Carcinogenesis vol.22 no.1 pp.17–25, 2001

CANCER BIOLOGY

Growth inhibition and induction of apoptosis in colorectal tumor cells by cyclooxygenase inhibitors

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Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit colorectal carcinogenesis and prevent or revert the growth of premalignant colonic polyps. They inhibit cyclooxygenase (COX) but recent data indicate that this is not the only or even the most important mechanism of inhibition in colorectal tumor cells. We have used colonic carcinoma and adenoma cell lines to study the effects of the NSAID sulindac sulfide, its COX-inactive metabolite, sulindac sulfone, and the isoenzyme-specific inhibitors SC58125, SC236 and SC58560 on tumor cell growth in relation to COX-2 expression and prostaglandin production. To establish the role of COX-2 in NSAID action, we constructed clones expressing different levels of COX-2 from SW480 cells. All five compounds inhibited DNA synthesis and/or induced apoptosis, each with a characteristic pattern. ID_{50}s were very similar in all the cell lines and were independent of COX expression, except for the COX-1 inhibitor SC58560, which was least effective in HT29/HI1, the cell line expressing the highest level of COX-1 (ID_{50} 70 μM; in other cells lines the ID_{50} was 15 μM). For all other compounds ID_{50} concentrations varied less than two-fold: 25–40, 40–90 and 150 μM for SC236, sulindac sulfide and sulindac sulfone, respectively. SC58125 was the weakest inhibitor, never causing >50% cell loss. All compounds modulated expression of Bcl-2 and Bak and activated caspase 3. Overexpression of COX-2 in SW480 cells protected them against induction of apoptosis by sulindac sulfide. The effect was restricted to clones producing high levels of prostaglandin E_2. In summary, our data indicate that both COX-dependent and COX-independent mechanisms are involved in NSAID-induced growth in colorectal tumor cells. The concentrations necessary to inhibit growth were higher than serum concentrations that can be obtained *in vivo*, indicating that the therapeutic effect of NSAIDs cannot be explained by a direct effect of NSAIDs on the epithelial cells alone. For therapeutic purposes, compounds using different targets could be used to minimize side effects while optimizing therapeutic effect.

Introduction

Regular consumption of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have been shown to reduce colon cancer risk by ~50% (1–3) and so have been used to prevent colorectal cancer in high-risk patients suffering from the hereditary syndrome of familial polyposis coli. Treatment not only inhibits the polyp growth but also causes reversion of the polyps (4,5). While the efficacy of these drugs has been effectively demonstrated, their mechanism of action is still far from clear. Their primary cellular target has been assumed to be the inducible cyclooxygenase isoenzyme COX-2 (1). This isoenzyme is overexpressed in many colorectal tumors causing increased prostaglandin (PG) levels in the tissue (6). Consequently, COX-2-specific inhibitors are under development as tumor-preventive drugs in the colon without the side effects caused by systemic inhibition of both COX isoenzymes (7,8).

Available data have indicated that NSAID treatment normalizes the rate of cell death of differentiated cells in early premalignant lesions, preventing polyp formation (9,10). In addition, reversion of preexisting polyps has been observed during NSAID treatment (for example see refs 4 and 11). This can only be explained by induction of cell death in the premalignant cell population of the adenomatous polyps. In contrast, *in vitro* studies have shown that malignant carcinoma cells are more sensitive to NSAID exposure than premalignant adenomas. This effect has also been observed in cells that do not express COX-2 at all (12).

Recent evidence suggests that the tumor-inhibitory efficacy of NSAIDs and similar drugs is not necessarily related to their COX-inhibitory potential. In a rat *in vivo* model, inhibition of tumorigenesis occurred at NSAID concentrations that did not inhibit PG synthesis (13). Furthermore, sulindac sulfone, a sulindac metabolite that does not inhibit COXs, affects polyp growth and tumor development (14–17).

Taken together, these data raise questions about cellular targets and the mechanism of action of NSAIDs and other COX inhibitors in colorectal tumor cells. While this has been shown in studies using one or a few tumor cell lines with different levels of COX-2 expression (12,16,18,19), a detailed analysis of COX-2-dependent and/or -independent effects in a defined system is still lacking. The present study aims to address such questions by investigating the growth effects of sulindac and of specific COX-1 and COX-2 inhibitors on colorectal carcinoma and adenoma cell lines. COX-2 overexpressing clones were constructed from SW480 cells, which initially do not produce detectable COX-2 and PG, to analyze the relationship between growth effects, COX-2 expression and PG production.

Abbreviations: COX, cyclooxygenase; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; MEM, minimal essential medium; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; PVDF, Polyvinylidendifluoride

Materials and methods

Cell lines

SW480 colon carcinoma cells were obtained from the American Type Culture Collection; HT29/HI1 cells were a gift from Dr E.Friedman (20) (Memorial Sloan Kettering Cancer Center, New York). The cell lines were kept under standard tissue culture conditions using minimal essential medium (MEM) containing 10% fetal calf serum (FCS). Under these conditions, SW480 cells grow with highly transformed morphology and a doubling time of ~24 h. VACO 235 adenoma cells were a gift from James K.V.Willson (21) (Ireland

Cancer Center, Case Western University, Cleveland, OH) and were cultured in MEM_{2+} [HEPES-buffered MEM containing 2% FCS, 2×10^{-10} M triiodo-L-thyronine, 1 μg/ml hydrocortisone, 10 μg/ml insulin, 2 μg/ml transferrin, 5×10^{-9} M selenite and 30 ng/ml epidermal growth factor (EGF); see ref. 26]. Their doubling time was ~60 h. The LT97 cell line was established from the mucosa of a familal polyposis patient, which consisted mainly of small tubular polyps (our unpublished data; M.Richter manuscript in preparation). They were maintained in MCDB203 medium containing 20% L15 medium(Sigma), 2% FCS, 2×10^{-10} M triiodo-L-thyronine, 1 μg/ml hydrocortisone (302 medium (Sigma)) supplemented with 10 μg/ml insulin, 2 μg/ml transferrin, 5×10^{-9} M selenite and 30 ng/ml EGF. Their doubling time under these conditions was ~96 h.

COX inhibitors

SC58125, SC236 and SC58560 were a gift from Searl (Skokie, IL); sulindac sulfide and sulindac sulfone were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Stock solutions were prepared in dimethylsulfoxide (DMSO) and stored at –20°C. Cells were plated at 5×10^4 cells/well in 24-well plates, left to attach for 24 h (SW480, HT29/HI1), 48 h (VACO235) or 96 h (LT97) and then exposed to inhibitors. Inhibitors were provided in HEPES-buffered MEM containing 1 mg/ml bovine serum albumin (BSA) for SW480, HT29/HI1 and VACO235 and in 203 basic medium for LT97 cells. Control media contained the appropriate volume of DMSO; DMSO content was limited to 6 μl/ml, which resulted in slight growth inhibition after 72 h.

Exposure to exogenous prostaglandins

PGE_2, $PGF_{2\alpha}$ or dimethyl-PGE_1were obtained from Sigma (St Louis, MO). They were kept as stock solutions in ethanol under a N_2 atmosphere at –80°C and diluted into MEM containing 1 mg/ml BSA immediately before use.

Growth parameters

Two assays were used to determine cell numbers. In most experiments cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). This compound is oxidized by cells with intact mitochondria and yields a purple product that can be solubilized with DMSO. Alternatively, Neutral Red uptake during a 2 h period from serum-free MEM containing 50 μg/ml Neutral Red was measured. This dye is taken up into the lysosomes of viable cells, from where it can be dissolved with 1% acetic acid in 70% ethanol.

