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Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells

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Abstract

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, is the only non-steroidal anti-inflammatory drug so far which has been approved by the FDA for adjuvant treatment of patients with familial adenomatous polyposis. The molecular mechanism responsible for the anticarcinogenic effects of celecoxib is still not fully understood. To investigate the extent to which the anticarcinogenic effect of celecoxib depends on COX-2 expression, we transfected human colon carcinoma cells (Caco-2) with the human COX-2 cDNA, in both sense and in antisense orientation, to generate cells which either overexpress COX-2 (human COX-2-sense, hCOX-2-s), express no COX-2 (human COX-2-antisense, hCOX-2-as) or express only very small amounts of COX-2 (control cells). Treatment of these cells with celecoxib dose-dependently (0–100 μM) reduced cell survival which was accompanied by an induction of a G_0/G_1 phase block and apoptosis. The effect of celecoxib treatment on both, cell survival and induction of apoptosis in hCOX-2-as cells was less marked than in the COX-2-expressing cells. Apoptosis was accompanied by an activation of caspase-3 and caspase-9 and cytochrome *c* release. In contrast, we observed no difference in sensitivity with regard to the induction of a cell cycle block between the different cell clones. The G_0/G_1 phase block caused by celecoxib correlated with a decrease in expression levels of cyclin A and cyclin B1 and an increase in the expression of the cell cycle inhibitory proteins $p21^{Waf1}$ and $p27^{Kip1}$ irrespective of the type of cell used. These data indicate that apoptosis-inducing effects of celecoxib partly depend on COX-2 expression of the cells, whereas induction of a cell cycle block occurred COX-2 independently. Thus, the anticarinogenic effects of celecoxib can be explained by both COX-2-dependent and -independent mechanisms.

Keywords: G_1/G_0 phase block; Apoptosis; NSAIDs; Cyclooxygenase; Colon cancer; Celecoxib

1. Introduction

Several epidemiological studies have shown that the regular intake of non-steroidal anti-inflammatory drugs (NSAIDs) decreases the risk of colon cancer [1–3]. NSAIDs inhibit cyclooxygenases which are key enzymes in the conversion of arachidonic acid to prostaglandins. Especially, for colon cancer cells but also for other tumour types an overexpression of COX-2 has been reported to occur [4,5]. This may result in a dysregulation of arachidonic acid metabolism and excessive production of prostaglandins. Furthermore, overexpression of COX-2 in tumour cells has also been implicated in various cancer-promoting effects, such as an alteration in cell adhesion to the extracellular matrix, upregulation of VEGF (vascular endothelial growth factor) and resistance to apoptosis-inducing stimuli [6–9]. According to these findings, the COX-2-selective NSAID, celecoxib, shows a chemopreventive effect both in animal tumour models and in patients [3,10,11]. Thus, it has been accepted that anticarcinogenic effects of NSAIDs are due to their ability to inhibit COX-2 [10]. On the other hand, however, it has been shown that celecoxib has an antitumourigenic effect in COX-2-deficient tumours in the nude mice model and also induces apoptosis in cells which do not express COX-2 [12–14]. Furthermore, some NSAID derivatives that do not inhibit COX activity retain their chemopreventive action [15–19]. Thus, these latter observations support the hypothesis that some of the antiproliferative effects of celecoxib are independent of COX-2 inhibition.

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Abbreviations: COX-2, cyclooxygenase-2; FCS, fetal calf serum; NSAID, non-steroidal anti-inflammatory drug; PARP, poly(ADP)-ribose polymerase; RT–PCR, reverse transcription–polymerase chain reaction; PGE_2, prostaglandin E_2; EIA, enzyme-linked immunoassay.

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In order to investigate the extent to which the anti-carcinogenic effect of celecoxib is dependent on COX-2 expression, we prepared Caco-2 cells which either over-express COX-2, express no COX-2 or express only very small amounts of COX-2. This was achieved by stable transfection of Caco-2 cells using human COX-2 cDNA in sense and antisense orientation, respectively. Using these cells it is possible to assess the effect of celecoxib as a function of COX-2 expression without the interference of other genetic factors. Celecoxib has been reported to induce apoptosis and a cell cycle block which is accompanied by changes in the expression level of various cell cycle regulating proteins [12]. In this study we, therefore, have compared the effects of celecoxib on the induction of apoptosis and cell cycle inhibition in Caco-2 cells expressing COX-2 (human COX-2-sense, hCOX-2-s) and Caco-2 cells which do not express COX-2 (human COX-2-antisense, hCOX-2-as) and focused on the expression levels of various cell cycle and apoptosis regulating proteins.

2. Material and methods

2.1. Cells and reagents

The human colon cancer cell line Caco-2 (ACC 169) was purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-I medium containing 10% fetal calf serum (FCS) at 37° in an atmosphere containing 5% CO_2. Celecoxib was synthesised by Berlin-Adlershof GmbH. The identity and purity of celecoxib was determined using high performance liquid chromatography (HPLC) [20] and was >99%.

