect the pre-G1 peak; (2) agarose gel electrophoresis of genomic DNA to detect DNA fragmentation; (3) AO staining to detect the typical morphological changes under fluorescence microscopy. The FACS analysis showed that both aspirin and indomethacin could induce apoptosis in AGS cells, with a typical subdiploid peak on the histogram (Figure 3A). Apoptosis induction followed a dose-dependent and non-linear manner (Figure 2). Compared with indomethacin, aspirin was less potent in inducing apoptosis of AGS cells. It failed to induce apoptosis at <0.1 mM. DNA fragmentation in AGS cells treated with aspirin (1 mM, 10 mM) and indomethacin (100–800 μM) was shown as a ladder pattern on agarose gel (Figure 4). The AO staining showed that both aspirin and indomethacin could induce apoptosis in AGS cells morphologically, which was characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and fragmentation (Figure 5).

Effect of 12-O-tetradecanoyl phorbol 13-acetate (TPA) on indomethacin-induced apoptosis and cell cycle

To study whether the PKC pathway is mediated in apoptosis induction by indomethacin, we investigated the effect of the PKC activator TPA, a pan-activator of PKC isoenzymes affecting all but the atypical class of PKCs (ζ, λ, μ) (Jarvis et al, 1994), on

Figure 3  The results of FACS analysis of AGS cells treated with aspirin and indomethacin with or without TPA. Cells were treated with aspirin, indomethacin and TPA alone or in combination for 24 h and their DNA content was determined by FACS, as described in Materials and methods. (A) DNA histogram. (a) control; (b) 1 mM aspirin; (c) 400 μM indomethacin; (d) 400 μM indomethacin + 100 nM TPA; (B) TPA on apoptosis and cell kinetics of indomethacin-treated AGS cells. The values are expressed as means ± s.d. (n > 3)
indomethacin-induced apoptosis of AGS cells. As a result, the proportion of cells in the G₀/G₁ phase was significantly decreased and that in G₂/M phase was increased after chronic treatment with TPA alone at 100 nM for 24 h (Figure 3B). Indomethacin-induced apoptosis was inhibited by co-incubation with 100 nM TPA, decreasing from 50.2±10% to 16.4±6.7%. In addition, cells at G₀/G₁ phase were reduced from 74.8±3.6% to 60.6±4.7% (P<0.05) and those at G₂/M phase was increased from 11.5±3.6% to 33.6±6.6% (P<0.01) (Figure 3B).

Effects of indomethacin on p53, p21waf1/cip1 and c-myc mRNA level

The mRNA level of p53, p21waf1/cip1 and c-myc was determined by Northern blotting in indomethacin-treated AGS cells. Cells exposed to 400 μM of indomethacin resulted in a progressive increase in c-myc mRNA levels noted as early as 2 h and reached a maximum at 8 h, whereas p53 and p21waf1/cip1 mRNA remained unchanged over time. Equal amounts of RNA were loaded in each lane, as determined by hybridization with the internal control GAPDH probe (Figure 6A).

Effects of indomethacin on p53, p21waf1/cip1 and c-myc protein level

The protein levels of p53, p21waf1/cip1 and c-myc were determined by Western blotting in indomethacin-treated AGS cells. Cells treated with 400 μM of indomethacin resulted in a marked increase in c-myc protein level noted as early as 4 h and reached a maximum at 8 h, whereas p53 and p21waf1/cip1 protein levels remained unchanged (Figure 6B).

Effect of TPA on p53, p21waf1/cip1 and c-myc mRNA level in indomethacin-treated AGS cells

Figure 7 shows that 100 nM TPA greatly inhibited the overexpression of c-myc mRNA induced by indomethacin (400 μM) at 8 h. However, p21waf1/cip1 mRNA was increased at 8 h, whereas p53 mRNA remained unchanged. Equal amounts of RNA were loaded in each lane, as determined by hybridization with the internal control GAPDH probe.

Down-regulation of c-myc with its antisense oligonucleotides on indomethacin-induced apoptosis

The role of c-myc proto-oncogene in indomethacin-induced apoptosis was confirmed using various concentrations of antisense oligonucleotides. Figure 8 shows that 2 μM c-myc antisense oligonucleotide could inhibit the indomethacin-induced increase in c-myc protein at 8 h, whereas sense oligonucleotide, as a random control, showed no effect on the c-myc overexpression. c-myc antisense oligonucleotide could inhibit the indomethacin-induced apoptosis. At 400 μM of indomethacin, the percentage of apoptotic cells decreased from 50.3±3.2% to 24.7±2.1% by the addition of 2 μM of antisense oligonucleotide. The same concentration of c-myc sense oligonucleotides showed no effect on apoptosis induced by indomethacin.

