Malignant Transformation and Antineoplastic Actions of Nonsteroidal Antiinflammatory Drugs (NSAIDs) on Cyclooxygenase-null Embryo Fibroblasts

By Xinping Zhang,* Scott G. Morham,‡ Robert Langenbach,§ and Donald A. Young*

*From the *Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642; the ‡Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27599-7525; and the §National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709*

Summary

In this study, we use primary embryonic fibroblasts derived from cyclooxygenase-deficient transgenic embryos to further investigate the role of the two cyclooxygenases, cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2), in the process of neoplastic transformation. Cells with either, neither, or both of the cyclooxygenases were transformed by Ha-*ras* and/or SV40. Our results show that when a cyclooxygenase enzyme is present, the transformed cells have marked increases in COX-2 and/or COX-1 expression. Nevertheless, each type of cell, deficient in either or both cyclooxygenases, can be readily transformed at almost equal efficiency. Different nonsteroidal antiinflammatory drugs (NSAIDs) were used to examine their possible antineoplastic effects on the transformed cells, which have various levels of expression of COX-1 or COX-2. Our results show that NSAIDs suppress the colony formation in soft agar in a dosage-dependent manner in the absence of the cyclooxygenase(s). Thymidine incorporation and apoptosis analyses further demonstrate that the NSAIDs are effective in the cyclooxygenase-null cells. Our findings with cyclooxygenase knockout cells confirm recent reports that some of the antiproliferative and antineoplastic effects of NSAIDs are independent of the inhibition of either COX-1 or COX-2. They also show that transformation is independent of the status of cyclooxygenase expression, suggesting that the involvement of the cyclooxygenases in tumorigenesis may occur at later steps.

Key words: cyclooxygenase • nonsteroidal antiinflammatory drugs • neoplastic transformation • transgenic knockout cells • *ras*

Two isoforms of cyclooxygenase (COX),[1] COX-1 and COX-2, have been identified (1–3). The two isozymes are regulated differently (1–5), and exhibit distinctive functional differences. COX-1 is expressed constitutively in many cell types, whereas COX-2 is a primary response gene whose expression may be induced by trauma, growth factors, tumor promoters, and a variety of cytokines (for a review, see reference 6). COX-2 was first discovered as an oncogene-responsive cyclooxygenase (7), and increased COX-2 expression has been found in a variety of tumors, including adenomatous polyps, colorectal cancers, and squamous carcinomas (8, 9). It has been shown that overexpression of the enzyme in colon cells prolongs the cell life span (10) and leads to resistance to apoptotic death, which some nonsteroidal antiinflammatory drugs (NSAIDs) that are inhibitors of cyclooxygenases and prostaglandin (PG) synthesis can reverse (11). Also, mice containing mutations in both the adenomatous polyposis coli (APC) gene ($APC^{A716-/+}$) and the COX-2 gene ($COX\text{-}2^{-/-}$) developed fewer intestinal polyps than mice with a functional COX-2 gene (12). A new selective COX-2 inhibitor, SC-58125, suppressed tumor growth in vivo and induced apoptosis in vitro in cell lines that express high levels of COX-2, but the drug was ineffective in HCT-116 cells, which have undetectable levels of cyclooxygenase expression (13). These studies place COX-2 in a central position of colon carcinogenesis and suggest that selective COX-2 inhibition may be a useful approach for chemoprevention or even treatment of the cancers, particularly those with high levels of COX-2 expression.

[1]*Abbreviations used in this paper:* COX, cyclooxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NSAID, nonsteroidal antiinflammatory drug; TPA, 12-*o*-tetradecanoylphorbol 13-acetate.

Despite the extensive investigation in this area, whether or not the cyclooxygenases and PGs produced by them are involved in the malignant transformation per se is unclear. The approach often used for understanding mechanisms has been to evaluate the effects of NSAIDs, but this suffers from the drawback of possible antineoplastic effects of NSAIDs that may be independent of their ability to inhibit cyclooxygenases (for a review, see reference 14), especially at the higher concentrations that are sometimes employed.

In an attempt to provide more definitive information about mechanisms, we studied malignant transformation in the cell lines we created from transgenic cyclooxygenase knockout embryos, which lack COX-1, COX-2, or both COX-1 and COX-2 (15, 16). We find that transformation by *ras* leads to large inductions of the cyclooxygenases when the genes are present. Nevertheless, we also find that all of these cells can be readily transformed in the absence of either or both cyclooxygenase enzymes. We applied several commonly used NSAIDs to the transformed knockout cells and found that the inhibitory effects of NSAIDs on cell growth, transformation, and induction of apoptosis also occur in the absence of either COX-1 or COX-2.

Taken together, our results confirm recent reports that some of the antiproliferative and antineoplastic effects of NSAIDs are independent of the inhibition of either COX-1 or COX-2. They also show that transformation is independent of the status of cyclooxygenase expression, suggesting that the involvement of the cyclooxygenases in tumorigenesis may occur at later steps.

Materials and Methods

Collection of Embryonic Fibroblasts from Cyclooxygenase-deficient Embryos. COX-$1^{+/-}$ or COX-$2^{+/-}$ pregnant heterozygous female mice (15, 16) were killed by CO_2 asphyxiation on day 18 after fertilization. Each embryo was taken from the uterus, separated, minced, and trypsinized twice with 0.1% trypsin-EDTA-DMEM for 15 min. Tissue debris were discarded, and cells were pooled by brief centrifugation and then suspended in DMEM containing 10% fetal bovine serum. Cells from each of the embryos were grown in the same medium at 37°C with 5% CO_2. PCR analysis was used to determine the genotypes of each embryo. Primers used are the following: for COX-1 genotyping, 5′-AGGAGATGGCTGCTGAGTTGG-3′ and 5′-AATCTGACTTTCTGAGTTGCC-3′ were used to detect the intact COX-1 exon 11; 5′-GCAGCCTCTGTTCCACATACAC-3′ and 5′-AATCTGACTTTCTGAGTTGCC-3′ were used to detect the targeted disruption of COX-1 exon 11 containing the neomycin gene. For COX-2 genotyping, primers 5′-ACACACTCTATCACTGGCAC-3′ and 5′-AGATTGTTGTCAGTATCTGCC-3′ were used to detect the endogenous COX-2 gene (the PCR product extending from exon 8 to exon 10); 5′-ACGCGTCACCTTAATATGCG-3′ and 5′-AGATTGTTGTCAGTATCTGCC-3′ were used to detect the targeted disruption of COX-2 exon 8 containing the neomycin gene. COX-$1^{-/-}$/COX-$2^{-/-}$ deficient cell lines were developed by breeding male COX-$1^{-/-}$/COX-$2^{+/-}$ mice and female COX-$1^{+/-}$/COX-$2^{+/-}$ mice; cells from these animals were isolated by an identical protocol as that described above.