2.2. Determination of free celecoxib concentrations in medium containing 10% FCS

10 mL DMEM with Glutamax-I medium containing 10% FCS and increasing concentrations of celecoxib (0, 20, 40, 60, 80, 100 μM) were centrifuged at 180,000 *g* and 37° for 15 hr using a Sorvall Discovery 90 SE ultracentrifuge (Kendro Laboratory Products). The supernatant containing free (non-protein bound) celecoxib was diluted 1:10 and 1:100 in acetonitrile/H_2O/ammonia solution 25% (50:50:0.1). Celecoxib concentrations were measured using liquid chromatography tandem mass spectrometry (LC/MS-MS) as described previously [20].

2.3. Stable transfection of Caco-2 cells with the human COX-2 cDNA

We cloned the human COX-2 cDNA (a generous gift from Dr. Timothy Hla, USA) in sense and antisense orientation in the expression vector pcDNA3 to generate the hCOX-2-s pcDNA3 vector and the hCOX-2-as pcDNA3 vector, respectively. Caco-2 cells were then transfected with the COX-2 expression vectors and the pcDNA3 control vector using the calcium phosphate coprecipitation method. Forty-eight hours after transfection, cells were reseeded 1:4 and subjected to selection using 1.5 mg/mL neomycin. Neomycin-resistant clones were isolated 10 days later and checked for COX-2 expression levels using the Western blot method. hCOX-2-s, hCOX-2-as and pcDNA control transfected cell clones were isolated and used in the experiments.

2.4. Colony forming assay

500 cells of the respective Caco-2 clones were seeded in 5 cm diameter dishes and incubated for 16 hr at 37° in an atmosphere containing 5% CO_2. Cells were then treated with increasing concentrations of celecoxib (0, 20, 30, 35, 40, 45 μM) and incubated further for 10 days. Cells were fixed with 100% methanol and stained with 5% crystal violet and 5% Giesma solution. Single cell colonies were counted using the Quantity one software (BIO-RAD). The number of colonies in the dish devoid of celecoxib was used as an index for a 100% survival rate (control) and this value was used to obtain the percentage survival rates for dishes containing celecoxib.

2.5. Detection of cell cycle arrest and apoptosis using flow cytometry

Caco-2 clones (hCOX-2-s, pcDNA control, hCOX-2-as) were seeded in concentrations of 5×10^5 cells per dish in medium containing 10% FCS and incubated for 24 hr at 37° in an atmosphere containing 5% CO_2. Cells were then synchronised by serum withdrawal for 48 hr and treated for 20 hr with increasing concentrations of celecoxib (0, 25, 50, 75, 100 μM) in medium containing 10% FCS. After trypsinisation, cells were harvested, washed twice with PBS and fixed with 80% ethanol. Cells were then incubated for 5 min with 0.125% Triton X-100, washed again and finally stained with 10 μg/mL ethidium bromide and 200 μg/mL RNaseA in PBS. For cell cycle analysis, 10,000 cells in the G_1 phase were determined using a flow cytometer (Beckton Dickinson FACSCalibur) and the CellQuest software (Beckton Dickinson). The percentage of apoptotic cells was calculated from the number of cells in sub-G_1 phase, representing fragmented cell vesicles.

2.6. Western blot analysis

Cells treated with increasing concentrations of celecoxib (0, 20, 40, 60, 80, 100 μM) for 20 hr were washed with 10 mL PBS, scraped in 1 mL PBS and centrifuged for 1 min at 11,000 *g*. After resuspending in buffer (10 mM Tris–HCl, pH 7.2, 1 mM EDTA, 1 mM mercaptoethanol, 5% glycine, 1 mM phenylmethylsulfonyl fluoride (PMSF),

5 mM dithiothreitol (DTT)), they were sonicated and centrifuged at 21,000 *g* for 10 min and the protein content in the supernatant was measured using the Bradford method. 30 μg of protein extract was separated electrophoretically on 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and proteins electroblotted onto a nitrocellulose membrane (Hybond-C, Amersham). Membranes were stained with 0.5% ponceau in 1% acetic acid to confirm equal loading. After overnight incubation of the membranes in blocking buffer (5% non-fat dried milk, 0.3% Tween 20 in PBS) or Odyssey blocking reagent (LI-COR Biosciences), diluted 1:1 in PBS, they were incubated for 4 hr with the respective primary antibody directed against COX-2 (1:1000 mouse monoclonal), poly(ADP)-ribose polymerase (PARP), 1:100 rabbit polyclonal, cyclin A (1:100 mouse monoclonal), cyclin B1 (1:100 rabbit polyclonal), p21 (1:100 rabbit polyclonal), p27 (1:1000 rabbit polyclonal), caspase-3 (1:100 rabbit polyclonal), caspase-9 (1:100 rabbit polyclonal) and cytochrome *c* (1:100 mouse monoclonal) diluted in blocking buffer. The COX-2 antibody was purchased from Cayman Chemical, the cytochrome *c* antibody from BD PharMingen, all other antibodies from Santa Cruz Biotechnology. Membranes were washed three times with 0.3% Tween 20 in PBS and then incubated with an IRDye800- or IRDye700-conjugated secondary antibody (BIOTREND Chemikalien GmbH) in blocking buffer or with a peroxidase-conjugated secondary antibody (Santa Cruz) for 1 hr. After extensive rinsing in 0.3% Tween 20 in PBS, protein–antibody complexes conjugated with peroxidase were treated with enhanced chemoluminiscence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's protocol and exposed to a chemoluminescence film. Protein–antibody complexes conjugated with IRDye800/700 were visualised on the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.7. Measurement of cytochrome c release using Western blot analysis