Neutral Red was washed out using first 70% ethanol and then PBS and fixed cells were then stained with 800 ng/ml Hoechst 33285. Nuclei with chromatin condensed at the nuclear margin or in the center of the nucleus as well as fragmented nuclei were classified as apoptotic. Apoptotic index was determined by counting 1000 cells per well from triplicate cultures. Mitotic figures were counted from the same fields as apoptotic nuclei. Both apoptotic and mitotic index are given as a percentage of total nuclei.

DNA synthesis was determined by measuring incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA using a cell proliferation kit (Boehringer Mannheim, Germany). BrdU 5 μg/ml was added to the medium for 2 h (for carcinoma cells) or 20 h (for adenoma cells). At the end of the treatment period, cells were fixed and BrdU uptake was determined by enzyme-linked immunosorbent assay (ELISA) using the kit reagents according to the manufacturer's instructions.

Immunoblotting

Cells were washed twice with ice-cold PBS and homogenized in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM $Na_4(PO_4)_2$, 25 μg/ml each of aprotinin and leupeptin). The insoluble fraction was removed by centrifugation for 15 min at 4°C and 10 000 g. Proteins (50 μg per lane) were analyzed by electrophoresis on 15% polyacrylamide gels and transferred to PVDF membranes. Membranes were probed with antibodies against Bcl-2 (1:1500; Oncogene Science, Cambridge, MA), Bak (1:1200, Oncogene Science) and pro-caspase 3 (1:1000; mAbC31720, Transduction Laboratories, Lexington, KY). For analysis of COX isoenzymes, proteins were separated on 10% gels and blots were probed with monoclonal antibodies against COX-2 and COX-1 (1:1000; Transduction Laboratories). Secondary antibody (anti-rabbit horseradish-peroxidase linked; Amersham, Arlington Heights, IL) was diluted 1:4000 and detected by an enhanced chemiluminescence western blotting detection system (Pierce, Rockford, IL).

COX-1 and COX-2 expression in tumor cells

mRNA was isolated from subconfluent cultures using Trizol (Gibco Life Technologies, Paisley, UK) and chloroform extraction. Aliquots (50 ng) were used to prepare and amplify cDNA using the reverse transcription–polymerase chain reaction (RT–PCR) Ready-To-Go kit from Pharmacia H (Peapack NJ). The following primer pairs were used: COX-1: sense, 5′-TGCCCAGCTCC-TGGCCCGCTT-3′; antisense, 5′-GTGCATCAACACAGGCGCCTCTTC; COX-2: sense, 5′-TTCAAATGAGATTGTGGGAAAATT-3′; antisense, 5′-AGATCATCTCTGCCTGAGTATCTT-3′; RPL6: sense, 5′-GGTCTAGA-AATGCCTAGGTATTA-3′; antisense, 5′-GAAGAATACGCCCAGTGAGG-ATGA- 3′.

Amplification of the target gene and RPL6 were performed in one reaction from four primers using cycles of 40 s denaturation at 94°C, 50 s annealing at 55°C and 30 s extension at 72°C. To assess gene expression of the target genes, reactions were performed for 23, 25, 27 and 29 cycles and products were separated on 8% acrylamide gels. Bands were stained with Vistra Green and visualized and quantified using a FluorImager and ImageQuant software (Molecular Dynamics).

Prostaglandin production

Cells were plated at 5×10^4 cells/well into 24-well plates. Twenty-four hours later, the cultures were exposed to treatment or control media containing 10 μM arachidonic acid to provide sufficient substrate for exactly 24 h. PGE_2 secreted into the medium was determined by competitive ELISA using a kit from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer's instructions. In short, medium from cells was added to 96-well plates coated with an anti-mouse antibody, mixed with a PG/acetylcholinesterase tracer and a monoclonal antibody against prostaglandin and incubated at 4°C overnight. Unbound PG/acetylcholinesterase was removed and washed extensively and bound acetylcholinesterase was detected by Ellman's reagent and measured at 414 nm. PG standard dilutions were prepared in MEM containing 1 mg/ml BSA and the PG content was determined from the standard curve.

COX-2-overexpressing clones

SW480 cells (5×10^6) were suspended in 800 μl of medium containing 10% FCS, 25 μg pcDNA3 carrying the COX-2 cDNA and 5 μg PEGFP-N3 and incubated for 3 min at room temperature. Controls were incubated with equal amounts of vector DNA and PEGFP-N3. DNA was transfected using electroporation at 250 V and 1500 μF using an Easyject+ (EquiBio, Kent, UK) 450V twin pulse electroporator. Immediately after the pulse, cells were transfered into two Petri dishes containing 10 ml of MEM supplemented with 10% FCS and left to attach. DNA uptake, as judged by green fluorescent protein (GFP) fluorescence, was ~30%. Geneticin (G418) was added to the medium to select transfected cells 4 days after transfection. G418-resistant clones were isolated after 2–3 weeks of selection and passaged whenever they were confluent.

Results

Expression of COX isoenzymes and prostaglandin synthesis in colorectal tumor cell lines

Expression of both COX isoenzymes was determined from sub-confluent cultures of SW480 and HT29/HI1 colon carcinoma cells and VACO235 and LT97 adenoma cells. Semi-quantitative analysis of COX-1 and COX-2 mRNA levels was performed by RT–PCR using 23–29 cycles. Each cell line expressed a distinct combination of the two isoenzymes, as shown in Figure 1a and b: SW480 expressed low levels of COX-1, but no detectable COX-2 mRNA, HT29/HI1 expressed both isoenzymes at significant levels, and VACO235 expressed high levels of COX-2 but hardly any COX-1.

Protein levels as shown by western blotting with isoenzyme-specific antibodies reflected the same isoenzyme pattern (Figure 1c). Production of the growth-stimulating prostglandin PGE_2 was measured by ELISA. It was 1144 ± 46 pg/10^6 cells for VACO235 and 23 ± 3.5 pg/10^6 cells for HT29/HI1 cells in 24 h, while PGE_2 in SW480 and LT97 cultures remained below the reliable detection level (<5 pg/10^6 cells).

Growth effects of NSAID and isoenzyme-specific COX inhibitors

To determine growth effects, we added the COX-2 inhibitors SC236 and SC125, the COX-1 inhibitor SC560, sulindac sulfide or its COX-inactive metabolite sulindac sulfone to the tissue culture medium of carcinoma and adenoma cell lines. Cell numbers were measured after 72 h using the MTT assay. The results showed a reduction of cell numbers to varying degrees with all compounds in all cell lines used (Figure 2). Differences in sensitivity between cell lines were only minor