Western and Northern Blot Analysis. For Western blot analysis, proteins were collected and dissolved in protein lysis buffers (10 mM Tris, pH 7.6, 1% Triton X-100, 100 mM NaCl, and 2 mM PMSF). 20 μg of protein was loaded and separated by 10% SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes and blotted with polyclonal antibody specific to murine COX-1 (provided by Dr. William Smith, Michigan State University, East Lansing, MI) and mAb specific to COX-2 (Transduction Labs). Membranes were also probed with anti–β-actin antibody (Santa Cruz Biotechnology) to normalize sample differences between the gel lanes. Immunodetection was done using the Enhanced Chemiluminescence Western blotting detection system (ECL kits) purchased from Amersham Pharmacia Biotech. For Northern blot analysis, total RNAs were extracted from the cell with 4 M guanidium thiocyanate homogenization buffer (4 M guanidium thiocyanate, 0.5% sodium laurel sarcosinate, 1% β-mercaptoethanol in 100 mM Tris-HCl, pH 7.5). 20 μg of the total RNA was loaded and then electrophoresed on formaldehyde agarose gels. The separated RNAs were transferred to Duralon UV membrane and hybridized with COX-1– or COX-2–specific cDNA probes. These membranes were later probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize the RNA loading of each sample.

Quantification of PG Production. Primary embryonic fibroblast cells were seeded in duplicate in 12-well plates and grown for 24–48 h in DMEM containing 10% fetal bovine serum. Cells were treated with 100 ng/ml phorbol ester (12-*o*-tetradecanoylphorbol 13-acetate [TPA]) or vehicle for 10 h. PGE2 accumulations in the medium were measured using RIA kits purchased from Amersham Pharmacia Biotech. Cyclooxygenase activities were quantified by adding 30 μM free AA to the culture and measuring PGE2 production after 15 min had elapsed. The quantity of cells per well was counted using a hemacytometer. The experiments were conducted using primary cultured cells from several different cyclooxygenase-deficient embryos, and similar data were recorded.

Transformation Assays. Early passage embryonic fibroblasts were plated in 100- or 60-mm dishes, and 10–20 μg of plasmids containing pEJ-*ras* and SV40 oncogenes were transfected at a 1:1 ratio into the cells by calcium-phosphate precipitation methods. The cells were allowed to grow for 2 d after transfection, then split in a 1:3 ratio into new tissue culture plates and cultured for an additional 2 wk. Plates were fixed with ethanol and subsequently stained with Giemsa dyes; foci formed on the plate were counted. Growing foci were also picked and allowed to grow to confluence to measure cyclooxygenase expressions.

Drug Studies. Cells expressing various levels of cyclooxygenase were plated at the same density in 0.33% soft agar with different NSAIDs: indomethacin (Sigma Chemical Co.), NS-398 (Cayman Chemicals), sulindac (Sigma Chemical Co.), ibuprofen (Sigma Chemical Co.), and piroxicam (Sigma Chemical Co.). The drugs were dissolved in DMSO, and identical volumes of top agar containing various concentrations of drugs were replenished at days 2 and 5. Cells were allowed to grow for 2 wk, and foci were counted. Duplicate plates were counted for each type of drug at each different concentration.

DNA Synthesis. DNA synthesis was estimated by [^{3}H]thymidine incorporation into cellular DNA. Cells in triplicate were plated in 24-well plates for 24 h and then treated with vehicle or a different NSAID for 24 h. [^{3}H]Thymidine (1 mCi/ml) was added and allowed to label the cells for 24 h. The cells were washed twice with cold DMEM, twice with 10% TCA, and three times with ethanol. The cells were then lysed in NaOH/SDS, and radioactivity was counted.

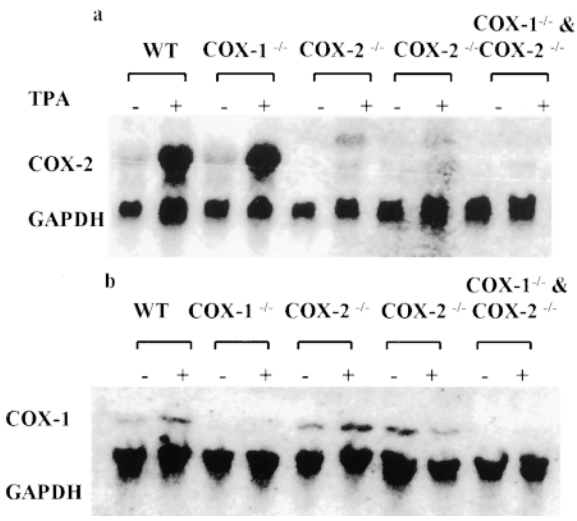

Figure 1. Northern blot analysis of cyclooxygenase mRNA levels in embryo fibroblasts upon TPA stimulation. Wild-type (WT), COX-$1^{-/-}$, COX-$2^{-/-}$, and COX-$1^{-/-}$/COX-$2^{-/-}$ cells were treated with 100 ng/ml TPA for 2 h. 20 μg of total RNA was loaded and then separated on formaldehyde agarose gels. The RNAs were then transferred to Duralon UV membranes and hybridized with COX-2 (a) or COX-1 (b) specific cDNA probes. The same membranes were also probed with GAPDH to examine the DNA loading.