After cells were exposed to 100 μM celecoxib for various times (0, 0.5, 1, 1.5, 2 hr), both floating and attached cells were collected and washed once with PBS and subsequently with buffer A (0.25 M sucrose, 30 mM Tris–HCl, pH 7.9, 1 mM EDTA). Cells were then resuspended in buffer A containing 1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin and homogenised with a glass Dounce homogenisator. After centrifugation for 10 min at 21,000 *g*, protein concentration of the supernatant was determined using the Bradford method. 30 μg of cytosolic protein extract was then applied to Western blot analysis as described above. Cytochrome *c* was detected using a mouse monoclonal antibody directed against human cytochrome *c* (Pharmingen) diluted 1:1000 in blocking buffer. The protocol was performed according to Li *et al*. [21].

2.8. Prostaglandin E2 enzyme-linked immunoassay (PGE_2-EIA)

Approximately 10^6 cells were incubated in 5 cm dishes for 24 hr at 37° in medium containing 10% FCS. Supernatant was removed and centrifuged briefly. The amount of PGE_2 in the supernatant was determined using the PGE_2 Correlate EIA™-Kit (Assay Designs Inc.) in accordance with the manufacturer's protocol. Parallel cells were harvested by trypsinisation and counted. The amount of PGE_2 in the supernatants [pg/mL] was related to the number of cells in the dishes [pg/10^6 cells].

2.9. Reverse transcription–polymerase chain reaction (RT–PCR) of COX-2

For extraction of total cellular RNA, approximately 5×10^6 cells were harvested in PBS and briefly centrifuged at 15,500 *g* for 1 min. Cells were then resuspended in 1 mL TRI reagent (150 mM ammonium thiocyanate, 300 mM guanidinium thiocyanate, 0.04 mM sodium acetate, 2% glycerol, 380 mg/L phenol, pH 5) and incubated for 10 min at room temperature. 200 μL chloroform were then added and the solution mixed and incubated for 15 min at room temperature. After centrifugation at 15,500 *g* and 4° for 15 min, the aqueous phase was separated into a new tube. One volume of isopropanol was added, the solution incubated for 10 min at room temperature and finally centrifuged at 4° and 15,500 *g* for 10 min. The supernatant was discarded and the pellet washed twice with 75% ethanol and dried. RNA pellets were resuspended in 40 μL DEPC water and treated with RNase-free DNaseI (Quiagen) according to the manufacturer's protocol in order to remove genomic DNA. The amount of RNA was determined using an Eppendorf-biophotometer.

Reverse transcription was performed with 500 ng of isolated RNA and an enhanced avian reverse transcription kit (Sigma-Aldrich GmbH). The RT reaction was carried out with random nonamer primers according to the manufacturer's protocol in which 50 ng of cDNA was applied to the Taqman® universal PCR Mastermix (P/N4304437, Applied Biosystems). A COX-2 specific probe (labelled with FAM, sequence: 5′-TTC TGA AAC CCA CTC CAA ACA CAG TGC-3′) and COX-2 specific primers (forward: 5′-GTT CCC ACC CAT CTC AAA AC-3′; reverse: 5′-CAA CGT TCC AAA ATC CCT TG-3′) were designed using the Primer Express Software (Applied Biosystems). The PCR was performed using a standard protocol recommended by the manufacturer with concentrations of primers optimised to 300 nM and probe concentration of 200 nM. Analysis was performed using the ABI PRISM® 7700 Sequence detection system (Applied Biosystems). For data analysis, the fit point method was employed. The relative amount of COX-2 mRNA in the different Caco-2 cell preparations was standardised against 18S

RNA and the relative amounts of COX-2 mRNA in hCOX-2-s and hCOX-2-as cells related to pcDNA control cells (set to 1). Fold increase of COX-2 mRNA in hCOX-2-s and hCOX-2-as cells was calculated using rel. mRNA $= 2^{-\Delta C_t(\text{COX-2})}/2^{-\Delta C_t(18S)}$, where $\Delta C_t(\text{COX-2}) = C_t^{\text{COX-2(hCOX-2-s,hCOX-2-as)}} - C_t^{\text{COX-2(pcDNA)}}$ and $\Delta C_t(18S) = C_t^{18S(\text{hCOX-2-s,hCOX-2-as})} - C_t^{18S(\text{pcDNA})}$.

2.10. Statistics

Data are presented as mean ± SEM. The SPSS 9.01 computer software was used for statistical analyses. IC_{80} values were analysed using a sigmoid E_{max} model and followed by subsequent submission to univariate ANOVA and t tests using a Bonferroni α-correction for multiple comparisons, α was set at 0.05.