DISCUSSION

It has been demonstrated for the first time in this study that both aspirin and indomethacin can inhibit the growth of AGS gastric
cancer cells in a concentration- and time-dependent fashion. Moreover, aspirin and indomethacin induced apoptosis in the AGS cancer cells. Indomethacin showed more potent growth inhibition on AGS cells compared with aspirin. Similarly, apoptosis was induced by indomethacin at a lower concentration than aspirin. These findings are consistent with those reported in colon cancer and other cell lines (Lu et al, 1995; Shiff et al, 1996). Apoptosis is often accompanied by growth arrest. Cell cycle checkpoints may act as a toggle switch between proliferation and death by sensing deregulation of the cell cycle (King and Cidlowski, 1995). In colon cancer cells, the apoptotic effect of NSAIDs correlated with the arrest of cells at G$_0$/G$_1$ phase (Hanif et al, 1996; Shiff et al, 1996). We failed to demonstrate any effect of aspirin or indomethacin on the AGS cell cycle phase distribution by using the FACS analysis. Thus, apoptosis may be the chief mechanism responsible for the inhibition of cell growth in NSAID-treated AGS cancer cells.

Although the role of NSAIDs in the induction of apoptosis of colon cancer cells are reported (Hanif et al, 1996; Shiff et al, 1996), the intracellular molecular events involved are still by far unknown. One potential molecular target for NSAIDs is cyclooxygenases (COXs), a key enzyme in prostaglandin biosynthesis. Interestingly, sulindac sulphone (an active metabolite of sulindac), which induced apoptosis in colon carcinoma cells HT29, is independent of inhibiting prostaglandin biosynthesis (Hanif et al, 1996). In our present study, we found that prolonged incubation with a PKC activator significantly reduced the apoptotic effect of indomethacin in the AGS cells. Thus, the protein kinase C pathway may be associated with NSAID-induced apoptosis in gastric cancer cells.

A high prevalence of p53 mutation has been detected in gastric cancer (Uchino et al, 1993; Triantafillou et al, 1996). In indomethacin-treated AGS cells, we did not observe any change in p53 mRNA and protein expression. The p53 target gene p21$^{waf1/cip1}$ also showed no change after the treatment with indomethacin, which might explain the unaltered cell cycle phase distribution. In HT-29 colon cancer cells, however, both sulindac and sulindac sulphone increased the level of p21$^{waf1/cip1}$ expression but reduced the level of mutant p53 (Goldberg et al, 1996). The differential regulation of tumour-suppressor genes in relation to apoptosis in cells from different gastrointestinal sites needs to be explored.

c-myc mRNA is overexpressed in most cases of primary gastric carcinoma. Metastatic lesions also have high levels of expression, which suggests that overexpression of c-myc may play a role in gastric carcinogenesis and tumour metastasis (Onoda et al, 1996). Our results showed that c-myc gene expression was up-regulated at both mRNA and protein level, preceding the onset of apoptosis after treatment with indomethacin. Down-regulation of the c-myc overexpression by antisense oligonucleotides inhibited the indomethacin-induced apoptosis of AGS cells, which suggested that c-myc was involved in indomethacin-induced apoptosis. Further evidence came from the down-regulation of c-myc after TPA co-treatment, which also reversed indomethacin-induced apoptosis. c-myc-mediated apoptosis in epithelial cells could be p53 dependent or independent (Sakamura et al, 1995). AGS cells express wild-type p53 (Matzaki et al, 1992). After treatment with indomethacin, p53 mRNA remained unchanged, although the c-myc mRNA was markedly increased. It has been shown that normal levels of wild-type p53 in the fibroblast system are sufficient to confer susceptibility to c-myc-mediated apoptosis (Wagner et al, 1995).
et al., 1994). Further study is needed to define the interaction of the two genes in apoptosis.

We found that TPA could reduce the proportion of AGS cells at G0/G1 phase and increase the cells at G2/M phase in the presence or absence of indomethacin. Usually, cells progress to late G1 phase before apoptosis takes place. Cell cycle arrest before this stage delays or blocks apoptosis, whereas arrest after this promotes apoptosis (Mekirantis et al., 1994). It may be possible that TPA inhibits indomethacin-induced apoptosis via modulation of cell cycle kinetics. In cells treated with PKC activator in the presence of indomethacin, p21waf1/cip1 protein expression was increased, but not accompanied by an increase in p53 protein expression. Therefore, activation/down-regulation of protein kinase C could lead to overexpression of p21waf1/cip1 in gastric cancer cells that is independent of p53. This finding agrees with previous studies showing that p21waf1/cip1 can be induced through p53-independent mechanisms by TPA in other cell types (Michieli et al., 1994). p21waf1/cip1 has been commonly associated with the G1 check point. However, it has been shown to accumulate in cells in G2/M phase in some cell types treated with TPA (Tchou et al., 1996). In our present study, overexpression of p21waf1/cip1 may be associated with the accumulation of cells at G2/M phase after TPA treatment.

We conclude from our experiments that both aspirin and indomethacin induce apoptosis in AGS gastric cancer cells, which may account for their growth inhibitory effects. The modulation of PKC activity and down-regulation of c-myc may block these effects. We believe that further understanding of the NSAID-induced apoptosis in gastric cancer can lead to some insight into the carcinogenesis and chemoprevention of the second most common cancer in the world.

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