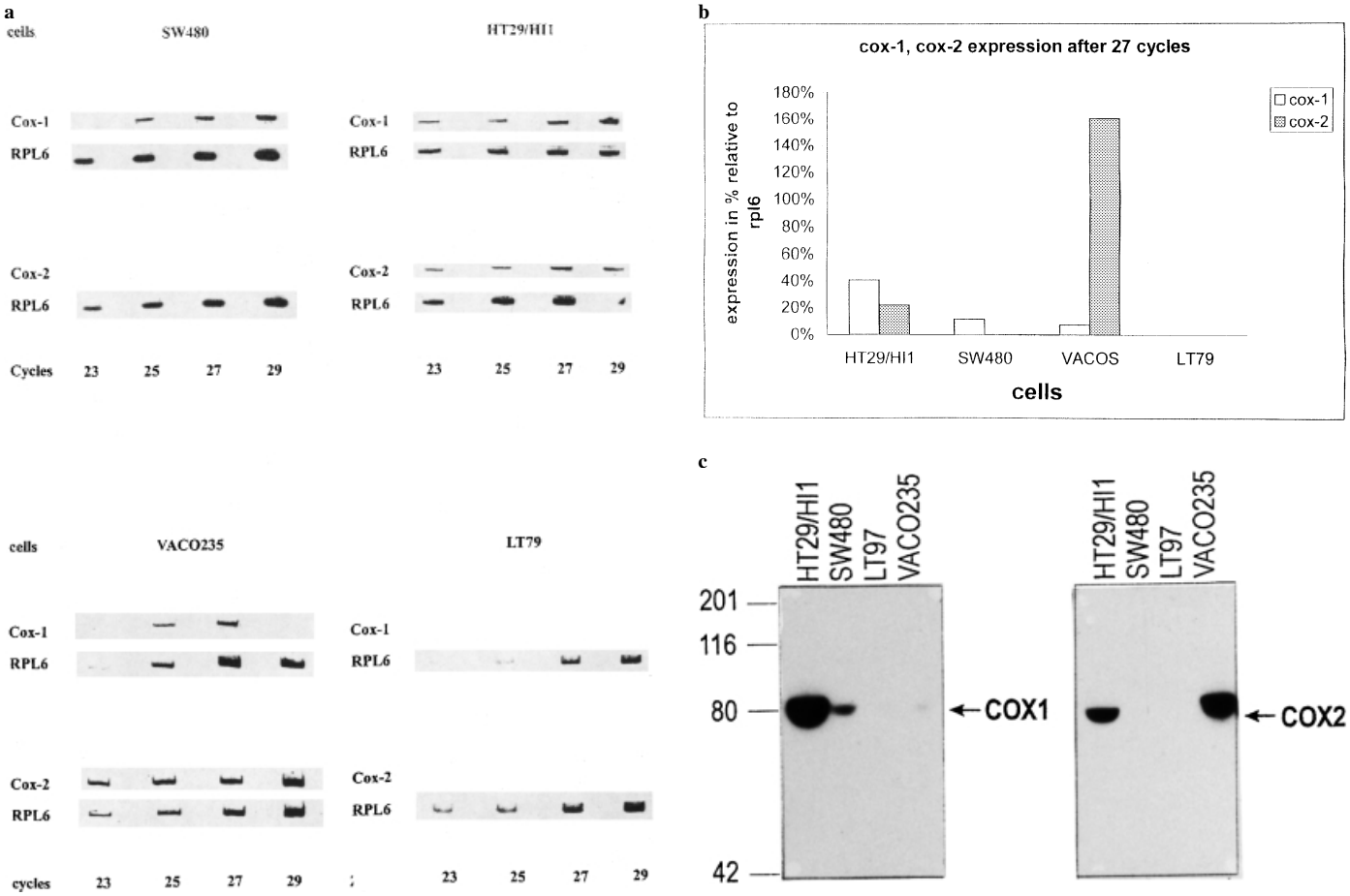

Fig. 1. Expression of cyclooxygenases in colorectal tumor cell lines. (**a**) RT–PCR was performed as described in Materials and methods and the products were analyzed on 8% acrylamide gels. Bands were stained with Vistra Green and their density was quantified using a Fluoroimager. (**b**) Band intensity of the housekeeping gene RPL6 was set at 100% for standardization. (**c**) Total cellular protein obtained from semiconfluent cultures was analyzed on 10% sodium dodecyl sulfate (SDS)–acrylamide gels and the COX-1 and COX-2 proteins were identified using monoclonal antibodies.

when SC236 was used: ID_{50} concentrations ranged between 25 and 40 μM, with LT97 adenoma cells being the most sensitive by a small margin (Figure 2a). SC125, the other COX-2 inhibitor, was much less effective. Only SW480 cells were affected to any significant degree (ID_{50} = 40 μM) while cell numbers for all other cell lines did not drop below 60% of control at 80 μM, the highest concentration that did not precipitate during the incubation period (Figure 2b). With SC560, there were major differences between cell lines, HT29/HI1 (the cell line with the highest expression of COX-1) being about five-fold less sensitive than SW480 or VACO235 (15 and 70 μM, respectively). LT97 adenoma cells were as insensitive as HT29/HI1 cells (Figure 2c). With the sulindac metabolites, low concentrations caused slight increases in cell number, especially with the VACO235 adenoma cells. At higher concentrations, cell loss was highest in LT97. With sulindac sulfide, ID_{50} was 25 μM in the LT97 cultures and 40–90 μM with all other cell lines (Figure 2d). With sulindac sulfone the differences were not as pronounced; the ID_{50} was ~150 μM for all cell lines (Figure 2e).

DNA synthesis was measured in parallel cultures after 24 h to assess whether a reduction of cell production contributed to the overall growth inhibition. It was also decreased but to a lesser degree than cell number; there were clear differences between cell lines (Figure 3): HT29/HI1 cells were hardly affected, with the lowest rate of BrdU incorporation being 60% of that in controls in the 25 μg/ml SC236 group (Figure 3a). Effects on SW480 cells were weak, just reaching 50% of control using SC125, SC560 or the sulindac metabolites. Stronger inhibition of DNA synthesis was only observed with sulindac sulfide and sulfone in VACO235 adenoma cells and with SC236 in SW480 and VACO235. In LT97 adenoma cultures, BrdU incorporation in control cultures (302 basic medium) was extremely low and hardly affected by exposure to SC125, SC560 or sulindac sulfone. After exposure to SC236 and sulindac sulfide, BrdU incorporation was lost together with the reduction in cell numbers in these cultures (compare Figures 2a and d and 3a and d).

In contrast, induction of apoptosis was a strong factor in NSAID-induced growth inhibition. Cultures were fixed after 24 h of exposure and stained with Hoechst 33285 dye. The incidence of apoptotic and mitotic nuclei was counted in 1000 cells each of duplicate cultures. The results obtained with SW480 cells are summarized in Table I: there was a dose dependent increase in the proportion of apoptotic nuclei in cultures with all four compounds. At the same time the incidence of mitosis decreased, approaching zero. Results obtained using the other cell lines were qualitatively similar (not shown).

To obtain further proof for the induction of apoptosis, we lysed SW480 cells after short-term exposure to SC236, SC560 or the sulindac metabolites. SC125 was not included because