3. Results

3.1. COX-2 expression

As a prerequisite for our experiments, we confirmed the different COX-2 expression levels of hCOX-2-s and hCOX-2-as clones using Western blot analysis (Fig. 1A), real time RT–PCR (Fig. 1B) and determination of PGE_2 levels (Fig. 1C). hCOX-2-s clones expressed high levels of COX-2 RNA and protein. pcDNA control clones showed only marginal COX-2 protein and hCOX-2-as clones showed no hCOX-2 protein expression, whereas the expression of COX-1 was equal in the various cell clones (data not shown). Since antisense mRNA just as sense mRNA can function as a template for RT–PCR, we observed increased amounts of COX-2 mRNA as compared to pcDNA cells in both cell clones, hCOX-2-s and hCOX-2-as. Because COX-2 is a key enzyme of the prostaglandin pathway, higher amounts of COX-2 may lead to an increase in prostaglandin production. We therefore determined the PGE_2 level in the supernatant of different hCOX-2-s, hCOX-2-as and pcDNA transfected Caco-2 cell clones using EIA (Fig. 1C). Overexpression of COX-2 led to a 3-fold increase in the production of PGE_2 in non-stimulated cells whereas hCOX-2-as and pcDNA control cells showed nearly the same basal PGE_2 level.

3.2. Cell survival

In order to investigate whether or not the sensitivity of colon cancer cells towards the selective COX-2 inhibitor celecoxib is dependent on the expression level of COX-2, we determined survival rates of hCOX-2-s, hCOX-2-as and pcDNA control cells in the colony forming assay. In Fig. 2 the means are shown of three separate experiments of different hCOX-2-s, hCOX-2-as and pcDNA Caco-2 cell clones. In all experiments, hCOX-2-s and pcDNA control clones were more sensitive to celecoxib

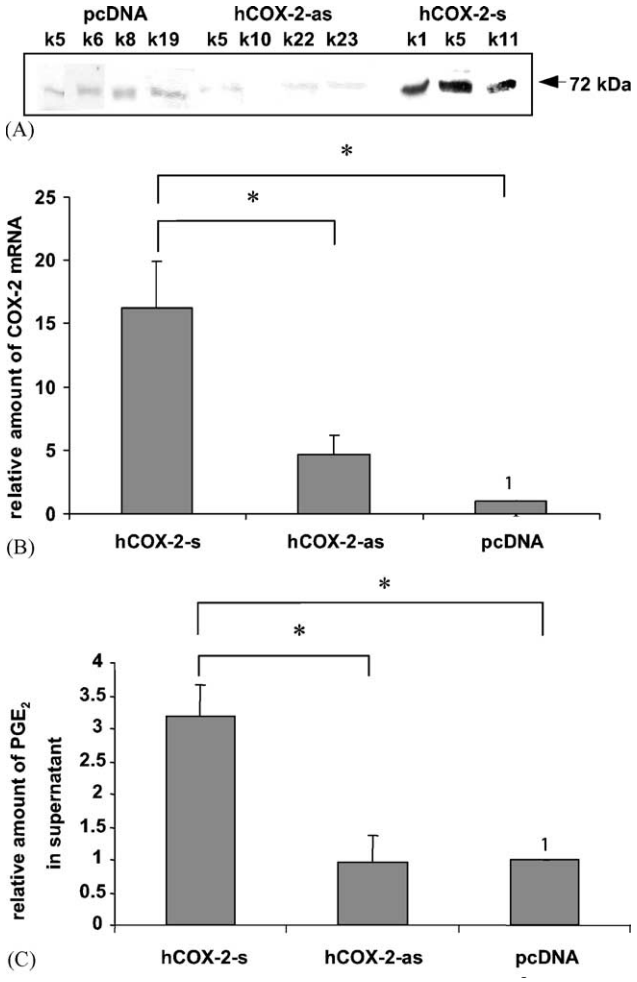

Fig. 1. (A) Western blot analysis of cyclooxygenase protein expression in Caco-2 hCOX-2-sense (hCOX-2-s), hCOX-2-antisense (hCOX-2-as) and pcDNA control cells. 50 μg of total protein extract per lane was loaded onto a 12% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Equal loading of the gel was checked by staining the membrane with ponceau solution. COX-2 protein expression was determined using a specific polyclonal antibody. (B) Determination of the relative amount of COX-2 mRNA in Caco-2 hCOX-2-s, hCOX-2-as and pcDNA control cells using Taqman® PCR. Total RNA was extracted with chloroform/phenol and DNA removed from the extract with RNAse-free DNAse. After reverse transcription, 50 ng of cDNA was applied to the Taqman® universal PCR Mastermix and PCR carried out using a standard protocol recommended by the manufacturer. The content of COX-2 mRNA in Caco-2 hCOX-2-s, hCOX-2-as and pcDNA control cells was standardised against 18S RNA and the amount of COX-2 mRNA in hCOX-2-s and hCOX-2-as cells expressed as a ratio of that in pcDNA cells. The mean of three independent experiments with different cell clones is shown. A significant difference in the relative amounts of COX-2 mRNA between the different cell clones is indicated with an asterisk, $P < 0.05$. (C) Relative amount of PGE_2 in the supernatant of hCOX-2-s and hCOX-2-as clones compared to pcDNA control cells. Measurement was performed using a PGE_2 Correlate EIA™-Kit according to the manufacturer's protocol. Parallel cells were harvested after trypsinisation, counted and the amount of PGE_2 related to the number of cells (pg PGE_2/10^6 cells). The mean of three independent experiments with different cell clones is shown. A significant difference in the relative amount of PGE_2 between the different cell clones is indicated with an asterisk, $P < 0.05$.