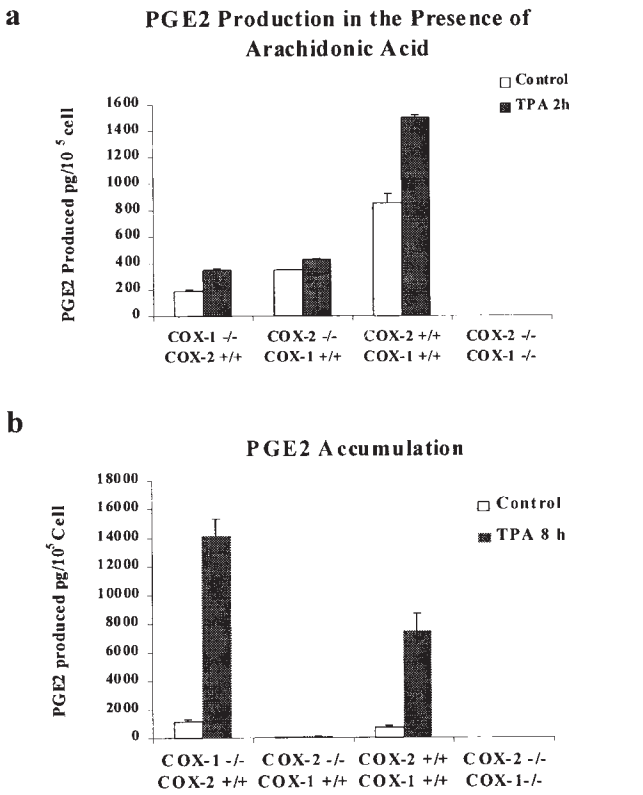

Figure 2. PG production in cyclooxygenase-null cells. (a) Wild-type, COX-$1^{-/-}$, COX-$2^{-/-}$, and COX-$1^{-/-}$/COX-$2^{-/-}$ cells were treated with or without 100 ng/ml TPA for 2 h. Media were removed, and 30 μM free AA was added into the culture. PGE2 generated within 15 min was quantified by RIA kits purchased from Amersham Pharmacia Biotech. (b) The same cells were treated with TPA for 8 h. Media were collected and measured directly by the RIA kit. Cell numbers in each well were counted in a hemacytometer.

DNA Fragmentation Analysis. Postconfluent cells were treated with each of the NSAIDs for 72 h. NSAID-treated and vehicle-treated cells were washed with PBS and then lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 1% SDS, and proteinase K. Lysates were digested for 3 h at 55°C and then extracted with phenol/chloroform. The genomic DNA was precipitated in ethanol and resolved on a 1.5% agarose gel at 60 V for 4 h.

Results

Cyclooxygenase-deficient Cell Lines Do Not Express Their Native COX-1 and/or COX-2 mRNA Transcripts. Cells from each embryo were collected and grown as described in Materials and Methods, the genotype of each cell line was identified by PCR, and total RNA was used for Northern blot analyses. As anticipated (Fig. 1, a and b), wild-type cells express both TPA-inducible COX-2 and constitutive COX-1 mRNA, whereas cells from COX-$1^{-/-}$ or COX-$2^{-/-}$ embryos each lack expression of their respective wild-type COX genes. Cells from COX-$1^{-/-}$/COX-$2^{-/-}$ deficient embryos lack expression of both COX-1 and COX-2 transcripts. A small amount of abnormal COX-2 transcript is detected by Northern blot analysis in COX-$2^{-/-}$ cells; however, Western blot analyses and measurements of PGE2 production confirm the disruption of the cyclooxygenase genes.

COX-$1^{-/-}$, COX-$2^{-/-}$, or COX-$1^{-/-}$/COX-$2^{-/-}$ Cells Demonstrate Deficiencies in PG Production from Each of Their Respective Cyclooxygenases. Reports have shown that COX-1 and COX-2 isozymes exhibit distinctive selectivity over a different source of arachidonic acid (AA). COX-2 is more efficient at using mitogen-activated endogenous AA, whereas COX-1 is more efficient at using exogenously added AA, or AA released by stimuli such as calcium ionophore (17, 18). Therefore, we investigated the production of PGE2 from these cells by measuring metabolism of either exogenous AA or endogenous AA. In the presence of 30 μM of added AA (Fig. 2 a), both COX-1– and COX-2–deficient cells produced less PGE2 than wild-type cells. TPA stimulation doubled the production of PGE2 in both wild-type and COX-2–containing cells. In the absence of added AA (Fig. 2 b), when 8-h PGE2 accumulation was measured, stimulation by TPA was found to dramatically increase PGE2 production in both wild-type and COX-2–containing (COX-$1^{-/-}$) cells, but the COX-$2^{-/-}$ cells produced almost undetectable levels of PGE2 even with an active COX-1 enzyme. TPA-activated AA releases were also measured by labeling the cells with ^{3}H-AA, and all of the cells, regardless of the presence of the enzymes, demonstrated similar induction of AA release in response to TPA or calcium ionophore (data not shown). Also, as expected, the COX-$1^{-/-}$/COX-$2^{-/-}$ cells do not produce detectable PGE2 either in the presence or the absence of AA or TPA. The failure to detect any PG production in the

Table I. *Focus Formation of Cyclooxygenase-null Cells Transformed by SV40 and/or EJ-ras*

Cell type	Experiment 1			Experiment 2		
	Control	SV40	EJ-*ras* + SV40	Control	EJ-*ras*	EJ-*ras* + SV40
Wild-type	0	13	150	0	0	50
	0	29	107	0	0	22
	0	–	166			
Cox-$2^{-/-}$	0	13	140	0	0	50
	0	20	99	0	0	49
	0	14	144			
Cox-$2^{+/-}$	0	14	142			
Cox-$1^{-/-}$	0	12	135	0	0	69
	0	4	98	0	0	63
Cox-$1^{-/-}$/Cox-$2^{-/-}$	0	3	110	0	0	28

Primary cultures of embryonic fibroblasts were collected as described in Materials and Methods. Cells of each type were transfected with 10–20 μg of each viral gene by calcium-phosphate precipitation methods. Foci were counted at 14 d after transfection as described.

COX-$1^{-/-}$/COX-$2^{-/-}$ cells confirms the inactivation of both genes. Previous studies by Kirtikara et al. (19) showed that COX-1 or COX-2 is increased in the null cells to compensate for the deficiency of PG production. Our data support this finding by showing that in the COX-$1^{-/-}$ cells, PGE2 production was increased both at the basal and the stimulated level (Fig. 2 b). However, we failed to detect any increased level of PGE2 production in the COX-$2^{-/-}$ cells compared with the wild-type cells.

