Cancer Chemother Pharmacol (2000) 45: 389–396

ORIGINAL ARTICLE

Chung-Tsen Hsueh · Chang-Fang Chiu
David P. Kelsen · Gary K. Schwartz

Selective inhibition of cyclooxygenase-2 enhances mitomycin-C-induced apoptosis

Received: 6 July 1999 / Accepted: 19 November 1999

Abstract *Purpose*: Cyclooxygenase-2 (COX-2) is involved in antiapoptosis signaling, and its induction may require activation of protein kinase C (PKC). Safingol (SAF), a PKC inhibitor, has been shown to enhance apoptosis induced by mitomycin-C (MMC) in human gastric cancer MKN-74 cells. The aim of this study was to identify the role of COX-2 in MMC-induced apoptosis in MKN-74 cells. *Methods*: Protein expression of COX-2 and Bcl-2 and activation of PKCα were examined by Western blot analysis. Apoptosis induction was examined by staining with bisbenzimide trihydrochloride (Hoechst-33258) of condensed chromatin, which characterizes the cells undergoing apoptosis. COX-2 mRNA levels were examined by Northern blot analysis. *Results*: After exposure for 1–2 h to 1 μg/ml MMC, upregulation of COX-2 and Bcl-2 protein expression was noted. The activation of PKCα occurred within 1 h of MMC exposure, and temporally preceded the induction of COX-2. Similar results were observed in cells exposed to the PKC activator, 3-phorbol 12-myristate 13-acetate. Cotreatment with SAF and MMC abolished the induction of COX-2 by MMC. Furthermore, NS-398, a selective COX-2 inhibitor, significantly enhanced MMC-induced apoptosis by fivefold from 4 ± 2% (MMC alone) to 20 ± 2% (MMC plus NS-398). There was no discernible change in COX-2 mRNA levels after a 2-h exposure to MMC but a twofold increase after a 24-h exposure. *Conclusions*: MMC upregulates COX-2 expression, which appears to be an antiapoptotic signal downstream of PKC. Selective inhibition of COX-2 can therefore provide a novel way to enhance MMC-induced apoptosis independent of inhibiting PKC.

Key words Gene expression · Safingol · Protein kinase C · Phorbol esters · Bcl-2

Abbreviations *COX* cyclooxygenase · *PKC* protein kinase C · *SAF* safingol · *PMA* 3-phorbol 12-myristate 13-acetate · *MMC* mitomycin-C

Introduction

COX-1 and COX-2 are the rate-limiting enzymes involved in the conversion of arachidonic acid to prostanoids [11, 53]. COX-1 is considered a housekeeping gene responsible for various physiological functions. In contrast, COX-2 is normally undetectable in most tissues, but can be rapidly induced by proinflammatory or mitogenic stimuli, including cytokines, growth factors, oncogenes, carcinogens and phorbol esters, and by overexpression or activation of PKC [7, 18, 26, 29, 35, 52]. It is noteworthy that COX-2 is also involved in tumorigenesis [10, 26, 38, 62], angiogenesis [33, 59] and metastasis [58] of cancer. Increased expression of COX-2 has been demonstrated in colorectal adenoma and carcinoma [12], gastric cancer [44], Barrett's esophagus and associated adenocarcinomas [63], esophageal carcinoma [64], pancreatic cancer [60], lung cancer [19], breast cancer [54], and cancer of the head and neck [4].

Forced expression of the COX-2 gene in rat intestinal epithelial cells results in an increased level of Bcl-2 protein and resistance to apoptosis induction [57]. Prostaglandin E_2, a major product of COX-2, has been

This work was supported in part by a Research Fellowship in Translational Cancer Research, American Association for Cancer Research-Amgen Inc. (to C.-T.H.) and National Cancer Institute grant CA67819 (to G.K.S.)

C.-T. Hsueh (✉) · C.-F. Chiu
Division of Hematology/Oncology,
Department of Internal Medicine,
China Medical College Hospital,
Taichung, Taiwan
e-mail: d7198@hpd.cmch.org.tw
Tel.: +886-4-2052121 ext. 2403; Fax: +886-4-2034608

D. P. Kelsen · G. K. Schwartz
Gastrointestinal Oncology Research Laboratory,
Division of Solid Tumor Oncology,
Department of Medicine,
Memorial Sloan-Kettering Cancer Center,
New York, NY 10021, USA

shown to inhibit apoptosis and increase Bcl-2 expression in a human colon cancer cell line [51]. Liu et al. have recently reported that NS-398, a selective COX-2 inhibitor, induces apoptosis and downregulates Bcl-2 expression in LNCaP prostate cancer cells [30]. In addition, COX-2 inhibitors have been shown to cause suppression of tumor growth in nude mice bearing gastric cancer xenografts by induction of apoptosis [48]. These findings indicate that COX-2 is involved in anti-apoptosis signaling and could be a target for apoptosis modulation in cancer chemotherapy.

PKC, a family of serine and/or threonine protein kinases, has been shown to play a major role in anti-apoptosis signaling [8, 24, 31, 50]. We have previously reported that SAF, a PKC inhibitor, enhances the cytotoxic effect of MMC in human gastric cancer cells by promoting drug-induced apoptosis [50]. Further study in this laboratory has suggested that activation of PKCα may be responsible for the resistance of MMC-induced apoptosis in MKN-74 gastric cancer cells, and SAF can block the activation of PKCα [8]. These studies have also demonstrated that short (i.e. 1- to 2-h) exposures to PMA, which activates PKC, can abrogate SAF-mediated enhancement of MMC-induced apoptosis and restore the activation of PKCα.