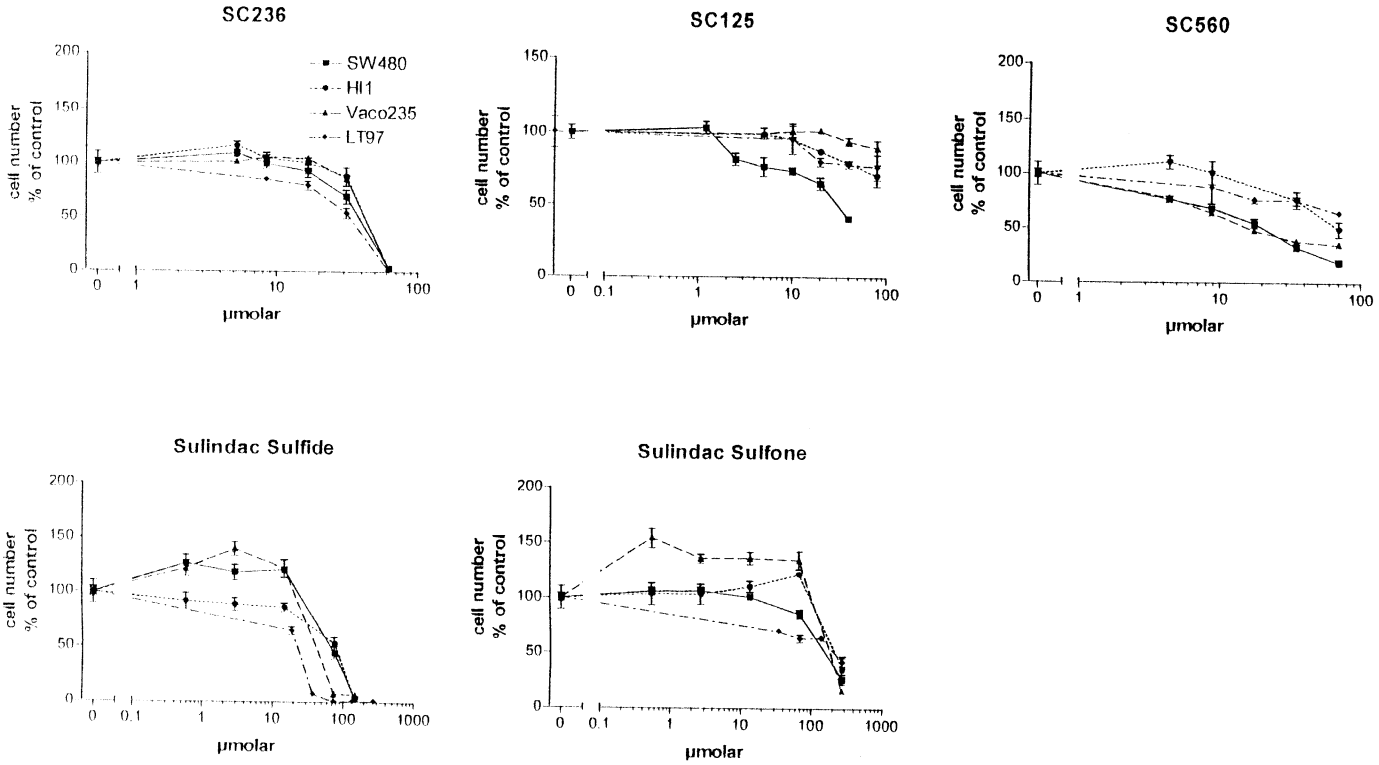

Fig. 2. Growth inhibition induced by COX inhibitors. Cells were plated as described in Materials and methods and exposed to inhibitors for 72 h. Cell number was determined using the MTT assay. The results are given as percentage of values for controls to facilitate comparison between different cell lines. Values comprise mean ± SEM from three or more experiments each done in triplicate.

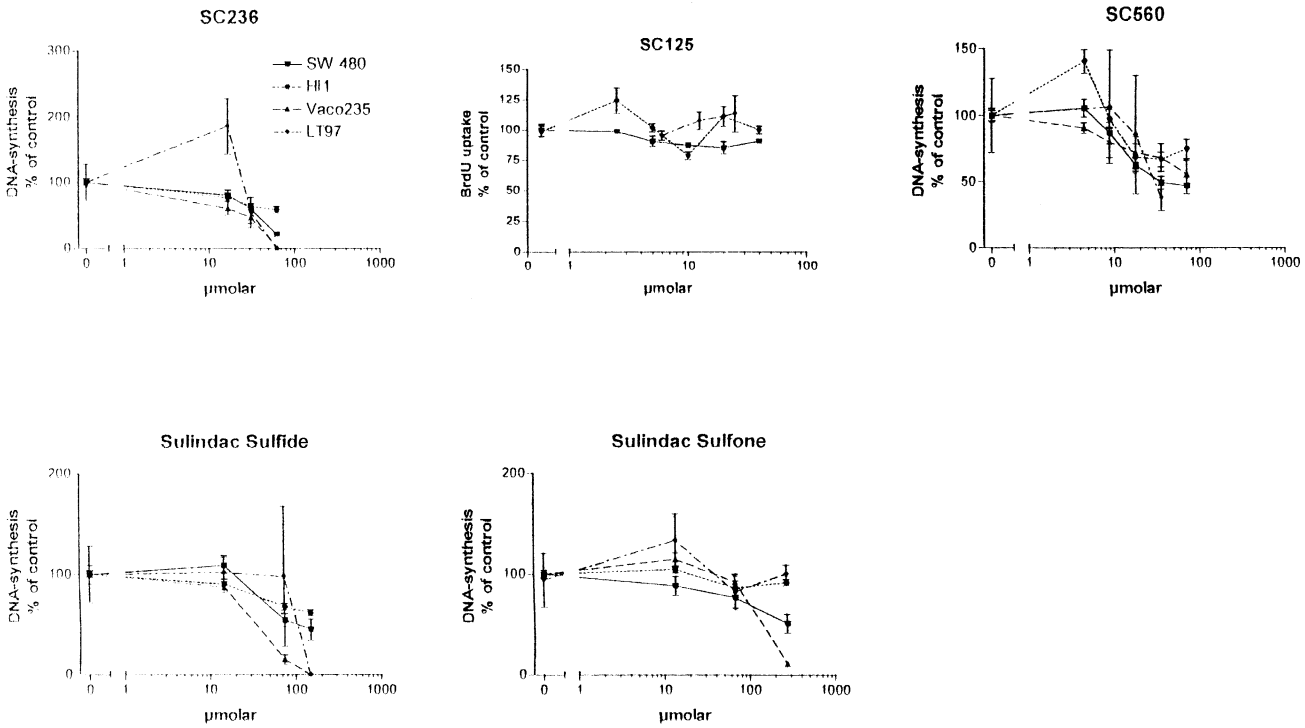

Fig. 3. DNA synthesis in inhibitor-exposed cultures. Tumor cell cultures were exposed to inhibitor compounds for 24 h and then incubated with 5 µg/ml BrdU for 2 h (SW480 and HT29/HI1) or for the final 20 h of exposure (VACO235 and LT97). BrdU incorporation into the DNA was measured by ELISA. Results are given as a percentage of control values for better comparison and comprise mean ± SEM from two or more experiments each done in triplicate assays.

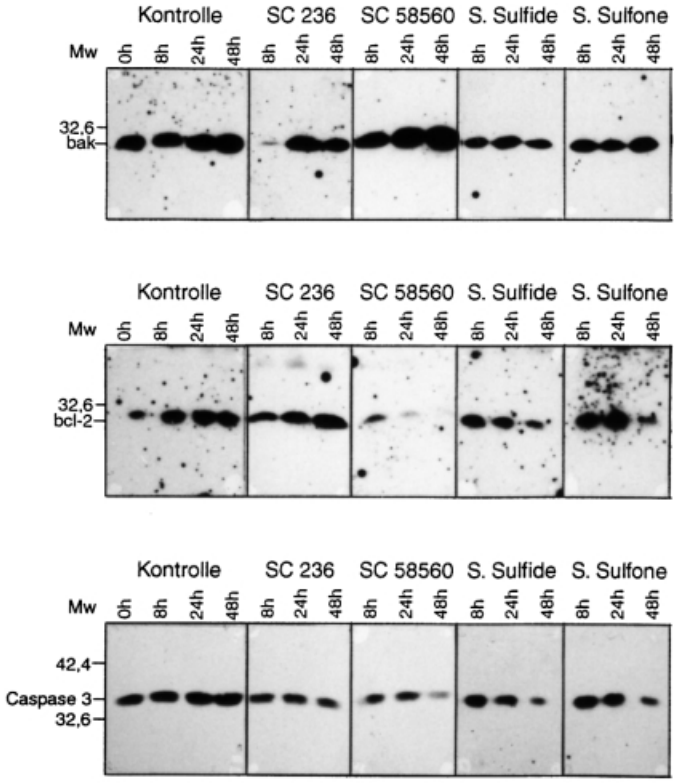

Fig. 4. Proteins related to apoptosis. Cultures were exposed to 30 μM SC236, 70 μM SC560 or sulindac sulfide or 270 μM sulindac sulfone for the times indicated on the blot. Total protein (50 μg) was analyzed on 15% SDS–acrylamide gels and Bcl-2, Bak and pro-caspase 3 were detected sequentially from the same blots using monoclonal antibodies.