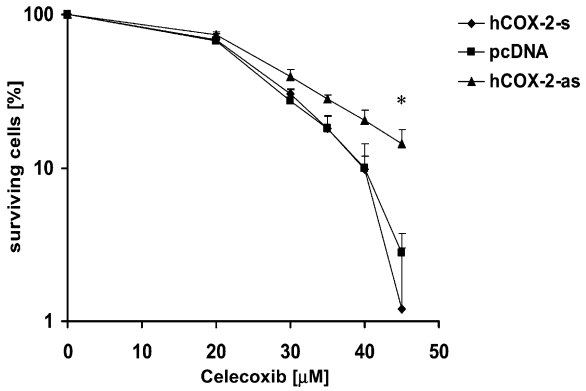

Fig. 2. Survival rate of hCOX-2-sense (hCOX-2-s), hCOX-2-antisense (hCOX-2-as) and pcDNA control cells after treatment with increasing concentrations of celecoxib. 500 cells were seeded per 5 cm dish. After an incubation time of 16 hr, cells were treated with celecoxib and incubated further until distinct single clones were detected. Cells were then fixed with methanol and stained. Single cell clones were counted and the number of untreated control cell clones set to 100% survival rate. The relative survival rate after treatment was expressed as a ratio of the number of untreated control cells. Data are mean ± SEM of three independent experiments with different cell clones. Statistically significant differences in survival rate of hCOX-2-as cells vs. pcDNA control cells are indicated with an asterisk, $P < 0.05$.

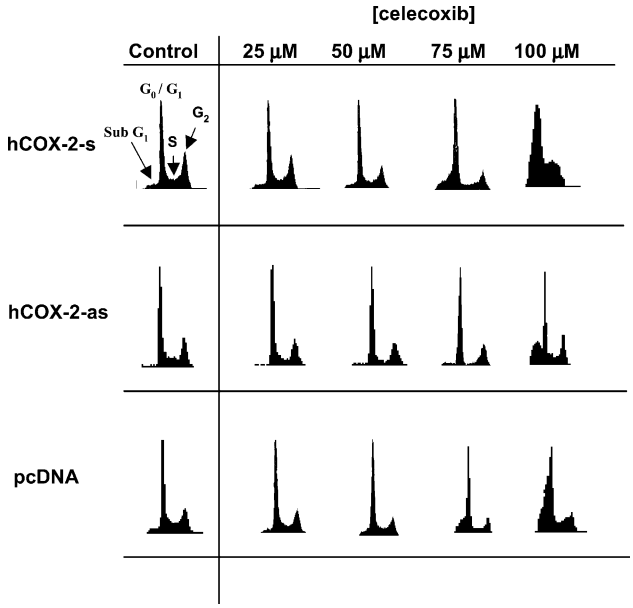

Fig. 3. Cell cycle analysis of hCOX-2-sense (hCOX-2-s), hCOX-2-antisense (hCOX-2-as) and pcDNA cells after treatment with increasing concentrations of celecoxib for 20 hr. Cells were harvested after trypsinisation, fixed with ethanol and the DNA content assessed using propidium iodide staining and fluorescence-activated cell sorter (FACS) analysis. A representative experiment from four separate experiments with different cell clones is shown.

treatment ($IC_{80} = 33.5 \pm 1.3$ μM and 32.7 ± 2.1 μM, respectively) than hCOX-2-as clones ($IC_{80} = 39.9 \pm 1.5$ μM). There were significant differences in sensitivity between hCOX-2-as and pcDNA control cells, $P < 0.05$.

3.3. Induction of apoptosis

In order to assess if the observed differences in survival rates between the COX-2 transfected Caco-2 cell clones are also reflected in the apoptosis rate of these cells after celecoxib treatment, we determined the percentage of cells in the sub-G_1 phase, representing the fraction of fragmented apoptotic cells, using flow cytometry. As shown in Fig. 3 and Table 1, hCOX-2-s and pcDNA control cells were more sensitive to the apoptosis induction potency of celecoxib than hCOX-2-as cells.