Activation of PKC has been shown to be required for induction of COX-2 [2, 32, 35, 46, 52]. In human normal and malignant cells, several studies have demonstrated that PKCα is involved in the induction of COX-2 expression [29, 32]. Using an antisense oligomer targeted against PKCα, the induction of COX expression by interleukin-1α can be prevented in human endothelial cells [32]. Moreover, further study has demonstrated that both interleukin-1α and PMA can induce COX-2 but not COX-1 in human endothelial cells, and the PKC inhibitors can block the COX-2 induction by interleukin-1α and PMA [3]. Given these observations, we elected to examine the role of COX-2 in the resistance of MMC-induced apoptosis in MKN-74 gastric cancer cells. We found that MMC induces COX-2, which appears to be an antiapoptotic signal downstream of PKC. Therefore, selective inhibition of COX-2 may provide a novel way to enhance MMC-induced apoptosis independent of inhibiting PKC.

Materials and methods

Cell culture and drug treatment

Early-passage human gastric cancer MKN-74 cells were established and characterized as described previously [37, 39]. All the cultures were maintained in standard MEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 20% heat-inactivated normal calf serum (Intergen) at 37 °C in a humidified atmosphere containing 5% CO_2. MMC was purchased from Bristol Laboratories. NS-398 was purchased from Cayman. SAF was obtained from Eli Lilly. PMA was purchased from Sigma. The cytotoxicity of each agent in MKN-74 cells for a 24-h exposure was determined by the Alamar blue assay [1], and the concentrations causing less than 20% growth inhibition was used in this study. At 24 h after passage, when cells were approximately 50–60% confluent, they were exposed to the indicated drug treatment. MMC 1 μg/ml was used in this study, and combination treatments were set up with NS-398 (100 μ*M*) or SAF (50 μ*M*) given 1 h before the addition of MMC. Cells were checked for mycoplasma contamination at least every 6 months with a GEN-Probe mycoplasma rapid detection kit (Fischer Scientific) and consistently tested negative.

Preparation of membrane and cytosolic protein extracts

Membrane and cytosolic protein extracts were prepared based upon published methods [34] with some modifications. Briefly, 2–4×10^6 MKN-74 cells were suspended in ice-cold lysis buffer (20 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 2 m*M* [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 10 m*M* β-mercaptoethanol, 0.5 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml aprotinin) at a concentration of 10^7 cells/ml. Cells were lysed by passing through a 27-gauge needle three times, and further homogenized by a motorized pestle for 50 strokes. The extent of cell lysis was examined under a microscope throughout the homogenization, and consistent results with more than 95% cell lysis were obtained. Cell homogenates were centrifuged at 27,000 *g* for 30 min at 4 °C. The supernatants were collected as cytosolic protein extracts. The pellets were resuspended in ice-cold lysis buffer plus 1% Triton-X for 30 min at 4 °C, and intermittently homogenized by a motorized pestle every 10 min. The undissolved debris was removed after centrifugation at 12,000 *g* for 10 min, and the clarified supernatants were collected as membrane protein extracts. Protein concentration was determined by Bio-Rad protein assay. For Western blot analysis, 30 μg of protein from each extract was used.

Preparation of whole cell lysates and Western blot analysis

Cells were washed twice with cold phosphate-buffered saline, and then lysed by scraping into a radioimmunoprecipitation assay buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing the protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 1 m*M* sodium orthovanadate). The lysate was left on ice for 30 min, passed through a 21-gauge needle twice, and then centrifuged at 15,000 *g* for 20 min in a microfuge at 4 °C. The clarified supernatant was collected and protein concentration was determined by the Bio-Rad protein assay. Whole cell lysate containing 50 μg protein from each sample was used in immunoblotting as previously described [17, 21]. The gels were then electroblotted onto PVDF membranes (Immobilon-P, Millipore). Ponceau S (Sigma) staining of the membranes was performed to assess the equivalence of sample loading and gel transfer followed by destaining with water as previously described [47].

Antibodies purchased from Santa Cruz Biotechnology (unless otherwise specified) were used to detect the proteins of interest: monoclonal PKCα (Upstate Biotechnology), goat polyclonal COX-2, and monoclonal Bcl-2. The horseradish peroxidase-conjugated antibodies against mouse and goat IgG were used as secondary antibodies. The detection of antibody binding was performed by using Pierce SuperSignal chemiluminescent detection reagents with the protocols recommended by the manufacturer, and blots were exposed to NEN Renaissance X-ray film with intensifying screens. The linear range signal intensity of each specific band on the fluorogram was quantitated by a densitometric scanning system and comparison of proteins of interest was performed after normalization to the densitometric scanning of the ponceau S staining.

Quantitative fluorescent microscopy

The quantitative fluorescent microscopy method for apoptosis determination involves staining with bisbenzimide trihydrochloride (Hoechst-33258) of condensed chromatin, which characterizes the cells undergoing apoptosis [15, 50]. After trypsinization, adherent

and non-adherent cells were washed with PBS, fixed in 3% paraformaldehyde, and then incubated at room temperature for 10 min. The fixative was removed and the cells were washed with PBS, resuspended in 20 µl PBS containing 8 µg/ml of bisbenzimide trihydrochloride, and incubated at room temperature for 15 min. Aliquots of the cells (10 µl) were placed on glass slides coated with 3-amino-propyl-triethoxysilane, and duplicate samples of 400 cells each were counted and scored for the incidence of apoptotic chromatin condensation using an Olympus BH-2 fluorescence microscope equipped with a BH2-DM2U2UV Dichetomic mirror cube filter.