Table I. Apoptosis and mitosis in SW480 carcinoma cells exposed to COX inhibitors

Substance	Concentration (μM)	Apoptosis (% of nuclei)	Mitosis (% of nuclei)
SC236	0.00	0.15 ± 0.05	0.85 ± 0.04
	16.50	0.70 ± 0.03	1.35 ± 0.09
	31.00	0.95 ± 0.07	0.40 ± 0.05
SC58125	0.00	0.15 ± 0.05	0.90 ± 0.06
	20.00	7.50 ± 0.10	0.85 ± 0.04
	40.00	9.80 ± 0.45	0.28 ± 0.09
SC58560	0.00	0.20 ± 0.07	1.10 ± 0.04
	35.50	1.20 ± 0.03	0.25 ± 0.03
	71.00	1.50 ± 0.05	0.00
Sulindac sulfide	0.00	0.10 ± 0.04	1.10 ± 0.04
	3.00	0.20 ± 0.07	1.75 ± 0.09
	15.00	0.30 ± 0.09	1.40 ± 0.08
	75.00	6.75 ± 0.13	0.00
Sulindac sulfone	0.00	0.15 ± 0.05	1.00 ± 0.03
	5.40	0.15 ± 0.08	0.80 ± 0.05
	27.00	0.10 ± 0.06	0.60 ± 0.09
	135.00	0.50 ± 0.10	0.30 ± 0.02

SW480 cells were seeded at 5×10^4 cells/well in Sonic Seal slides Nunc (Naperville, IL) and exposed to inhibitors. Cell number was determined by Neutral Red uptake and the resulting fixed cultures were stained with Hoechst 33285. Nuclear morphology was determined by fluorescence microscopy and incidence of apoptosis counted from 1000 cells per well in duplicate (mean ± SD).

its growth effects were only minor. Lysates were analyzed by western blotting using antibodies against Bcl-2, Bak and pro-caspase 3 (Figure 4). Surprisingly, SC236 had the least effect

Fig. 5. PGE_2 production during exposure to inhibitors. Cells were plated and exposed to inhibitors as for the growth experiments (Figures 2 and 3) except that exactly 0.5 ml of medium was used per well and media were collected after exactly 24 h. PGE_2 content of the conditioned media was determined by ELISA. Results are mean ± SD of triplicate assays and two experiments.

in this regard: a drop in Bcl-2 was evident at 8 h followed by a recovery later, Bak levels remained constant and pro-caspase was reduced from 8 h onwards, indicating proteolytic activation of the enzyme, but the effect was less than that with SC560. The latter compound was the most active in this analysis, inducing a lasting loss of Bcl-2 from 8 h onwards, increase in Bak protein and a time-dependent reduction in pro-caspase. Both sulindac compounds caused a slow decrease in Bcl-2 and pro-caspase, which only became obvious after 24 and 48 h, while Bak levels remained constant.

PG production under growth-inhibitory conditions was determined by ELISA in VACO235 and HT29/HI1 cells. As expected, PGE_2 production was inhibited but not abolished by all compounds except sulindac sulfone. In HT29/HI1 cells, PGE_2 production was reduced to 50% of that in controls; in VACO235 cells, with their 40-fold higher baseline production, inhibition was less but still significant (Figure 5).

To substantiate a role of PG in growth and survival of colorectal tumor cells, we tried to prevent the effects of PGs by adding them either together with the inhibitor or after a short treatment and washing out of the compound. Using PGE_2, $PGF_{2\alpha}$ and the stabilized analog dimethyl-PGE_1, none of the combinations used was sufficient to prevent growth inhibition (data not shown).

Construction of COX-2-overexpressing cell lines

Comparison of inhibitor sensitivities of different cell lines and the absence of an effect of PG on induction of apoptosis casts doubt on any crucial role of COX in the process. However, we could not exclude the possibility that the lack of effects of PG were due to insufficient stability and/or uptake into the correct cellular compartments. We therefore constructed COX-2-overexpressing clones by transfecting SW480 cells with an expression vector containing the COX-2 cDNA governed by a cytomegalovirus (CMV) promotor. We isolated 30 clones with different levels of COX-2 expression and PGE_2 production (Figure 6) and five clones transfected with the control plasmid. COX-2 clones showed a large spectrum of growth alterations, mainly consisting of growth retardation and increased serum dependency. None of these effects was related to the level of COX expression (Table II).

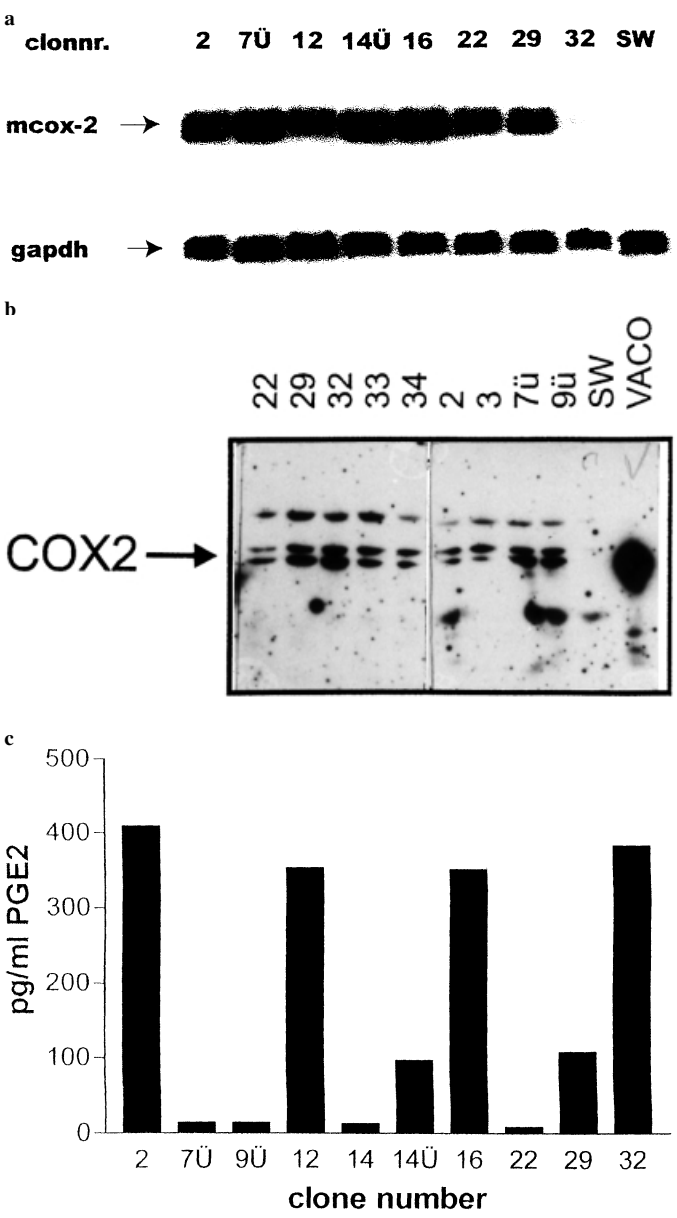

Fig. 6. COX-2 expression in transfected clones. (**a**) Total RNA was isolated from semi-confluent cultures and COX-2 expression was estimated by RT–PCR for 25 cycles as described in the legend to Figure 1. Products were analyzed on 1% agarose–TBE gels. (**b**) Total protein from parallel cultures was analyzed by western blotting using a monoclonal antibody against COX-2. (**c**) PGE_2 production in the presence of 10 μM arachidonic acid to provide sufficient substrate over 24 h was measured as in Figure 5.