Table 1
Cell cycle distribution of the hCOX-2-sense, hCOX-2-antisense and pcDNA control cells after treatment for 20 hr with various concentrations of celecoxib

Celecoxib (μM)	Sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2 (%)
hCOX-2-sense				
Control	3.71 ± 0.67	45.21 ± 2.79	14.78 ± 1.67	32.03 ± 3.67
25	6.14 ± 1.65	51.63 ± 2.95	11.87 ± 0.13	30.13 ± 4.13
50	6.1 ± 1.27	56.02 ± 1.04*	11.92 ± 0.39	27.16 ± 2.28
75	17.43 ± 0.57*	52.07 ± 2.02	9.42 ± 1.32*	18.11 ± 1.84*
100	32.5 ± 0.77*	38.01 ± 2.27	10.45 ± 1.18	18.27 ± 3.06*
hCOX-2-antisense				
Control	3.76 ± 0.76	52.43 ± 1.54	14.76 ± 0.69	27.76 ± 1.33
25	3.86 ± 0.94	54.62 ± 1.2	14.12 ± 1.44	27.32 ± 1.91
50	3.33 ± 0.31	58.16 ± 2.57	11.37 ± 1.49	24.06 ± 2.85
75	6.07 ± 0.94	59.28 ± 1.85*	7.92 ± 0.32*	21.86 ± 3.77
100	19.19 ± 2.93*	46.94 ± 3.48	9.81 ± 1.78*	18.26 ± 5.49
pcDNA				
Control	4.55 ± 0.52	56.52 ± 1.61	15.5 ± 1.38	22.16 ± 0.59
25	3.38 ± 0.87	58.5 ± 1.77	15.53 ± 0.61	21.03 ± 1.33
50	6.7 ± 1.1	68.9 ± 5.54	10.62 ± 2.96	13.22 ± 2.54*
75	18.81 ± 4.08*	62.59 ± 3.79	5.09 ± 0.85*	12.88 ± 2.16*
100	31.17 ± 2.58*	41.79 ± 5.57	9.43 ± 1.34*	11.56 ± 1.44*

The mean values from four independent experiments with different cell clones are shown. Statistically significant changes caused by celecoxib vs. untreated control cells are indicated with an asterisk, $P < 0.05$.

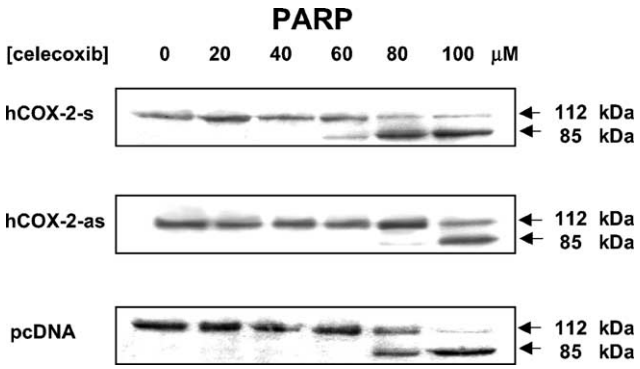

Fig. 4. Detection of PARP cleavage in hCOX-2-sense (hCOX-2-s), hCOX-2-antisense (hCOX-2-as) and pcDNA control cells using Western blot analysis after treatment with increasing concentrations of celecoxib. Cells were seeded in 10 cm dishes and treated for 20 hr with the concentrations indicated. 50 μg of total protein extract were separated on a 12% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane. PARP (112 kDa) and the apoptotic cleavage product of PARP (85 kDa) were detected using a rabbit polyclonal anti-PARP antibody. A representative experiment from three separate experiments with different cell clones is shown.

As a second marker for apoptosis we assessed PARP cleavage, using Western blot analysis. PARP is a 112 kDa nuclear protein that is specifically cleaved by activated caspase-3 and caspase-6 into an 85 and a 29 kDa apoptotic fragment. Figure 4 shows the Western blot analysis of PARP cleavage after treatment of cells for 20 hr with various concentrations of celecoxib. PARP cleavage in hCOX-2-s (60 μM) and pcDNA control (80 μM) cells occurred at lower concentrations of celecoxib than in hCOX-2-as cells (100 μM).

We next investigated the activation of caspase-3, caspase-9 and cytochrome *c* release, which are executors of apoptosis (Fig. 5A–C). Activation of caspase-3 and caspase-9 and cytochrome *c* release occurred soon (0.5–2 hr) after the addition of celecoxib (80–100 μM) and these changes were also more pronounced in hCOX-2-s and pcDNA control cells than in hCOX-2-as cells.

3.4. Influence of celecoxib on cell cycle distribution and expression of cyclin A and cyclin B1 as well as on the cell cycle inhibitory proteins $p21^{Waf1}$ and $p27^{Kip1}$

Celecoxib is known to cause a cell cycle block in the G_0/G_1 phase which is accompanied by a decrease in the expression of cyclin A and cyclin B1 and an increase in the expression of the cell cycle inhibitor proteins $p21^{Waf1}$ and $p27^{Kip1}$ [12]. Using flow cytometry, we could demonstrate that treatment of cells with celecoxib (0–75 μM) caused a concentration-dependent decrease in the number of cells in the S and G_2/M phase in hCOX-2-s, hCOX-2-as and in pcDNA Caco-2 clones with no significant differences between the different cell clones (Table 1).