Northern blot analysis

Total cellular RNA preparation, Northern blot analysis and preparation of prehybridization and hybridization buffers were carried out as previously described [20]. To assess the equivalence of sample loading and gel transfer, 30 µg RNA from each sample was treated with ethidium bromide prior to loading [45]. Samples were then visualized by UV illumination of both the gels after electrophoresis and the nylon filters after capillary transfer. RNA blots were prehybridized at 42 °C for 12 h, then hybridized to COX-2 ^{32}P-labeled cDNA probe of specific activity greater than 10^9 cpm/µg at 42 °C for 12–16 h. A 1.2-kbp COX-2 cDNA fragment (purchased from Cayman) was used in preparing ^{32}P-labeled probes by a Multiprime DNA labeling system (Amersham). Unbound probe was removed by washing blots with two changes of a solution containing 2 × SSC and 0.2% SDS for 30 min each at room temperature, and with two changes for 30 min each of 0.2 × SSC and 0.2% SDS at 60 °C. Blots were exposed to Kodak X-OMAT AR film with intensifying screens. Specific bands of interest (about 4.5-kb COX-2 mRNA) were quantitated with a Fuji BAS 2500 Phosphor Imaging System.

Statistical analysis

All experiments were performed at least twice with similar results, and the results of one representative experiment are shown. The statistical significance (*P*-value) of the experimental results was determined by the two-sided Student's *t*-test.

Results

MMC and PMA activate PKCα and induce the protein expressions of COX-2 and Bcl-2

Exponentially growing MKN-74 cells were exposed to 1 µg/ml MMC, and at different time-points cells were collected and fractionated into membrane and cytosolic extracts. Western blots were performed using PKCα, COX-2 and Bcl-2 antibodies. As shown in Fig. 1, after a 1-h exposure to MMC, there was activation of PKCα, which was demonstrated by translocation of PKCα from the cytosolic to the membrane fraction. COX-2 is a membrane-bound protein residing at both the endoplasmic reticulum and the nuclear envelope [36]. COX-2 protein expression was gradually upregulated to about five- to eightfold after a 2-h exposure to MMC when compared to basal expression, and remained persistently induced after a 27-h exposure. COX-1 protein expression was not increased after MMC exposure (data not shown). The expression of Bcl-2 protein, an antiapoptotic protein residing mainly in the outer membrane of mitochondria [5, 25], was increased two- to threefold by

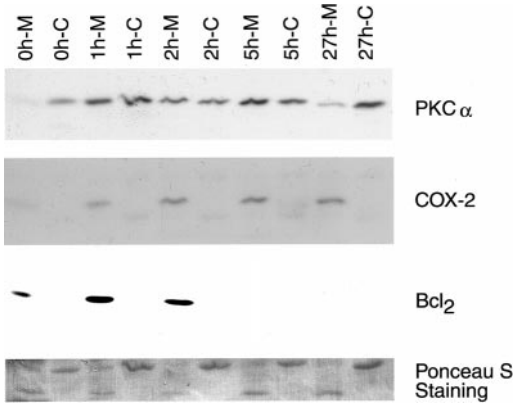

Fig. 1 Effects of MMC on the membrane translocation of PKCα and expression of COX-2 and Bcl-2 proteins. MKN-74 cells were treated with 1 µg/ml MMC for 0 (control), 1, 2, 5 and 27 h, and subsequently membrane (*M*) and cytosolic (*C*) lysates were prepared and Western blot analysis performed as described in Materials and methods. Ponceau S staining of the membrane is displayed in the lower panel to demonstrate equal loading and even transfer of the gel

MMC after the initial 1–2-h exposure, and the expression of Bcl-2 was no longer detectable after prolonged exposure.

Similar results on the activation of PKCα, and induction of COX-2 and Bcl-2 protein expression were observed when MKN-74 cells were exposed to the PKC activator PMA (Fig. 2). After the short (1- to 2-h) exposure to PMA, PKCα was activated, and the protein expression of COX-2 and Bcl-2 was induced. With the prolonged (24-h) exposure to PMA, there was depletion of activated PKCα and loss of COX-2 induction, as opposed to persistent COX-2 induction after prolonged exposure to MMC as shown in Fig. 1. These results indicate that after cellular exposure to MMC or PMA, the levels of COX-2 and Bcl-2 proteins are temporally

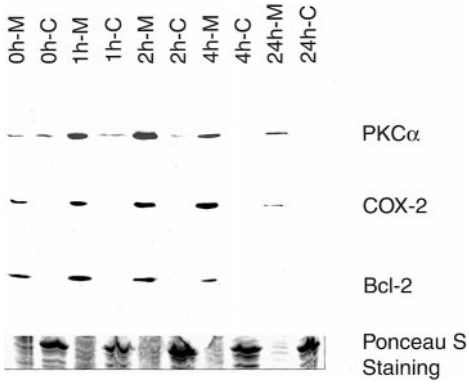

Fig. 2 Effects of PMA on the membrane translocation of PKCα and expression of COX-2 and Bcl-2 proteins. MKN-74 cells were treated with 10 n*M* PMA for 0 (control), 1, 2, 4 and 24 h, and subsequently membrane (*M*) and cytosolic (*C*) lysates were prepared and Western blot analysis performed as described in Materials and methods. Ponceau S staining of the membrane is displayed in the lower panel to demonstrate equal loading and even transfer of the gel

up-regulated following activation of PKCα. The induction of COX-2 protein may be due to the activation of PKCα.