To determine the effect of COX-2 expression on the sensitivity towards COX inhibitors, we repeated the experiment shown in Figure 2 with a panel of clones displaying different levels of COX-2 expression and PGE_2 production. We constructed dose–response curves for all compounds, comparing overexpressors with control clones. Figure 7 summarizes data from three independent experiments that protected against sulindac sulfide in clones 2 and 32, which produce high amounts of PGE_2 (Figure 7d). Protection against the COX-2 inhibitor SC125 was observed in clone 32 (Figure 7b), but there was no protection against other inhibitor compounds, including the COX-2 inhibitor SC236. Low and intermediate expressors were at least as sensitive as controls towards all compounds.

Analysis of apoptosis-associated proteins showed an increase of Bcl-2 expression in overexpressing clones, but also in three of five control clones (not shown). This increase was observed regardless of whether the cells were protected from apoptosis or not and exposure to inhibitor compounds still reduced the amount of Bcl-2 (Figure 8).

Discussion

All the compounds tested—the NSAID sulindac sulfide, its metabolite sulindac sulfone, the specific COX-2 inhibitors SC125 and SC236 and the COX-1 inhibitor SC560—inhibited growth of colorectal tumor cells. Both induction of apoptosis and inhibition of proliferation (DNA synthesis) were part of the overall effect of all five compounds, but each had its characteristic pattern of effects. The effects were independent of the activity and specificity of the compound towards COX isoenzymes. Even the COX-inactive metabolite sulindac sulfone had qualitatively the same effect, although higher concentrations were necessary (the ID_{50} was 200 μM as compared with 40–90 μM for the sulfide). This generally confirms data from other groups showing growth inhibition and/or induction of apoptosis in various tumor cell lines (18,22–24). The sensitivity of the different cell lines towards inhibitors was not related to their COX-1 or COX-2 expression, with one exception: HT29/HI1 cells that had the highest expression of COX-1 were less sensitive towards the COX-1 inhibitor than any other cell line used. Strong selectivity for malignant over benign cell lines, as demonstrated by Elder *et al*. using the aspirin metabolite salicylic acid (12), was not observed. On the contrary, the early adenoma cell line LT97 was the most sensitive line towards both sulindac derivatives and towards SC236.

Inhibition of DNA synthesis must play only a minor role in growth inhibition by the tested compounds, as DNA synthesis by the two carcinoma cell lines never dropped below 50% of that in controls. Only the VACO235 adenoma cells displayed strong inhibition of proliferation (DNA synthesis). Induction of apoptosis was stronger, as shown by the increase in the proportion of apoptotic cells in the treated cultures. Apoptosis-related proteins were only analyzed in SW480 cells, where loss of Bcl-2, induction of Bak and activation of caspase 3 occurred, again in different individual patterns for the individual compounds. The strongest reaction for all three markers was observed in SC560-treated cultures. Both sulindac metabolites caused loss of Bcl-2 and some activation of caspase 3. Surprisingly, SC236—the strongest growth inhibitor—caused nothing but a slight transient decrease in Bcl-2 at 8 h and slight activation of caspase at 48 h. In this case, induction and execution of apoptosis are probably caused by another mechanism, which is currently under investigation.

Overexpression of COX-2 in SW480-protected cells from apoptosis induced by sulindac sulfide, but not that induced by the sulfone metabolite. The protective effect was restricted to the two clones with the highest PG production, indicating that at least part of the effect of NSAIDs on cell growth is mediated by COX-2 inhibition and decreased PG levels, as postulated previously (25). Another component of the overall effect might well be shared with sulindac sulfone, which induced apoptosis at three to five times higher concentrations than the sulfide. With the COX-2 inhibitor SC125, protection was observed in the COX32 clone, which produces high levels of PG.

SC236 effects, on the other hand, were not affected by increased COX-2 expression and PG levels or by exogenous

Table II. COX2 expression and growth in overexpressors

Clone number	Amount of protein[a]	mRNA expression[b]	PG production (pg/10^6 cells)[c]	Doubling time (h)[d]	Serum (h)[d] dependency[e]
2	+++	450	409.3 ± 41	36	0.56
7ü	++	236	14.0 ± 0.7	nd	nd
9ü	++	nd	14.0 ± 1.3	nd	0.54
12	+	164	353.2 ± 6.6	30	0.66
13	++	nd	nd	nd	nd
14ü	+	350	96.6 ± 4.8	nd	nd
16	-	444	350.8 ± 42	30	0.54
22	++	276	8.0 ± 0.4	26	0.56
29	+++	273	107.4 ± 7.5	24	0.66
32	++++	32	382.8 ± 76.4	36	0.53
33	++	nd	nd	nd	nd
34	++	nd	nd	nd	nd
Co	–	0	<5	24	0.75
Co	–	0	<5	60	0.66
SW480	–	0	<5	24	0.72
VACO	+++++	nd	1364.8 ± 68.2	72	nd

[a]Semiquantitative evaluation of protein level from western blotting using 50 µg each of total cellular protein.
[b]Evaluation of mRNA expression using RT–PCR and calculated relative to the housekeeping gene GAPDH (25 cycles; compare Figure 6). Measurements for VACO235 were done in an earlier experiment using RPL6 as the housekeeping gene (Figure 1b).
[c]Mean ± SD of triplicates.
[d]Cells were plated at 2.5×10^4 cells/well in 24-well plates and cell number determined on days 1, 3, 5, 7 and 9. Doubling times were assessed during the logarithmic growth phase.
[e]Cells were plated at 5×10^4 cells/well and changed to serum-free MEM or MEM containing 10% FCS 24 h later. Cell numbers were determined 72 h after the change in medium and the quotient of cell number in serum-free MEM and cell number in FCS is given.