Neoplastic Transformation by EJ-ras Plus SV40 Is Independent of Cyclooxygenase Expression. To examine the role of cyclooxygenases in neoplastic transformation, we conducted transformation assays in primary cultured embryonic cells. The oncogene *ras* was used in this study because its expression contributes to many malignancies, and several studies, including our unpublished data, have demonstrated that transformation by *ras* induces COX-2 gene expression (20). The expression of a mutant *ras* gene usually does not by itself lead to a transformed phenotype in primary cultured cells; additional mutations in other protooncogenes such as c-*myc*, or inactivation of tumor suppressor genes such as p53, are required to achieve a full malignant transformation (21). Thus, we evaluated the contribution of the cyclooxygenases to the transforming activity of activated EJ-*ras* by adding wild-type SV40 viral gene to complete the transformation. As shown in Table I, transfection of EJ-*ras* alone did not induce any foci formation, and transfection of SV40 alone induced only a few small foci. These foci from SV40 transformation failed to grow in the 0.33% soft agar within 2 wk. In contrast, SV40 plus EJ-*ras* cooperatively led to dramatically increased foci formation, and these transformed cells developed a fully transformed phenotype, as shown by their loss of contact inhibition and their rapid growth in soft agar and nude mice. By comparing the wild-type cells and the cells deficient in either or both of the cyclooxygenases, our results demonstrate that cells lacking either or both of the cyclooxygenases can be readily transformed by SV40 plus *ras* at almost equal frequencies. Both cyclooxygenase-null cells and wild-type cyclooxygenase-containing cells showed similar morphology and growth behavior in soft agar. This result indicates that cyclooxygenases are not required for transformation per se, nor specifically for the operation of the *ras*-dependent transformation pathway.

Expression of the cyclooxygenase isozymes in transformed embryonic fibroblasts was analyzed using Western blots. As shown in Fig. 3 a, six of the seven transformed clones showed moderate or marked increases of COX-2 expression, and three of them had increases in the expression of both enzymes. However, one of the clones showed unchanged expression of both cyclooxygenases, again suggesting that fibroblasts can be transformed without altering the cyclooxygenase pathway. Interestingly, we found that the embryo fibroblasts immortalized only by SV40 expressed less cyclooxygenase, both COX-1 and COX-2, than the untransformed cells (Fig. 3, b and c). Further studies confirmed that the corresponding cyclooxygenase product, PGE2, was dramatically reduced in the cells that were transformed by SV40 alone (data not shown). Isoenzyme expression was also measured in cells lacking either cyclooxygenase (Fig. 3, c and d). In the cells lacking COX-1, transformation by EJ-*ras* plus SV40 led to an increased basal expression of COX-2 (Fig. 3 d, lanes 3–6), and in cells lacking COX-2, the expression of COX-1 was also markedly increased (Fig. 3 c, lanes 7–10). The results from the Western blot analyses are consistent with our Northern blot analyses, further confirming the genotype of each cell line we used in these studies. Taken together, these experiments illustrate that both cyclooxygenases can be selectively altered by oncogene *ras* or viral gene SV40; these actions are oncogene specific and might involve the alterations of signal transduction pathways that lead to the ex-

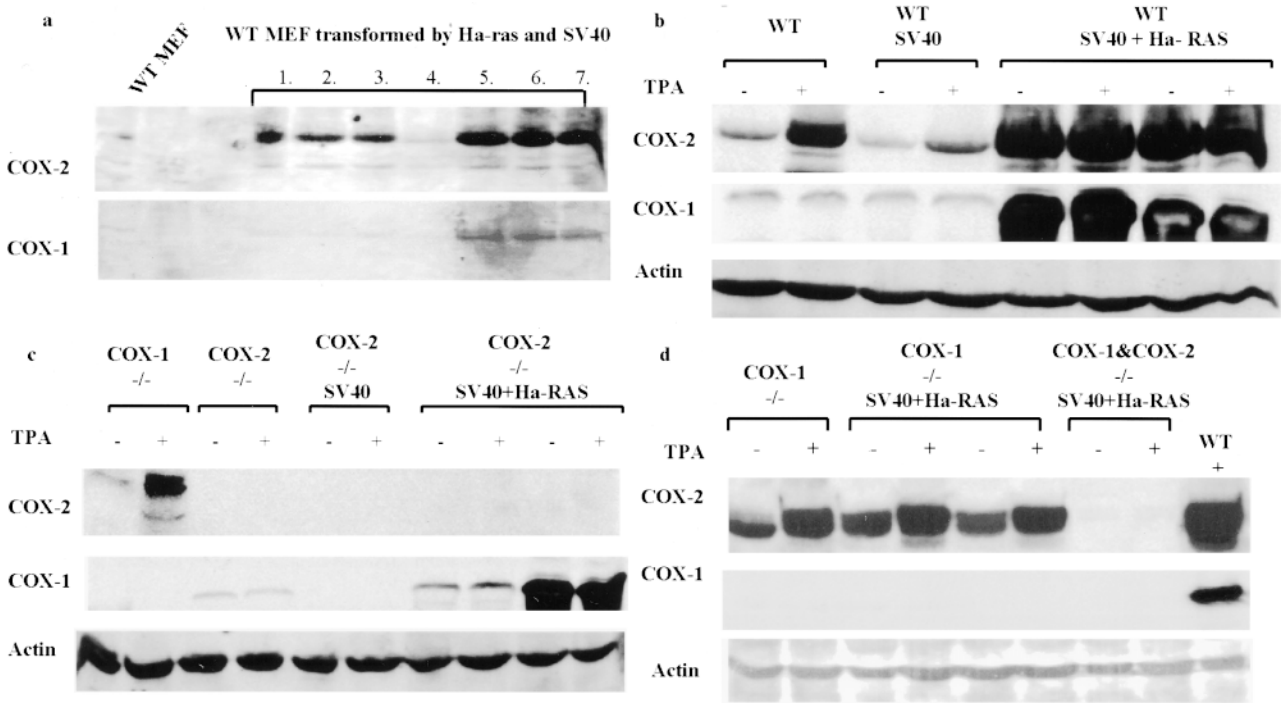

Figure 3. Western blot analysis of cyclooxygenase in wild-type and cyclooxygenase-null cells transformed by SV40 and/or *ras*. Postconfluent untransformed or transformed MEF cells were treated with or without TPA for 6 h, and the cells were lysed in Triton X-100 as described in Materials and Methods. Isoenzyme-specific antibodies were used to detect levels of cyclooxygenase expression in wild-type (WT) or cyclooxygenase-null cells. The same membrane was used to examine protein loadings. (a) Western blot analysis was performed using wild-type cells transformed by SV40 and *ras*. Colonies were selected randomly and expanded for the analysis. (b) Western blot analysis using wild-type cells exhibited decreased expression of both cyclooxygenases in SV40-transformed cells and dramatically increased expression of both cyclooxygenases in SV40- and *ras*-transformed cells. (c) Western blot analysis demonstrated the lack of the COX-2 protein in COX-$2^{-/-}$ cells. Transformation by SV40 decreased the expression of COX-1, whereas transformation by SV40 plus *ras* resulted in increased expression of COX-1 protein. (d) Western blot analysis was performed using COX-$1^{-/-}$ cells and COX-$1^{-/-}$/COX-$2^{-/-}$ cells. It demonstrated the lack of COX-1 proteins in COX-1 cells and the lack of both COX-1 and COX-2 proteins in the double knockout cells. Transformation by SV40 plus *ras* led to increased expression of COX-2 in the COX-$1^{-/-}$ cells.

pression of both cyclooxygenases. However, despite the changes in cyclooxygenase gene expression, transformation does occur in the absence of both cyclooxygenases.