Inhibition of PKC suppresses the induction of COX-2 by MMC

To further confirm that COX-2 is a downstream anti-apoptotic signal for PKC, we examined the expression of COX-2 in MKN-74 cells after inhibition of PKC by SAF. SAF, the L-*threo* enantiomer of dihydrosphingosine, is a PKC-specific inhibitor that inhibits PKC enzyme activity at micromolar concentrations [49, 50]. Whole-cell lysates from cells exposed for 24 h to no drug, 1 μg/ml MMC, 50 μ*M* SAF, and 50 μ*M* SAF plus 1 μg/ml MMC were used for immunoblotting analysis of COX-2 protein levels (Fig. 3). SAF alone suppressed COX-2 expression by about 80% when compared to the control cells. MMC alone induced COX-2 by five- to eightfold, and cotreatment with SAF and MMC suppressed the COX-2 induction by about 80%. These results further substantiate the notion that PKC is an upstream regulator of COX-2 expression.

Selective inhibition of COX-2 enhances MMC-induced apoptosis

To further characterize the role of COX-2 in apoptosis signaling, NS-398, a selective COX-2 inhibitor, was used in combination with MMC to determine whether MMC-induced apoptosis could be enhanced by inhibition of COX-2. NS-398 is a sulfonamide derivative that inhibits COX-2 with an IC_{50} of as low as 30 n*M*, and does not affect COX-1 activity at concentrations exceeding 100 μ*M* [14]. MKN-74 cells were exposed to the following conditions for 24 h: no drug, 100 μ*M* NS-398, 1 μg/ml MMC, and 100 μ*M* NS-398 in combination with 1 μg/ml MMC. Apoptosis was evaluated by quantitative fluorescent microscopy of the nuclear changes induced by apoptosis, as determined by bisbenzimide trihydrochloride (Hoechst-33258) staining of condensed nuclear chromatin (Fig. 4). Minimal apoptosis induction was observed when cells were exposed to NS-398 (2 ± 1%) or MMC (4 ± 2%) alone. However, significant enhancement of apoptosis induction was noted in the NS-398 and MMC cotreatment group (20 ± 2%) when compared to either NS-398 ($P < 0.02$) or MMC ($P < 0.03$) alone.

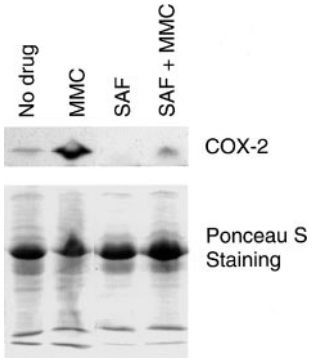

Fig. 3 Effects of MMC and SAF on COX-2 protein expression. MKN-74 cells were exposed to no drug (control), 1 μg/ml MMC, 50 μ*M* SAF, and the combination of 50 μ*M* SAF plus 1 μg/ml MMC for 24 h, and subsequently whole cellular lysates were prepared and Western blot analysis performed as described in Materials and methods. Ponceau S staining of the membrane is displayed in the lower panel to demonstrate equal loading and even transfer of the gel

MMC induces COX-2 mRNA expression only after prolonged exposure

To further examine the molecular mechanism responsible for the induction of COX-2 expression by MMC, Northern blot analysis for COX-2 mRNA expression was performed in MKN-74 cells after exposure to 1 μg/ml MMC for different times. As shown in Fig. 5, there was about a twofold increase in COX-2 mRNA level after a 24-h exposure to MMC. However, COX-2 mRNA levels remained relatively unchanged for the first 2 h of exposure to MMC. In conjunction with Western blot analysis results shown in Fig. 1, these results imply that the induction of COX-2 expression by short (e.g. 1- to 2-h) exposures to MMC is mediated at the level of posttranscription and/or translation. The upregulation of COX-2 expression after prolonged (e.g. 24-h) exposure to MMC occurs at the level of posttranscription and/or transcription.

Discussion

The current study demonstrates that COX-2 is induced by MMC in MKN-74 cells. A similar COX-2 induction has been observed when a different DNA alkylating agent such as cisplatin, or a different cell line such as human breast cancer MCF-7, was used [5]. Upon exposure of cells to DNA-damaging agents, induction of COX-2, which appears to be triggered by activation of PKC, contributes to the cellular resistance to apoptosis induction. Previously, we have shown potentiation of apoptosis by treatment with the PKC-specific inhibitor SAF in MMC-treated MKN 74 cells [50]. Here we demonstrated that, independently of inhibition of PKC, selective inhibition of COX-2 significantly enhances MMC-induced apoptosis. A preliminary report by Nokihara et al. also indicates that COX-2 is induced after exposure to anticancer drugs (etoposide, doxorubicin and vincristine) in human lung cancer cells, and a selective inhibitor of COX-2 increases the sensitivity to these anticancer agents [41]. Taken together, selective inhibition of COX-2 can potentially provide a novel way to enhance chemosensitivity in the treatment of solid tumor malignancies. In further studies it is planned to use