Results from Western blot analysis showing the expression levels of cyclin A and cyclin B1, which in association with the respective cyclin-dependent kinases, are the main

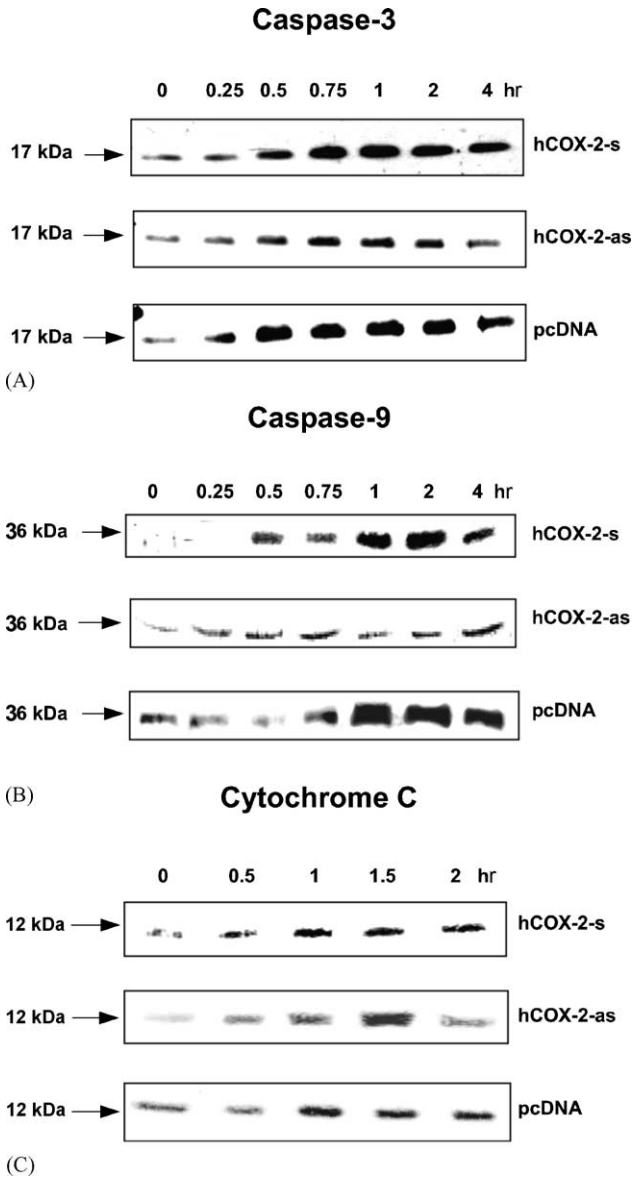

Fig. 5. (A) and (B) Western blot analysis of caspase activation after treatment of Caco-2 hCOX-2-sense (hCOX-2-s), hCOX-2-antisense (hCOX-2-as) and pcDNA control cells with 100 μM celecoxib. For Western blot analysis, 50 μg of total protein extract were separated onto a 12% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was then incubated with antibodies recognising subunits of caspase-3 and caspase-9 generated by activating cleavage of the whole caspase enzyme. Equal loading of the gel was checked by staining the membrane with ponceau solution. A representative experiment from three separate experiments with different cell clones is shown. (C) Western blot analysis of cytochrome *c* release into cytosol after treatment of Caco-2 hCOX-2-s, hCOX-2-as and pcDNA control cells with 100 μM celecoxib for various times. 30 μg protein were separated onto a 12% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane which was incubated with a specific monoclonal mouse antibody directed against human cytochrome *c*. Equal loading of the gel was checked by staining the membrane with ponceau solution. A representative experiment from two separate experiments with different cell clones is shown.

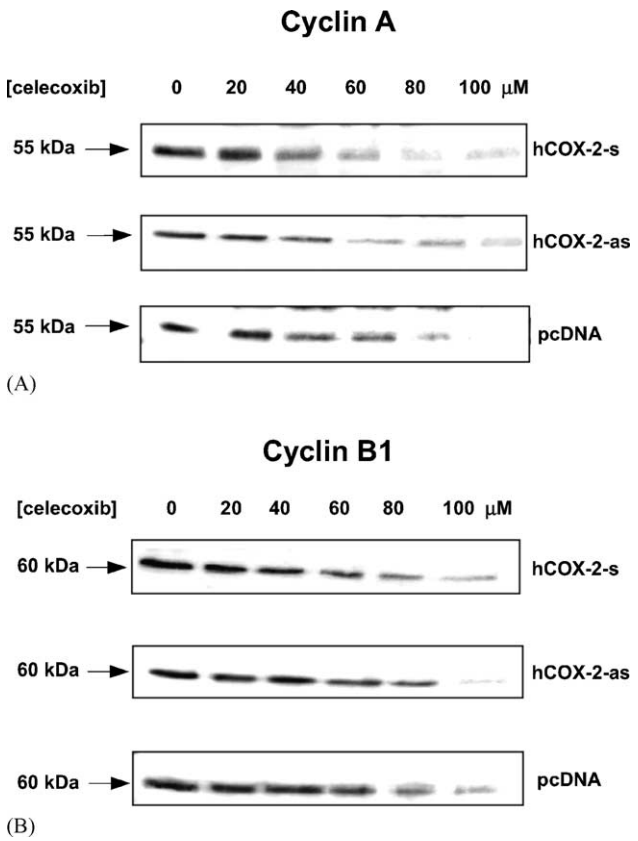

Fig. 6. Western blot analysis of cyclin A and cyclin B1 protein expression in Caco-2 clones after celecoxib treatment. Colon carcinoma cells were treated with increasing concentrations of celecoxib for 20 hr. For Western blot analysis 50 μg of total protein extract were separated onto a 12% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane which was incubated with specific antibodies directed against cyclin A and cyclin B1. Equal loading of the gel was checked by staining the membrane with ponceau solution. A representative experiment from two separate experiments with different cell clones is shown.