NSAIDs Inhibit the Growth of Transformed Cells and also Induce Apoptosis Regardless of the Presence or Absence of Cyclooxygenase Isozymes. Clinical and experimental evidence strongly suggests that NSAIDs are anticarcinogenic, antiproliferative, and antineoplastic. Although some evidence suggests that the effects of NSAIDs are achieved via their known inhibitory effects on COX-2, recent data from several independent groups demonstrate that some of the actions may be COX independent. Cells in which either or both cyclooxygenase genes have been disrupted offer distinct advantages for evaluation of the antineoplastic activities of these drugs. For this purpose, we examined the actions of several NSAIDs on transformed cells with different deficiencies in the expression of the cyclooxygenase isozymes. The antineoplastic activities of the drugs were evaluated by their influence on colony formation in soft agar, [^{3}H]thymidine incorporation, and by DNA fragmentation analysis. NSAID dosages were varied from 1 to 200 μM, and these dosages are compatible with those used in several studies where antineoplastic effects were achieved (11, 22–25). The therapeutic plasma concentration for NSAID therapy can be achieved with up to 10 μM indomethacin, 20 μM sulindac, 20 μM piroxicam, or 300 μM ibuprofen (26). However, it is reported that the local concentration of NSAIDs in certain organs such as intestines can be built up to an even higher level (27). Therefore, the dosages we used in these studies include both the physiological dosages and the higher dosages that might be achievable in certain organs and tumor tissues in vivo.

We evaluated NSAID actions by seeding the same number of cells in soft agar containing various concentrations of different NSAIDs. All of the NSAIDs used (NS-398, sulindac, indomethacin, piroxicam, and ibuprofen) reduced the size and the number of colonies derived from the cells containing either, neither, or both cyclooxygenase genes in a dosage-dependent manner, with the inhibition being statistically significant at the higher dosages (Fig. 4). Several cell clones of each cyclooxygenase-deficient cell line were used in this study. Quantitative differences in colony formation were observed in the presence of various NSAIDs, but these differences were not correlated with the presence or the absence of cyclooxygenase expression, as seen in Fig. 4. Thymidine incorporation was used to examine DNA synthesis of the cells in the presence of different NSAID doses. At the 20 and 100 μM doses of a COX-2–selective

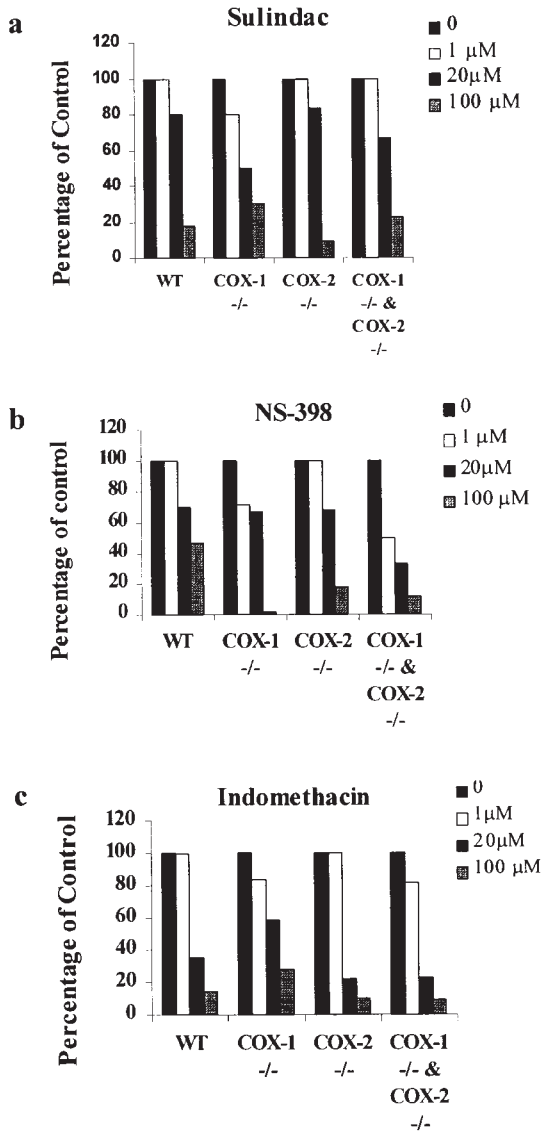

Figure 4. Inhibition of colony formation by NSAIDs in soft agar. Wild-type (WT), COX-1$^{-/-}$, COX-2$^{-/-}$, and COX-1$^{-/-}$/COX-2$^{-/-}$ cells were plated at the same density in 0.33% soft agar and grown for 2 wk as described in Materials and Methods, with the indicated concentrations of the NSAIDs indomethacin (Sigma Chemical Co.), NS-398 (Cayman Chemicals), and sulindac (Sigma Chemical Co.). Different cyclooxygenase-deficient cell clones were used in this study, and the data presented are representative. Each value is an average of duplicate plates.