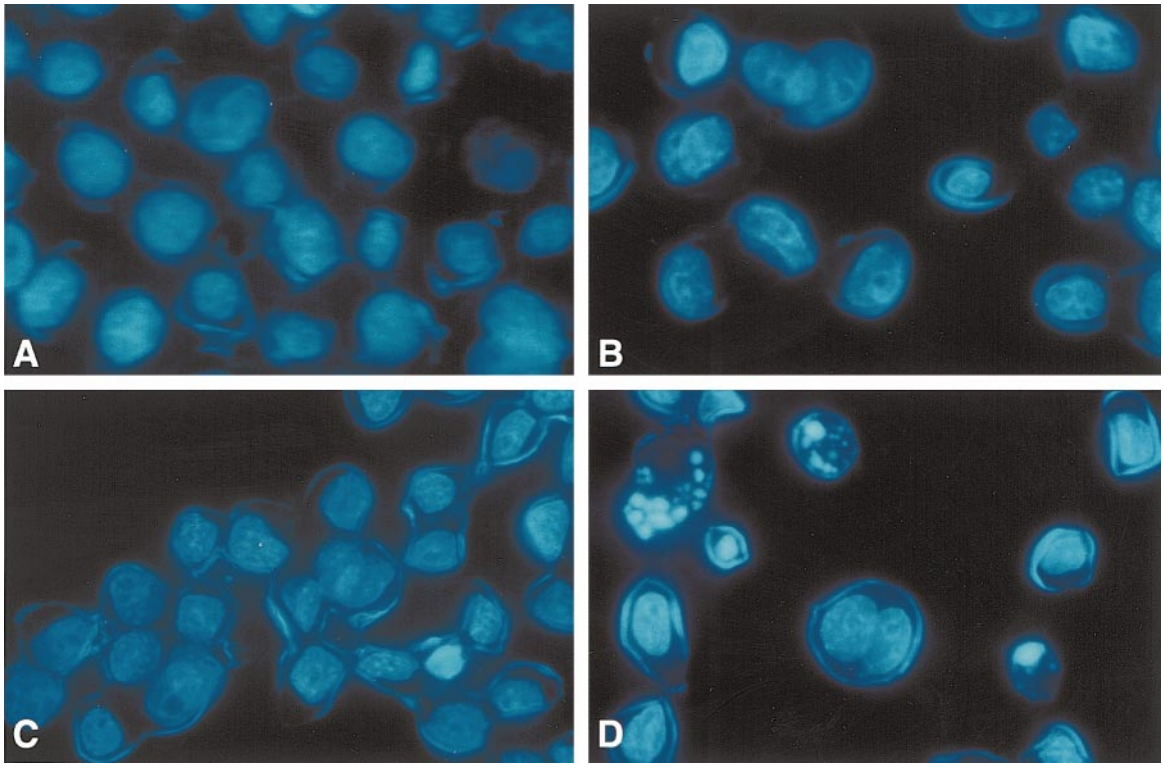

Fig. 4A–D Effects of NS-398 on apoptosis induction by MMC. The photomicrographs show representative fields of MKN-74 cells stained with bisbenzimide trihydrochloride (Hoechst-33258) to evaluate nuclear chromatin condensation (i.e. apoptosis induction) after a 24-h treatment with no drug (**A**), 100 μ*M* NS-398 (**B**), 1 μg/ml MMC (**C**), and the combination of 100 μ*M* NS-398 plus 1 μg/ml MMC (**D**)

similar schema in a panel of solid tumor cell lines with a wide variety of chemotherapeutic agents and combinations.

Activation of PKC has been shown to be required for induction of COX-2 [2, 32, 35, 46, 52]. An antisense oligomer targeted against PKCα has been shown to prevent induction of COX expression by interleukin-1α in human endothelial cells [32]. Additionally, both interleukin-1α and PMA can induce COX-2 but not COX-1 in human endothelial cells, and the PKC inhibitors can block COX-2 induction by interleukin-1α and PMA [3]. Liu and Rose have reported their preliminary findings that PMA induces COX-2 expression in the human breast cancer cell line, MDA-MB-231 [29]. In the same study, calphostin-C, a PKC inhibitor, was shown to suppress basal COX-2 expression at certain concentrations. It has previously been shown in our laboratory that the activation of PKCα might be responsible for the resistance of MMC-induced apoptosis in MKN-74 cells, and that abolishing the activation of PKCα by SAF leads to significant enhancement of MMC-induced apoptosis [8, 50]. In the current study, we have identified that the activation of PKCα precedes COX-2 induction after treatment with MMC or PMA. SAF alone represses basal COX-2 expression, and cotreatment of SAF and MMC suppresses COX-2 induction by MMC. It is very likely that PKC, especially PKCα, is the upstream regulator of COX-2 expression. Although COX-2 exhibits consensus sequences for PKC phos-