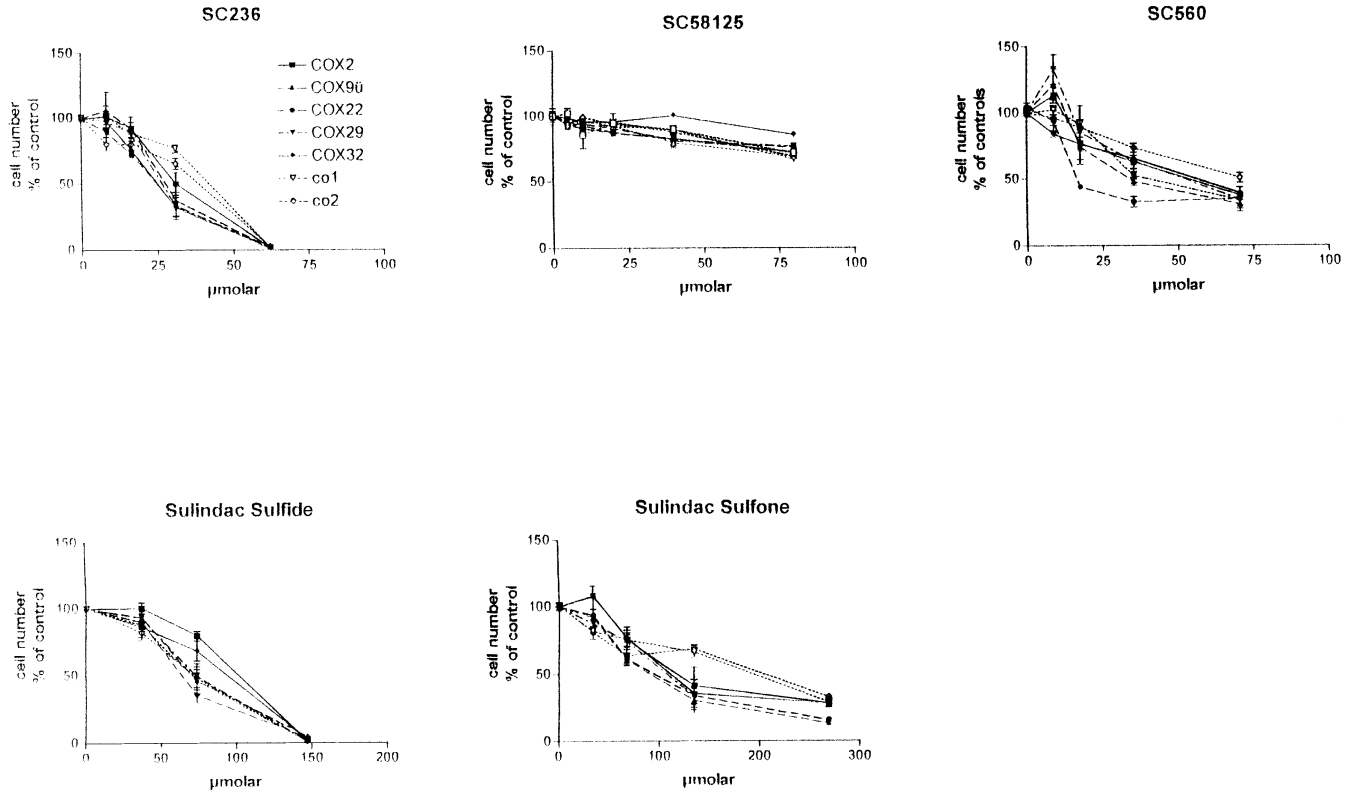

Fig. 7. Induction of apoptosis in COX-2-overexpressing clones. Cells were plated as in Figure 2 and exposed to inhibitors for 72 h. Cell number was determined using the MTT assay. The results are given as a percentage of control values and are the mean ± SEM from three or more experiments each using triplicate assays.

PG substitution. Therefore, we have to assume that even though the drug is a highly effective COX-2 inhibitor and was the most potent growth inhibitor used in this study, these two attributes are independent of one another. Analysis of apoptosis-related proteins also supports the assumption that SC236 uses other mechanisms: while ⩾50% of cells were lost within 24 h of SC236 exposure and the fraction of apoptotic cells in the population increased, as seen with other drugs, those markers of apoptosis that were modulated by sulindac and even more strongly by SC560 were barely affected by SC236. This clearly

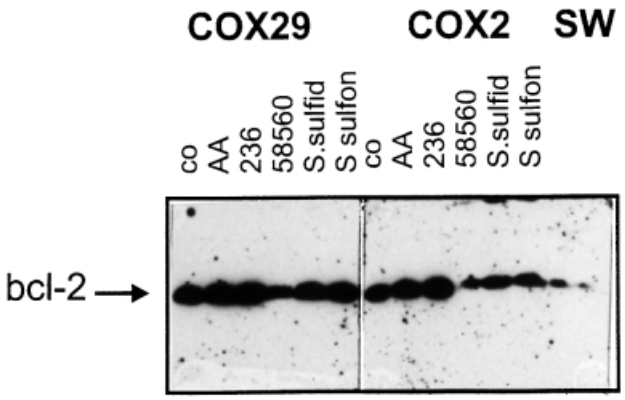

Fig. 8. Bcl-2 in COX-2-overexpressing clones. Cultures exposed to 30 μM SC236, 70 μM SC560 or sulindac sulfide or 270 μM sulindac sulfone in the presence of 10 μM arachidonic acid or control media with and without fatty acids were lysed after 24 h of exposure and analyzed by western blotting as described in Figure 4.

puts SC236 in a separate mechanistic group using different cellular targets. Possible candidates in this regard include modulation of growth factor pathways (26), inhibition of NF-κB activation (27) or modulation of stress-activated kinase (28). These possibilities are currently under investigation.

As for the COX-1 inhibitor SC560, the lesser sensitivity of HT29/HI1 cells that express the highest level of COX-1 indicates that this isoenzyme is involved in the cellular effect. However, we have not constructed COX-1-overexpressing clones, and instead concentrated on the isoenzyme that is implicated in tumor development. We therefore cannot make any definite statement where COX-1 is concerned.

In vivo experiments in colon carcinogenesis (29) and in skin tumor promotion in the mouse (30) have provided strong indications that COX-2 is causally involved in tumor development. *In vitro* studies like this one do not generally support this conclusion. No effects on cell number or apoptosis were observed using concentrations of <5–10 μM, the concentrations that can be obtained in the tissue *in vivo*. This is in agreement with earlier observations (for example, see refs 12, 16, 18, 19 and 27) and indicates that direct effects on the colorectal epithelial cells may not be the only or even the main effect of NSAIDs *in vivo*. Indirect mechanisms, e.g. involving macrophages or effects on angiogenesis (31–33), may be more important than has been recognized yet. This assumption is also supported by the observation that in tissue sections of colorectal tumors, COX-2 protein levels do not correlate with the incidence of apoptosis (O.Habel and B. Marian, unpublished observation). Lower concentrations may only be effective by indirect mechanisms, involving interactions with different cell types (as discussed above) or with components of the colonic lumen. In this context it is interesting to note that a selective COX-2 inhibitor was found to sensitize colorectal cells towards the growth inhibitory effect of butyrate, an endogenous degradation product of dietary fiber (34). This effect critically depended on COX-2 expression. The effective dose was 10 μM and thus in the dose range that inhibits COX-2 and is likely to be relevant to the human situation.

At the cellular level, our data indicate that different COX inhibitors inhibit cell growth by different pathways using different cellular targets which are not necessarily related to their COX-inhibitory activity or specificity. While the mechanisms of action of these compounds still need clarification, our results open alternatives for tumor prevention strategies. Compounds with different mechanisms of action should also display different side effects and a combination of such compounds could be used to minimize side effects while maximizing the therapeutic outcome.

Acknowledgement

This project was supported by the Jubiläumsfonds der Österreichischen Nationalbank project 6634.