NSAID, NS-398, [^{3}H]thymidine incorporation of all four cell lines examined was reduced by 15–68% regardless of the presence or absence of the two isozymes (Fig. 5). At these doses, where PGE2 production was reduced by 95% in the wild-type cells and the COX-1$^{-/-}$ or COX-2$^{-/-}$ cell lines, further addition of PGE2 up to 10 μM failed to restore DNA synthesis (data not shown). Finally, we examined the apoptotic responses of the various deficient cells upon treatment with the different NSAIDs. At higher doses (indomethacin 200 μM, sulindac 200 μM, piroxicam 200 μM, NS-398 100 μM, and ibuprofen 1 mM), these

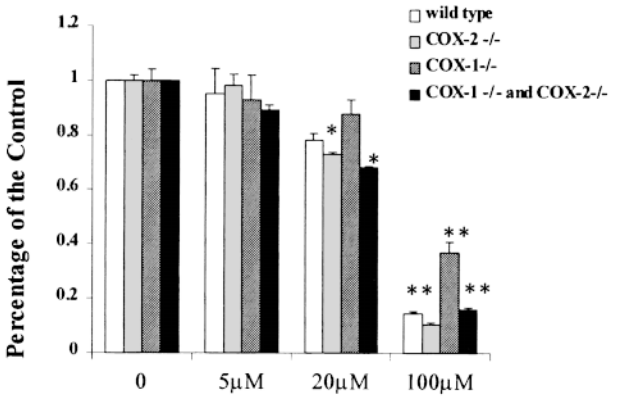

Figure 5. Thymidine incorporation of cyclooxygenase-null cells treated with NS-398, a COX-2–selective NSAID. DNA synthesis was estimated by [^{3}H]thymidine incorporation into cellular DNA as described in Materials and Methods. Each value is an average of triplicate wells, and the *t* test was used to show statistical differences (*$P < 0.05$, **$P < 0.01$).

drugs potently induced apoptosis in postconfluent cells at 48–72 h (Fig. 6). The cell death was induced in cells with neither COX-1 nor COX-2 expression. Based on these results, we conclude that the antiproliferative and antineoplastic actions of NSAIDs in these cells at these concentrations are independent of cyclooxygenase inhibition.

Discussion

The activating mutations of *ras* occur in ~30% of all human tumors. In squamous carcinomas, these mutations appear at early or intermediate stages of neoplasia. In colon cancers, *ras* gene mutations were found in 50% of adenocarcinomas and larger adenomas (28). To initiate studies on the involvement of the cyclooxygenases in transformation and malignancy, we developed cells that lack COX-1, COX-2, or both, and studied *ras*-induced malignant transformation. We found that transformation by SV40 alone downregulated the expressions of both cyclooxygenases. However, addition of the activated *ras* oncogene induced malignant transformation accompanied by an increased expression of either or both cyclooxygenase enzymes. Although most of the clones exhibit increased expression of either or both cyclooxygenases, some do not express either

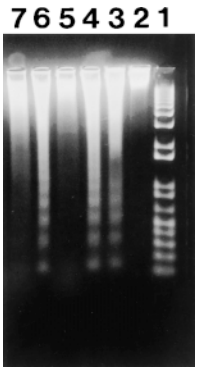

Figure 6. Induction of DNA fragmentation in cyclooxygenase-null cells (COX-1$^{-/-}$/COX-2$^{-/-}$) by NSAID treatment. Postconfluent cells were treated with different NSAIDs for 72 h, and genomic DNA was purified and resolved on the 1.5% agarose gel as described in Materials and Methods. Lane 1, 1-kb DNA ladder; lane 2, control postconfluent cells with no treatment of NSAIDs; lanes 3–7, the same cells treated, respectively, with sulindac (200 μM), NS-398 (100 μM), ibuprofen (1 mM), piroxicam (200 μM), or indomethacin (200 μM).

of the genes at increased levels. This result by itself suggests that the expression of either cyclooxygenase does not have a direct implication on mechanisms of transformation. Furthermore, our transformation experiments conducted in the cyclooxygenase-null cells provide still stronger evidence that transformation can occur in the absence of either of the cyclooxygenases, and that cyclooxygenase overexpression is not necessary to induce neoplastic transformation.

Nevertheless, there is abundant evidence suggesting that high levels of cyclooxygenase activity do play a critical role in some malignancies. Recent reports show that forced overexpression of cyclooxygenase enzymes increases metastatic potential, invasiveness, and angiogenesis of tumor cells both in vitro and in vivo (29–31). Moreover, carcinogenesis is a chronic, sequential, and progressive process that usually involves accumulation of mutations of protooncogenes and tumor suppressor genes in later steps. Mechanisms involving cyclooxygenases in mutagenesis have been suggested (32, 33). COX-2 overexpression also leads to resistance to apoptosis and increases in the cellular life-span (10, 11); these phenotypic changes may result in the additional development of mutations leading to more malignant behavior. Elevated cyclooxygenase expression is also known to increase malondialdehyde generation, providing another mechanism to foster higher cellular mutation rates (34). So while activation of *ras* by itself is not usually sufficient for malignancy, the elevations in cyclooxygenases induced by the activation of *ras* could well predispose the cells to mutations in *p53* or other genes that participate in the later steps towards high-grade malignancy. It is also noteworthy that in addition to COX-2, COX-1 expression is often induced by the activated *ras* oncogene, suggesting that both cyclooxygenases may serve some overlapping roles in tumorigenesis. Further studies are required to clarify the functional differences and relative importance of the two isozymes in cell malignancy and in the different stages of carcinogenesis.

NSAIDs such as sulindac and indomethacin have been shown to be beneficial for the prevention and in some cases for the treatment of certain cancers, although the underlying mechanisms are still unknown. Inhibition of cellular proliferation and/or induction of apoptosis are reported to be the mechanisms that cause the regression of tumors in vivo (35). The antiproliferative and apoptotic effects of NSAIDs have been documented in a variety of cells and cell lines, including colon cancer cells, breast cancer cells, and fibroblasts (22, 23). Considerable recent data suggest that some of the NSAIDs actions may not be entirely explained by inhibition of cyclooxygenases. First, some NSAID-related compounds, which are not cyclooxygenase inhibitors, can also induce antineoplastic changes in both the cell cycle and cellular apoptotic responses (36, 37). Second, the antineoplastic or anticarcinogenic activity of NSAIDs may not coincide with the inhibition of PG production in some tumor cells and animals (38). Finally, Hanif et al. (39) found that sulindac and piroxicam inhibit the growth of and induce apoptosis in HCT-15 cells, which were shown to produce neither COX-1, COX-2, nor PGs. Elder et al. (40) also showed that NS-398 induced apoptosis in S/KS cells, which lack COX-2 protein. In contrast, Murphy et al. (41) showed that the growth of the cells expressing COX-2 was not suppressed by COX-2–selective inhibitors. In this study, we further illustrate that a selective knockout of cyclooxygenase activity does not change the ability of NSAIDs to inhibit transformation or to induce apoptosis. These results confirm that inhibition of cyclooxygenase is not the only mechanism responsible for the anticarcinogenic and antineoplastic activity of NSAIDs.