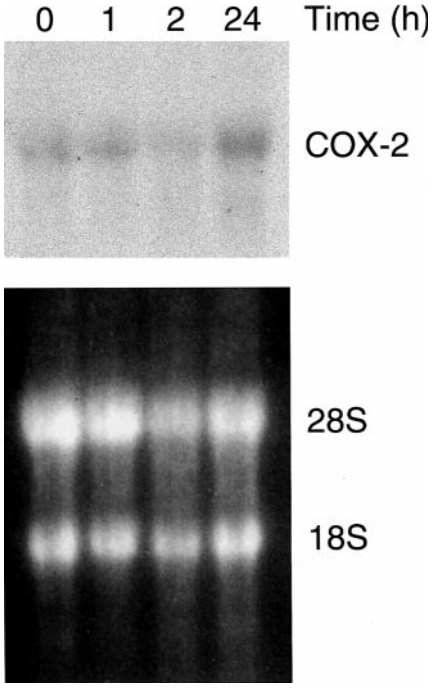

Fig. 5 Effects of MMC on COX-2 mRNA expression. COX-2 mRNA levels in MKN-74 cells exposed to 1 μg/ml MMC for 0, 1, 2 and 24 h were determined by Northern blot analysis as described in Materials and methods. The photograph of the nylon filter exhibiting ethidium bromide fluorescence of 28 S and 18 S ribosomal RNAs is displayed in the lower panel, demonstrating equal loading and even transfer of the gel

phorylation, direct interaction between PKC and COX-2 has not been identified [61]. Moreover, overexpression of PKCζ, an atypical isoform lacking the phorbol ester binding site, has been shown to induce COX-2 expression in rat mesangial cells [35]. To better understand the interaction between various PKC isoforms and COX-2, experiments will be carried out using an antisense approach to suppress the gene expression of different PKC isoforms. The effects on COX-2 induction after exposure to MMC or PMA will be examined under these circumstances.

Although in this study, we were not able to identify any significant apoptosis induction by COX-2 inhibitor alone, COX-2 inhibitors have been shown to cause suppression of tumor growth in nude mice bearing gastric cancer xenografts by induction of apoptosis [48]. Several lines of evidence suggest that COX-2 is an upstream regulator of Bcl-2 [30, 51, 57]. Liu et al. have reported that NS-398 induces apoptosis and downregulates the expression of the active Bcl-2 protein in human prostate cancer cells, LNCaP [30]. It has also been shown that prostaglandin E2, which is a main product of COX-2, can induce Bcl-2 expression and prevent apoptosis induction by COX-2 inhibitor in human colon cancer cells [51]. In the present study, we showed Bcl-2 induction in conjunction with COX-2 induction within 1 to 2 h of exposure to MMC or the PKC activator PMA. Moreover, selective inhibition of COX-2 leads to enhancement of MMC-induced apoptosis. The signal cascade between the COX-2 and the apoptosis-related signals such as Bcl-2 and Bax remains to be established. We are planning a series of studies using COX-2 inhibitor, COX-2 antisense oligomer and overexpression of COX-2 by a gene transfer technique to further characterize the role of COX-2 in apoptosis signaling.

COX-2 is an intermediate early response gene to various stimuli such as growth factors, cytokines, tumor promoters, hormones and bacterial endotoxin. In response to various stimuli, COX-2 gene expression can be regulated at different levels [53]. Many studies have identified upregulation of COX-2 expression caused by enhanced transcription in carcinoma and normal cells [23, 27, 28, 55]. However, regulation of COX-2 gene expression at posttranscriptional or translational levels has been uncovered by discordant expression levels of COX-2 mRNA and protein under certain circumstances such as nitric oxide production and chronic hypoxia [6, 22, 56]. Dean et al. have reported that p38 mitogen-activated protein kinase regulates COX-2 mRNA stability in lipopolysaccharide-treated human monocytes [9]. Several studies have demonstrated that dexamethasone suppresses COX-2 expression by destabilizing its mRNA [13, 40, 43]. Moreover, IL-1α has been shown to prolong COX-2 mRNA half-life in human endothelial cells [42].

Our present study suggests that the upregulation of COX-2 expression by a short exposure to MMC is mediated at posttranscriptional/translational level in conjunction with PKCα activation. Interestingly, PKCα recently has been shown to regulate IκBα expression posttranscriptionally by facilitating its mRNA export from the nucleus [16]. Our findings also indicate that prolonged exposure to MMC increases COX-2 mRNA expression, which may very well contribute to the persistently elevated COX-2 protein levels despite the depletion of activated PKCα. Further study is warranted to fully characterize the molecular mechanisms responsible for upregulation of COX-2 expression by MMC and the role of PKCα.

Increased expression of COX-2 has been demonstrated in many solid tumor malignancies. Based on the findings presented here and the preliminary findings of Nokihara et al. [41], it is conceivable that induction of COX-2 plays an important role in the resistance to chemotherapy-induced apoptosis in most of the solid tumor malignancies. COX-2 is also involved in cancer angiogenesis [33, 59] and metastasis [58]. Therefore, clinical use of COX-2 inhibitors in combination with chemotherapy may not only enhance chemosensitivity but also inhibit the angiogenic and metastatic potential of cancer. With these rationales, we are currently planning clinical trials to incorporate COX-2 inhibitors into chemotherapeutic regimens for the treatment of solid tumor malignancies.

