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 colon 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. ID50 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/H11, the cell line expressing the highest level of COX-1 (ID50 70 µM; in other cell lines the ID50 was 15 µM). For all other compounds ID50 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 E2. 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.

Materials and methods

Cell lines

SW480 colon carcinoma cells were obtained from the American Type Culture Collection; HT29/H11 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.Willson (21) (Ireland...
Cancer Center, Case Western University, Cleveland, OH) and were cultured in MEM supplemented with 10% FCS, 2 × 10⁻⁴ M triiodo-L-thyronine, 1 µg/ml hydrocortisone, 10 µg/ml insulin, 2 µg/ml transferrin, 5 × 10⁻⁴ M selenite and 30 ng/ml epithelial growth factor (EGF); see ref. 26). The doubling time was ~60 h. The LT97 cell line was established from the mucosa of a familial 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 × 10⁻⁴ M selenite and 30 ng/ml EGF. Their doubling time under these conditions was ~96 h.

**COX inhibitors**

SCS8125, SC236 and SC58560 were a gift from Searl (Skokie, IL); sulindac sulfide and sulindac sulfate 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 × 10⁵ cells/well in 24-well plates, left to attach for 24 h (SW480, HT29/H11), 48 h (VACO235) or 96 h (LT97) and then exposed to inhibitors. Inhibitors were provided in DMSO-buffered MEM containing 1 mg/ml bovine serum albumin (BSA) for SW480, HT29/H11 and VACO235 and in 203 basic medium for LT97 cells. Control media contained the appropriate volume of DMSO; DMSO content at 414 nm. PG standard dilutions were prepared in MEM containing 10% FCS, to provide sufficient substrate for exactly 24 h. PGE₂ 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/acyticholinesterase tracer and a monoclonal antibody against prostaglandin and incubated at 4°C overnight. Unbound PG/acyticholinesterase was removed and washed extensively and bound acetylcholinesterase was detected by Eillman’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.

**Prostaglandin production**

Cells were plated at 5 × 10⁶ cells/well into 24-well plates. Twenty-four hours later, the cultures were exposed to treatment or control media containing 10 µg/ml acetic acid to provide sufficient substrate for exactly 24 h. PGE₂ 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/acyticholinesterase tracer and a monoclonal antibody against prostaglandin and incubated at 4°C overnight. Unbound PG/acyticholinesterase was removed and washed extensively and bound acetylcholinesterase was detected by Eillman’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.

**Growth effects of NSAID and isoenzyme-specific COX inhibitors**

Expression of both COX isoenzymes was determined from sub-confluent cultures of SW480 and HT29/H11 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/H11 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**

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

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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: ID₅₀ 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 (ID₅₀ = 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/H11 (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/H11 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 sulfone, ID₅₀ 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 ID₅₀ 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/H11 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/H1) 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.
NSAID-induced growth of colorectal tumor cells

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.

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

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (µM)</th>
<th>Apoptosis (% of nuclei)</th>
<th>Mitosis (% of nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC236</td>
<td>0.00</td>
<td>0.15 ± 0.05</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>16.50</td>
<td>0.70 ± 0.03</td>
<td>1.35 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>31.00</td>
<td>0.95 ± 0.07</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>SC58125</td>
<td>0.00</td>
<td>0.15 ± 0.05</td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>20.00</td>
<td>0.75 ± 0.10</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>40.00</td>
<td>0.98 ± 0.45</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>SC58560</td>
<td>0.00</td>
<td>0.20 ± 0.07</td>
<td>1.10 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>35.50</td>
<td>1.20 ± 0.03</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>71.00</td>
<td>1.50 ± 0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>0.00</td>
<td>0.10 ± 0.04</td>
<td>1.10 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>0.20 ± 0.07</td>
<td>1.75 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>15.00</td>
<td>0.30 ± 0.09</td>
<td>1.40 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>75.00</td>
<td>6.75 ± 0.13</td>
<td>0.00</td>
</tr>
<tr>
<td>Sulindac sulfone</td>
<td>0.00</td>
<td>0.15 ± 0.05</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>5.40</td>
<td>0.15 ± 0.08</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>27.00</td>
<td>0.10 ± 0.06</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>135.00</td>
<td>0.50 ± 0.10</td>
<td>0.30 ± 0.02</td>
</tr>
</tbody>
</table>

SW480 cells were seeded at $5 \times 10^4$ 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 procaspase 3 (Figure 4). Surprisingly, SC236 had the least effect 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.

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) promoter. 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).
COX-2 inhibitor SC236. Low and intermediate expressors the COX-32 clone, which produces high levels of PG.

We repeated the experiment shown Overexpression of COX-2 in SW480-protected cells from semi-confluent cultures and COX-2 expression was estimated by RT–PCR for 25 cycles as described in the legend to Figure 1. Total protein was analyzed by western blotting using a monoclonal antibody against COX-2.

(c) PGE₂ 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 PGE₂ 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 PGE₂ (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 ID₅₀ 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/H11 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 medicine.
Table II. COX2 expression and growth in overexpressors

<table>
<thead>
<tr>
<th>Clone number</th>
<th>Amount of protein</th>
<th>mRNA expression</th>
<th>PG production (pg/10^6 cells)</th>
<th>Doubling time (h)</th>
<th>Serum (h) dependency</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>+++</td>
<td>450</td>
<td>409.3 ± 41</td>
<td>36</td>
<td>nd</td>
</tr>
<tr>
<td>7u</td>
<td>+</td>
<td>236</td>
<td>14.0 ± 0.7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>9u</td>
<td>+</td>
<td>nd</td>
<td>14.0 ± 1.3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>164</td>
<td>353.2 ± 6.6</td>
<td>30</td>
<td>0.66</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>14u</td>
<td>+</td>
<td>350</td>
<td>96.6 ± 4.8</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>16</td>
<td>-</td>
<td>444</td>
<td>350.8 ± 42</td>
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<td>8.0 ± 0.4</td>
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<tr>
<td>Co</td>
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<td>&lt;5</td>
<td>60</td>
<td>0.66</td>
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<tr>
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<td>0</td>
<td>&lt;5</td>
<td>24</td>
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</tr>
<tr>
<td>SW480</td>
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<td>0</td>
<td>&lt;5</td>
<td>24</td>
<td>0.72</td>
</tr>
<tr>
<td>VACO</td>
<td>+++</td>
<td>nd</td>
<td>1364.8 ± 68.2</td>
<td>72</td>
<td>nd</td>
</tr>
</tbody>
</table>

*aSemiquantitative evaluation of protein level from western blotting using 50 µg each of total cellular protein.
*bEvaluation 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).
*cMean ± SD of triplicates.
*dCells 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.
*eCells 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
put 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/H11 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 concentra-
tions 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 strat-

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.

egies. 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


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