NSAID actions that are independent of cyclooxygenase have been reviewed by Abramson and Weissmann (42). New evidence suggests that many of the traditional NSAIDs have targets other than cyclooxygenases. A recent report by Herrmann et al. (24) demonstrated that sulindac sulfide inhibits the *ras* signaling apparatus, potentially via an eicosanoid-independent pathway. Lehmann et al. (43) demonstrate that many NSAIDs are ligands of peroxisome proliferation–activated receptors (PPARs) α and γ. Chan et al. (25) demonstrated that the elevation of the PG precursor AA could be involved in the induction of apoptotic response by the NSAIDs (i.e., sulindac and indomethacin) through production of ceramide. However, due to the higher concentration of NSAIDs used in their study, it is not clear whether the induction of AA is a result of cyclooxygenase enzyme inhibition or direct production of AA through activation of phospholipases. NSAIDs are a large group of structurally diverse compounds; therefore, differences in the actions of individual NSAIDs are also expected. For example, aspirin inhibits the activation of the transcription factor NF-κB, whereas indomethacin does not (44). Recently COX-2–specific inhibitors were demonstrated to have potent antiproliferative effects and to induce apoptosis in cultured cells (20, 45, 46). Whether or not actions exerted by these drugs are mediated through inhibition of COX-2 needs additional investigation.

We have also observed in our studies that there are differences between cell lines in their sensitivity to NSAIDs that are independent of cyclooxygenase expression (Fig. 5). Thus, it seems likely that the differences in the sensitivity of tumor cell lines to NSAIDs as reported in the literature may also be independent of cyclooxygenase expression, particularly when NSAIDs are used at high concentrations.

Finally, the effects of NSAIDs on apoptosis, cell growth, and DNA synthesis studied here are those often cited to account for the antitumor activity of this class of compounds, yet in accord with the results of others, our data show that NSAID inhibition of the cyclooxygenases does not account for the antiproliferative and apoptotic actions seen in transformed embryonic fibroblasts. Our results further demonstrate that although cellular transformation by *ras* and SV40 usually leads to a dramatic increase in the expression of COX-1 and COX-2 when present, neither these inductions nor the presence of either of the enzymes is required for neoplastic transformation per se. These results suggest that the involvement of cyclooxygenase expression in neoplastic growth may occur at steps beyond those involved in transformation.

This study is supported by U.S. Public Health Service grant RO1 AM-16177 and institutional funds.

Address correspondence to Donald Young, Division of Endocrinology and Metabolism, Department of Medicine, Box 693, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642. Phone: 716-275-1625; Fax: 716-256-2789; E-mail: don_young@urmc.rochester.edu

Submitted: 27 January 1999 Revised: 21 June 1999 Accepted: 22 June 1999

References

1. O'Banion, M.K., H.B. Sadowski, V.D. Winn, and D.A. Young. 1991. A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. *J. Biol. Chem.* 266:23261–23267.
2. O'Banion, M.K., V. Winn, and D.A. Young. 1992. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. USA*. 89:4888–4892.
3. Kujubu, D.A., B.S. Fletcher, B.C. Varnum, R.W. Lim, and H.R. Herschman. 1991. Structure and expression of TIS21, a primary response gene induced by growth factors and tumor promoters. *J. Biol. Chem.* 266:12866–12872.
4. Pritchard, K.A., Jr., M.K. O'Banion, J.M. Miano, N. Vlasic, U.G. Bhatia, D.A. Young, and M.B. Stemerman. 1994. Induction of cyclooxygenase-2 in rat vascular smooth muscle cells *in vitro* and *in vivo*. *J. Biol. Chem.* 269:8504–8509.
5. Winn, V.D., M.K. O'Banion, and D.A. Young. 1993. Anti-inflammatory glucocorticoid action: inhibition of griPGHS, a new cyclooxygenase. *J. Lipid Mediat.* 6:101–111.
6. Herschman, H.R. 1992. Prostaglandin synthase 2. *Biochim. Biophys. Acta*. 1229:125–140.
7. Han, J.-w., H. Sadowski, D.A. Young, and I.G. Macara. 1990. Persistent induction of cyclooxygenase in p60 v-src-transformed 3T3 fibroblasts. *Proc. Natl. Acad. Sci. USA*. 87: 3373–3377.
8. Eberhart, C.E., R.J. Coffey, A. Radhika, F.M. Giardiello, S. Ferrenbach, and R.N. DuBois. 1994. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*. 107:1183–1188.
9. Loftin, C.D., and T.E. Eling. 1996. Prostaglandin synthase 2 expression in epidermal growth factor-dependent proliferation of mouse keratinocytes. *Arch. Biochem. Biophys.* 330: 419–429.
10. DuBois, R.N., J. Shao, M. Tsujii, H. Sheng, and R.D. Beauchamp. 1996. G1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. *Cancer Res.* 56:733–737.
11. Tsujii, M., and R.N. DuBois. 1995. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell.* 83:493–495.
12. Oshima, M., J.E. Dinchuk, S.L. Kargman, H. Oshima, B. Hancock, E. Kwong, J.M. Trzaskos, J.F. Evan, and M.M. Taketo. 1996. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell.* 87:803–809.
13. Sheng, H.M., J. Shao, S.C. Kirkland, P. Isakson, R.J. Coffey, J. Morrow, R.D. Beauchamp, and R.N. DuBois. 1996. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J. Clin. Invest.* 99:2254–2259.
14. Shiff, S.J., and B. Rigas. 1997. Nonsteroidal anti-inflammatory drugs and colorectal cancer: evolving concepts of their chemopreventive actions. *Gastroenterology*. 113:1992–1998.
15. Langenbach, R., S.G. Morham, H.F. Tiano, C.D. Loftin, B.I. Ghanayem, P.C. Chulada, J.F. Mahler, C.A. Lee, E.H. Goulding, and K.D. Kluckman. 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell.* 83:483–492.
16. Morham, S.G., R. Langenbach, C.D. Loftin, H.F. Tiano, N. Vouloumanos, J.C. Jennette, J.F. Mahler, K.D. Kluckman, A. Ledford, and C.A. Lee. 1995. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell.* 83:473–482.
17. Chulada, P.C., C.D. Loftin, V.D. Winn, D.A. Young, H.F. Tiano, T.E. Eling, and R. Langenbach. 1996. Relative activities of retrovirally expressed murine prostaglandin synthase-1 and -2 depend on source of arachidonic acid. *Arch. Biochem. Biophys.* 330:301–313.
18. Reddy, S.T., and H.R. Herschman. 1994. Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. *J. Biol. Chem.* 269:15473–15480.
19. Kirtikara, K., S.G. Morham, R. Raghow, S.J. Laulederkind, T. Kanekura, S. Goorha, and L.R. Ballou. 1998. Compensatory prostaglandin E2 biosynthesis in cyclooxygenase 1 or 2 null cells. *J. Exp. Med.* 187:517–523.
20. Sheng, G.G., J. Shao, H. Sheng, E.B. Hooton, P.C. Isakson, J.D. Morrow, R.J. Coffey, Jr., R.N. DuBois, and R.D. Beauchamp. 1997. A selective cyclooxygenase 2 inhibitor suppresses the growth of H-ras-transformed rat intestinal epithelial cells. *Gastroenterology*. 113:1883–1891.
21. Land, H., L.F. Parada, and R.A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*. 304:596–602.
22. Lu, X., W. Xie, D. Reed, W.S. Bradshaw, and D.L. Simmons. 1995. Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts. *Proc. Natl. Acad. Sci. USA*. 92:7961–7965.
23. Shiff, S.J., M.I. Koutsos, L. Qiao, and B. Rigas. 1996. Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis. *Exp. Cell Res.* 222:179–188.
24. Herrmann, C., C. Block, C. Geisen, K. Haas, C. Weber, G. Winde, T. Moroy, and O. Muller. 1998. Sulindac sulfide inhibits Ras signaling. *Oncogene*. 17:1769–1776.
25. Chan, T.A., P.J. Morin, B. Vogelstein, and K.W. Kinzler. 1998. Mechanisms underlying nonsteroidal anti-inflammatory drug-mediated apoptosis. *Proc. Natl. Acad. Sci. USA*. 95: 681–686.
26. McEvoy, G.K. 1997. AHFS Drug Information 97. American Society of Health Systems Pharmacists, Bethesda, MD. 2955 pp.
27. Duggan, D.E., K.F. Hooke, and S.S. Hwang. 1980. Kinetics