References

1. Ahmed SA, Gogal RM Jr, Walsh JE (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]thymidine incorporation assay. J Immunol Methods 170: 211
2. Barry OP, Kazanietz MG, Pratico D, FitzGerald GA (1999) Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. J Biol Chem 274: 7545
3. Blanco A, Habib A, Levy-Toledano S, Maclouf J (1995) Involvement of tyrosine kinases in the induction of cyclo-oxygenase-2 in human endothelial cells. Biochem J 312: 419
4. Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslow RA, Koki AT, Woerner BM, Masferrer JL, Dannenberg AJ (1999) Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res 59: 991
5. Chen-Levy Z, Nourse J, Cleary ML (1989) The Bcl-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14,18) translocation. Mol Cell Biol 9: 701
6. Chida M, Voelkel NF (1996) Effects of acute and chronic hypoxia on rat lung cyclooxygenase. Am J Physiol 270: L872
7. Coffey RJ, Hawkey CJ, Damstrup L, Graves-Deal R, Daniel VC, Dempsey PJ, Chinery R, Kirkland SC, DuBois RN, Jetton TL, Morrow JD (1997) Epidermal growth factor receptor activation induces nuclear targeting of cyclooxygenase-2, basolateral release of prostaglandins, and mitogenesis in polarizing colon cancer cells. Proc Natl Acad Sci U S A 94: 657
8. Danso D, Farsi K, Greaney C, Spriggs D, Kelsen DK, Schwartz GK (1997) The protein kinase C inhibitor safingol restores mitomycin-C-induced loss of PKCα and PKC∈ expression in association with the induction of apoptosis. Proc Am Assoc Cancer Res 38: A1305
9. Dean JLE, Brook M, Clark AR, Saklatvala J (1999) p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. J Biol Chem 274: 264

10. DuBois RN, Radhika A, Reddy BS, Entingh AJ (1996) Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology 110: 1259
11. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Vandeputte LBA, Lipsky PE (1998) Cyclooxygenase in biology and disease. FASEB J 12: 1063
12. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107: 1183
13. Evett GE, Xie W, Chipman JG, Robertson DL, Simmons DL (1993) Prostaglandin G/H synthase isoenzyme 2 expression in fibroblasts: regulation by dexamethasone, mitogens, and oncogenes. Arch Biochem Biophys 306: 169
14. Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, Otomo S (1994) NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins 47: 55
15. Haimovitz-Friedman A, Kan CC, Ehleiter D, Persaud RS, McLoughlin M, Fuks Z, Kolesnick RN (1994) Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J Exp Med 180: 525
16. Han YQ, Meng T, Murray NR, Field AP, Brasier AR (1999) Interleukin-1-induced nuclear factor-kappa B-I kappa B alpha autoregulatory feedback loop in hepatocytes – a role for protein kinase C alpha in post-transcriptional regulation of I kappa B alpha resynthesis. J Biol Chem 274: 939
17. Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, New York
18. Herschman HR (1996) Prostaglandin synthase 2. Biochim Biophys Acta 1299: 125
19. Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M, Mitsudomi T, Sugiura T, Takahashi T (1998) Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res 58: 3761
20. Hsueh CT, Dolnick BJ (1994) Regulation of folate-binding protein gene expression by DNA methylation in methotrexate-resistant KB cells. Biochem Pharmacol 47: 1019
21. Hsueh C-T, Kelsen D, Schwartz GK (1998) UCN-01 suppresses thymidylate synthase gene expression and enhances 5-fluorouracil-induced apoptosis in a sequence-dependent manner. Clin Cancer Res 4: 2201
22. Hughes FJ, Buttery LDK, Hukkanen MVJ, O'Donnell A, Maclouf J, Polak JM (1999) Cytokine-induced prostaglandin E2 synthesis and cycloxygenase-2 activity are regulated both by a nitric oxide-dependent and -independent mechanism in rat osteoblasts in vitro. J Biol Chem 274: 1776
23. Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T (1995) Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. J Biol Chem 270: 24965
24. Jarvis WD, Fornari FA Jr, Browning JL, Gewirtz DA, Kolesnick RN, Grant S (1994) Attenuation of ceramide-induced apoptosis by diglyceride in human myeloid leukemia cells. J Biol Chem 269: 31685
25. Jong D de, Prins FA, Mason DY, Reed JC, Ommen GB van, Kluin PM (1994) Subcellular localization of the Bcl-2 protein in malignant and normal lymphoid cells. Cancer Res 54: 256
26. Kelley DJ, Mestre JR, Subbaramaiah K, Sacks PG, Schantz SP, Tanabe T, Inoue H, Ramonetti JT, Dannenberg AJ (1997) Benzo[a]pyrene up-regulates cyclooxygenase-2 gene expression in oral epithelial cells. Carcinogenesis 18: 795
27. Kim Y, Fischer SM (1998) Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells. Regulatory role of CCAAT/enhancer-binding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. J Biol Chem 273: 27686
28. Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, White RL, Prescott SM (1996) Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. Proc Natl Acad Sci U S A 93: 4816
29. Liu X-H, Rose DP (1997) Involvement of protein kinase C in COX-2 expression, activation, and subcellular translocation in a human breast cancer cell line. Proc Am Assoc Cancer Res 38: A4164
30. Liu XH, Yao S, Kirschenbaum A, Levine AC (1998) NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates Bcl-2 expression in LNCaP cells. Cancer Res 58: 4245
31. Lucas M, Sanchez-Margalet V (1995) Protein kinase C involvement in apoptosis. Gen Pharmacol 26: 881
32. Maier JA, Ragnotti G (1993) An oligomer targeted against protein kinase C alpha prevents interleukin-1 alpha induction of cyclooxygenase expression in human endothelial cells. Exp Cell Res 205: 52
33. Masferrer JL, Settle S, Zweifel BS, Edwards DA, Ornberg RL, Raz A, Seibert K (1998) Cyclooxygenase-2 inhibitors as novel antiangiogenic agents. Proc Am Assoc Cancer Res 39: A1855
34. McHugh M, Cheng Y-C (1979) Demonstration of a high affinity folate binder in human cell membranes and its characterization in cultured human KB cells. J Biol Chem 254: 11312
35. Miller BW, Baier LD, Morrison AR (1997) Overexpression of protein kinase C-zeta isoform increases cyclooxygenase-2 and inducible nitric oxide synthase. Am J Physiol 273: C130
36. Morita I, Schindler M, Regier MK, Otto JC, Hori T, DeWitt DL, Smith WL (1995) Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. J Biol Chem 270: 10902
37. Motoyama T, Watanabe H (1983) Carcinoembryonic antigen production in human gastric cancer cell lines in vitro and in nude mice. Gann 74: 679
38. Mullerdecker K, Koppschneider A, Marks F, Seibert K, Furstenberger G (1998) Localization of prostaglandin h synthase isoenzymes in murine epidermal tumors – suppression of skin tumor promotion by inhibition of prostaglandin h synthase-2. Mol Carcinog 23: 36
39. Nabeya Y, Loganzo F Jr, Maslak P, Lai L, Oliveira AR de, Schwartz GK, Blundell ML, Altorki NK, Kelsen DP, Albino AP (1995) The mutational status of p53 protein in gastric and esophageal adenocarcinoma cell lines predicts sensitivity to chemotherapeutic agents. Int J Cancer 64: 37
40. Newton R, Seybold J, Kuitert LM, Bergmann M, Barnes PJ (1998) Repression of cyclooxygenase-2 and prostaglandin E2 release by dexamethasone occurs by transcriptional and post-transcriptional mechanisms involving loss of polyadenylated mRNA. J Biol Chem 273: 32312
41. Nokihara H, Nishioka Y, Shinohara T, Yanagawa H, Sone S (1999) Critical role of cyclooxygenase-2 in sensitivity of human lung cancer cells to anticancer agents. Proc Am Assoc Cancer Res 40: A2824
42. Ristimaki A, Garfinkel S, Wessendorf J, Maciag T, Hla T (1994) Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. J Biol Chem 269: 11769
43. Ristimaki A, Narko K, Hla T (1996) Down-regulation of cytokine-induced cyclooxygenase-2 transcript isoforms by dexamethasone: evidence for post-transcriptional regulation. Biochem J 318: 325
44. Ristimäki A, Honkanen N, Jänkälä H, Sipponen P, Härkönen M (1997) Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res 57: 1276
45. Rosen KM, Villa-Komaroff L (1990) An alternative method for visualization of RNA in formaldehyde agarose gels. Focus 12: 23
46. Rzymkiewicz DM, Tetsuka T, Daphna-Iken D, Srivastava S, Morrison AR (1996) Interleukin-1beta activates protein kinase C zeta in renal mesangial cells. Potential role in prostaglandin E2 up-regulation. J Biol Chem 271: 17241

47. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York
48. Sawaoka H, Kawano S, Tsuji S, Tsujii M, Gunawan ES, Takei Y, Nagano K, Hori M (1998) Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. Am J Physiol 274: G1061
49. Schwartz GK, Jiang J, Kelsen D, Albino AP (1993) Protein kinase C: a novel target for inhibiting gastric cancer cell invasion. J Natl Cancer Inst 85: 402
50. Schwartz GK, Haimovitz-Friedman A, Dhupar SK, Ehleiter D, Maslak P, Lai L, Loganzo F Jr, Kelsen DP, Fuks Z, Albino AP (1995) Potentiation of apoptosis by treatment with the protein kinase C-specific inhibitor safingol in mitomycin C-treated gastric cancer cells. J Natl Cancer Inst 87: 1394
51. Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN (1998) Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res 58: 362
52. Simonson MS, Wolfe JA, Konieczkowski M, Sedor JR, Dunn MJ (1991) Regulation of prostaglandin endoperoxide synthase gene expression in cultured rat mesangial cells: induction by serum via a protein kinase-C-dependent mechanism. Mol Endocrinol 5: 441
53. Smith WL, Garavito RM, DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271: 33157
54. Subbaramaiah K, Telang N, Ramonetti JT, Araki R, DeVito B, Weksler BB, Dannenberg AJ (1996) Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res 56: 4424
55. Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang M, Pezzuto JM, Dannenberg AJ (1998) Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem 273: 21875
56. Tetsuka T, Baier LD, Morrison AR (1996) Antioxidants inhibit interleukin-1-induced cyclooxygenase and nitric-oxide synthase expression in rat mesangial cells. Evidence for post-transcriptional regulation. J Biol Chem 271: 11689
57. Tsujii M, DuBois RN (1995) Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 83: 493
58. Tsujii M, Kawano S, DuBois RN (1997) Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci U S A 94: 3336
59. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN (1998) Cyclooxygenase regulates angiogenesis induced by colon cancer cells [published erratum appears in Cell (1998) 94(2): following 271]. Cell 93: 705
60. Tucker ON, Dannenberg AJ, Yang FK, Zhang F, Teng LS, Daly JM, Soslow RA, Masferrer JL, Woerner BM, Koki AT, Fahey TJ (1999) Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res 59: 987
61. Vezza R, Habib A, Li H, Lawson JA, FitzGerald GA (1996) Regulation of cyclooxygenases by protein kinase C. Evidence against the importance of direct enzyme phosphorylation. J Biol Chem 271: 30028
62. Williams CS, Luongo C, Radhika A, Zhang T, Lamps LW, Nanney LB, Beauchamp RD, DuBois RN (1996) Elevated cyclooxygenase-2 levels in Min mouse adenomas. Gastroenterology 111: 1134
63. Wilson KT, Fu S, Ramanujam KS, Meltzer SJ (1998) Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Res 58: 2929
64. Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K (1999) Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res 59: 198