References

1. DuBois,R.N. and Smalley,W.E. (1996) Cyclooxygenase, NSAIDs, and colorectal cancer. *J. Gastroenterol.*, **31**, 898–906.
2. Heath,C.W. Jr, Thun,M.J., Greenberg,E.R., Levin,B. and Marnett,L.J. (1994) Nonsteroidal antiinflammatory drugs and human cancer. Report of an interdisciplinary research workshop. *Cancer*, **74**, 2885–2888.
3. Giovannucci,E., Rimm,E.B., Stampfer,M.J., Colditz,G.A., Ascherio,A. and Willett,W.C. (1994) Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. *Ann. Intern. Med.*, **121**, 241–246.
4. Giardiello,F.M., Hamilton,S.R., Krush,A.J., Piantadosi,S., Hylind,L.M., Celano,P., Booker,S.V., Robinson,C.R. and Offerhaus,G.J. (1993) Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *New Engl. J. Med.*, **328**, 1313–1316.
5. Giardiello,F.M., Spannhake,E.W., Du Bois,R.N., Hylind,L.M., Robinson,C.R., Hubbard,W.C., Hamilton,S.R. and Yang,V.W. (1998) Prostaglandin levels in human colorectal mucosa: effects of sulindac in patients with familial adenomatous polyposis. *Digest. Dis. Sci.*, **43**, 311–316.
6. Eberhart,C.E., Coffey,R.J., Radhika,A., Giardiello,F.M., Ferrenbach,S. and Du Bois,R.N. (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*, **107**, 1183–1188.
7. Nishimura,G., Yanoma,S., Mizuno,H., Kawakami,K. and Tsukuda,M. (1999) A selective cyclooxygenase-2 inhibitor suppresses tumor growth in nude mouse xenografted with human head and neck squamous carcinoma cells. *Jpn J. Cancer Res.*, **90**, 1152–1162.
8. Reddy,B.S., Rao,C.V. and Seibert,K. (1996) Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. *Cancer Res.*, **56**, 4566–4569.
9. Pasricha,P.J., Bedi,A.,K.O.C., Rashid,A., Akhtar,A.J., Zahurak,M.L., Piantadosi,S., Hamilton,S.R. and Giardiello,F.M. (1995) The effects of sulindac on colorectal proliferation and apoptosis in familial adenomatous polyposis. *Gastroenterology*, **109**, 994–998.
10. Bedi,A., Pasricha,P.J., Akhtar,A.J., Barber,J.P., Bedi,G.C., Giardiello,F.M., Zehnbauer,B.A., Hamilton,S.R. and Jones,R.J. (1995) Inhibition of apoptosis during development of colorectal cancer. *Cancer Res.*, **55**, 1811–1816.
11. Keller,J.J., Offerhaus,G.J., Polak,M., Goodman,S.N., Zahurak,M.L., Hylind,L.M., Hamilton,S.R. and Giardiello,F.M. (1999) Rectal epithelial apoptosis in familial adenomatous polyposis patients treated with sulindac. *Gut*, **45**, 822–828.
12. Elder,D.J., Hague,A., Hicks,D.J. and Paraskeva,C. (1996) Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: enhanced apoptosis in carcinoma and *in vitro*-transformed adenoma relative to adenoma relative to adenoma cell lines. *Cancer Res.*, **56**, 2273–2276.
13. Charalambous,D., Skinner,S.A. and O'Brien,P.E. (1998) Sulindac inhibits colorectal tumour growth, but not prostaglandin synthesis in the rat. *J. Gastroenterol Hepatol.*, **13**, 1195–1200.
14. Alberts,D.S., Hixson,L., Ahnen,D., Bogert,C., Einspahr,J., Paranka,N., Brendel,K., Gross,P.H., Pamukcu,R. and Burt,R.W. (1995) Do NSAIDs exert their colon cancer chemoprevention activities through the inhibition of mucosal prostaglandin synthetase? *J. Cell. Biochem.*, **22** (suppl.), 18–23.
15. Reddy,B.S., Kawamori,T., Lubet,R.A., Steele,V.E., Kelloff,G.J. and Rao,C.V. (1999) Chemopreventive efficacy of sulindac sulfone against colon cancer depends on time of administration during carcinogenic process. *Cancer Res.*, **59**, 3387–3391.
16. Piazza,G.A., Rahm,A.L., Krutzsch,M. *et al.* (1995) Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. *Cancer Res.*, **55**, 3110–3116.
17. Piazza,G.A., Alberts,D.S., Hixson,L.J. *et al.* (1997) Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res.*, **57**, 2909–2915.
18. Elder,D.J., Halton,D.E., Hague,A. and Paraskeva,C. (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.

19. Picariello,L., Brandi,M.L., Formigli,L., Orlandini,S.Z., Dolara,P., Caderni,G., Raimondi,L. and Tonelli,F. (1998) Apoptosis induced by sulindac sulfide in epithelial and mesenchymal cells from human abdominal neoplasms. *Eur. J. Pharmacol.*, **360**, 105–112.
20. Hafez,M.M., Infante,D., Winawer,S. and Friedman,E. (1990) Transforming growth factor β1 acts as an autocrine-negative growth regulator in colon enterocytic differentiation but not in goblet cell maturation. *Cell Growth Differ.*, **1**, 617–626.
21. Willson,J.K., Bittner,G.N., Oberley,T.D., Meisner,L.F. and Weese,J.L. (1987) Cell culture of human colon adenomas and carcinomas. *Cancer Res.*, **47**, 2704–2713.
22. Erickson,B.A., Longo,W.E., Panesar,N., Mazuski,J.E. and Kaminski,D.L. (1999) The effect of selective cyclooxygenase inhibitors on intestinal epithelial cell mitogenesis. *J. Surg. Res.*, **81**, 101–107.
23. Lim,J.T., Piazza,G.A., Han,E.K. *et al*. (1999) Sulindac derivatives inhibit growth and induce apoptosis in human prostate cancer cell lines. *Biochem. Pharmacol.*, **58**, 1097–1107.
24. Sheng,G.G., Shao,J., Sheng,H., Hooton,E.B., Isakson,P.C., Morrow,J.D., Coffey,R.J. Jr, Du Bois,R.N. and Beauchamp,R.D. (1997) A selective cyclooxygenase 2 inhibitor suppresses the growth of H-ras-transformed rat intestinal epithelial cells. *Gastroenterology*, **113**, 1883–1891.
25. Du Bois,R.N., Giardiello,F.M. and Smalley,W.E. (1996) Nonsteroidal anti-inflammatory drugs, eicosanoids, and colorectal cancer prevention. *Gastroenterol Clin. North Am.*, **25**, 773–791.
26. Winde,G., Lugering,N., Glodny,B., Schmid,K.W., Muller,O., Senninger,N. and Osswald,H. (1998) Decreased HER-2 tyrosine kinase expression in rectal mucosa of FAP patients following low-dose sulindac chemoprevention. *Cancer Lett.*, **134**, 201–207.
27. Yamamoto,Y., Yin,M.J., Lin,K.M. and Gaynor,R.B. (1999) Sulindac inhibits activation of the NF-ΦB pathway. *J. Biol. Chem.*, **274**, 27307–27314.
28. Shin,E.A., Kim,K.H., Han,S.I., Ha,K.S., Kim,J.H., Kang,K.I., Kim,H.D. and Kang,H.S. (1999) Arachidonic acid induces the activation of the stress-activated protein kinase, membrane ruffling and H_2O_2 production via a small GTPase, Rac1. *FEBS Lett.* **452**, 355–359.
29. Oshima,M., Dinchuk,J.E., Kargman,S.L., Oshima,H., Hancock,B., Kwong,E., Trzaskos,J.M., Evans,J.F. and Taketo,M.M. (1996) Suppression of intestinal polyposis in Apc Δ716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, **87**, 803–809.
30. Muller Decker,K., Kopp Schneider,A., Marks,F., Seibert,K. and 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. Carcinogen.*, **23**, 36–44.
31. Bamba,H., Ota,S., Kato,A., Adachi,A., Itoyama,S. and Matsuzaki,F. (1999) High expression of cyclooxygenase-2 in macrophages of human colonic adenoma. *Int. J. Cancer*, **83**, 470–475.
32. Masferrer,J.L., Koki,A. and Seibert,K. (1999) COX-2 inhibitors. A new class of antiangiogenic agents. *Ann. N Y Acad. Sci.*, **889**, 84–86.
33. Uefuji,K., Ichikura,T. and Mochizuki,H. (2000) Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer. *Clin. Cancer Res.*, **6**, 135–138.
34. Crew,T.E., Elder,D.J.E. and Paraskeva,C. (2000) A cyclooxygenase-2 (COX-2) selective non-steroidal anti-inflammmatory drug enhances the growth inhibitory effect of butyrate in colorectal carcinoma cells expressing COX-2 protein: regulation of COX-2 by butyrate. *Carcinogenesis*, **21**, 69–77.

Received April 28, 2000; revised July 17, 2000; accepted September 7, 2000