of the tissue distributions of sulindac and metabolites. Relevance to sites and rates of bioactivation. *Drug Metab. Dispos.* 8:241–246.
28. Bos, J.L. 1989. *ras* oncogenes in human cancer: a review. *Cancer Res.* 49:4682–4689.
29. Beauchamp, R.D., M. Tsujii, S. Kawano, and R.N. DuBois. 1997. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc. Natl. Acad. Sci. USA*. 94:3336–3340.
30. Narko, K., A. Ristimaki, M. MacPhee, E. Smith, C.C. Haudenschild, and T. Hla. 1997. Tumorigenic transformation of immortalized ECV endothelial cells by cyclooxygenase-1 overexpression. *J. Biol. Chem*. 272:21455–21460.
31. Tsujii, M., S. Kawano, S. Tsuji, H. Sawaoka, M. Hori, and R.N. DuBois. 1998. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*. 93:705–716.
32. Eling, T.E., and J.F. Curtis. 1992. Xenobiotic metabolism by prostaglandin H synthase. *Pharmacol. Ther*. 53:261–273.
33. Marnett, L.J. 1994. Generation of mutagens during arachidonic acid metabolism. *Cancer Metastasis Rev*. 13:303–308.
34. Fink, S.P., G.R. Reddy, and L.J. Marnett. 1997. Mutagenicity in *Escherichia coli* of the major DNA adduct derived from the endogenous mutagen malondialdehyde. *Proc. Natl. Acad. Sci. USA*. 94:8652–8657.
35. Sawaoka, H., S. Kawano, S. Tsuji, M. Tsujii, E.S. Gunawan, Y. Takei, K. Nagano, and M. Hori. 1998. Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. *Am. J. Physiol*. 274: G1061–G1067.
36. Piazza, G.A., A.L. Rahm, M. Krutzsch, G. Sperl, N.S. Paranka, P.H. Gross, K. Brendel, R.W. Burt, D.S. Alberts, and R. Pamukcu. 1995. Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. *Cancer Res*. 55:3110–3116.
37. Thompson, H.J., C. Jiang, J. Lu, R.G. Mehta, G.A. Piazza, N.S. Paranka, R. Pamukcu, and D.J. Ahnen. 1997. Sulfone metabolite of sulindac inhibits mammary carcinogenesis. *Cancer Res.* 57:267–271.
38. Carter, C.A., M.M. Ip, and C. Ip. 1989. A comparison of the effects of the prostaglandin synthesis inhibitors indomethacin and carprofen on 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis in rats fed different amounts of essential fatty acid. *Carcinogenesis*. 10:1369–1374.
39. Hanif, R., A. Pittas, Y. Feng, M.I. Kutsos, L. Qiao, L. Staiano-Coico, S.I. Shiff, and B. Riggs. 1996. 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.
40. Elder, D.J.E., D.E. Halton, A. Hague, and C. Paraskeva. 1997. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin. Cancer Res*. 3:1679–1683.
41. Murphy, V.J., Z. Yang, K.A. Rorison, and G.S. Baldwin. 1998. Cyclooxygenase-2 selective antagonists do not inhibit growth of colorectal carcinoma cell lines. *Cancer Lett*. 122: 25–30.
42. Abramson, S.B., and G. Weissmann. 1989. The mechanisms of action of nonsteroidal anti-inflammatory drugs. *Arthritis Rheum*. 32:1–9.
43. Lehmann, J.M., J.M. Lenhard, B.B. Oliver, G.M. Ringold, and S.A. Kliiewer. 1997. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* 272:3406–3410.
44. Frantz, B., and E.A. O'Neill. 1995. The effect of sodium salicylate and aspirin on NF-kappa B. *Science*. 270:2017–2019.
45. Liu, X.H., S. Yao, A. Kirschenbaum, and A.C. Levine. 1998. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. *Cancer Res.* 58:4245–4249.
46. Kusuhara, H., H. Matsuyuki, M. Matsuura, T. Imayoshi, T. Okumoto, and H. Matsui. 1998. Induction of apoptotic DNA fragmentation by nonsteroidal anti-inflammatory drugs in cultured rat gastric mucosal cells. *Eur. J. Pharmacol*. 360: 273–280.