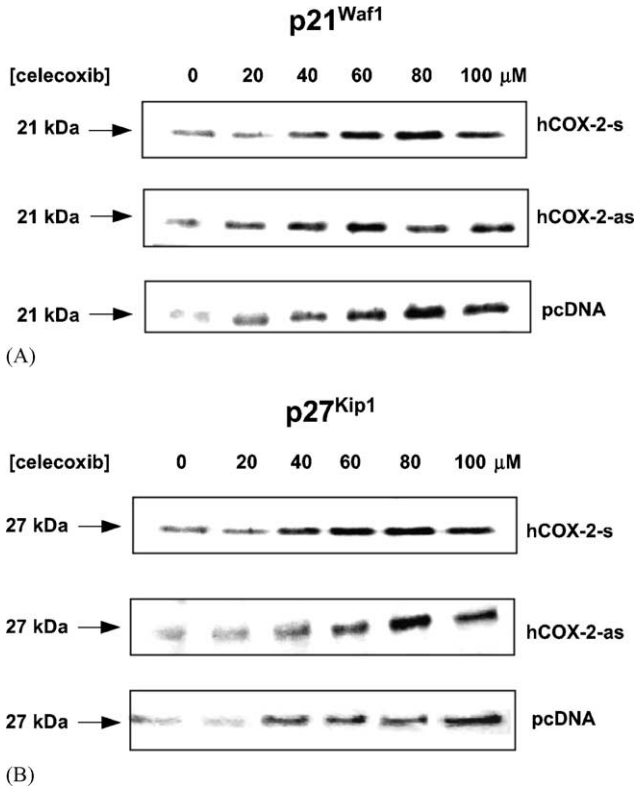

Fig. 7. Western blot analysis of $p21^{Kip1}$ (A) and $p27^{Waf1}$ (B) protein expression in hCOX-2-sense (hCOX-2-s), hCOX-2-antisense (hCOX-2-as) and pcDNA control cells after treatment for 20 hr with increasing concentrations of celecoxib. For Western blot analysis, 50 μg of total protein extract was separated onto a 12% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane which was incubated with specific antibodies directed against $p21^{Kip1}$ or $p27^{Waf1}$. Equal loading of the gel was checked by staining the membrane with ponceau solution. A representative experiment from three separate experiments with different cell clones is shown.

positive regulators in the progression of cells through the S and G_2/M phase, were in accord with the flow cytometry data. There was a decrease in cyclin A and cyclin B1 in hCOX-2-s, hCOX-2-as and pcDNA Caco-2 cell clones after 20-hr incubation with celecoxib at concentrations between 40 and 100 μM (Fig. 6A and B). Furthermore, the expression levels of the cell cycle inhibiting proteins $p21^{Waf1}$ and $p27^{Kip1}$ were increased after 20 hr celecoxib treatment at concentrations between 40 and 100 μM (Fig. 7A and B). There were no differences in the sensitivity of COX-2-overexpressing or COX-2-deficient cells neither in the case of downregulation of cyclin A or cyclin B1 nor in upregulation of the cell cycle inhibitors p21 and p27 after celecoxib treatment.

4. Discussion

In this study, we analysed the anticarcinogenic effects of the selective COX-2 inhibitor celecoxib using Caco-2 cell clones transfected with hCOX-2 cDNA in antisense orientation, sense orientation and with the empty pcDNA3 vector in order to obtain an insight into the role of COX-2 inhibition for its anticarcinogenic effects. We were able to show that COX-2-deficient Caco-2 cells (hCOX-2-as), when compared with hCOX-2-s and pcDNA control cells, are less sensitive to the apoptosis-inducing efficacy of celecoxib as determined by PARP cleavage, activation of caspase-3 and caspase-9, and cytochrome *c* release. Results similar to these have been reported by Li *et al.* [22] and Chang and Weng [23] who examined the apoptosis-inducing effects of the COX-2-selective inhibitor NS-398 in 15 human colon cancer cell lines and in lung cancer cells, respectively, and by Hsu *et al.* [24] who observed an enhanced apoptosis rate with celecoxib in prostate cancer cells overexpressing COX-2. However, we were unable to detect a difference in the sensitivity of the various Caco-2 cell clones in terms of induction of a G_1 cell cycle block and these data are in line with findings described by us previously [12].

COX-2-derived prostaglandins have been associated with antiapoptotic as well as proliferation-promoting

effects [7] and PGE_2 in particular, has been shown to act as an antiapoptotic agent and to promote cell cycle progression [1,25–28]. Since celecoxib is able to inhibit both cell proliferation *in vitro* and tumour growth *in vivo* [10,29], it appears at first glance that the pronounced apoptotic effects of celecoxib, observed in our Caco-2 hCOX-2-s cell clones when compared with COX-2-antisense cells (see Figs. 3–5), are associated with the inhibition of COX-2 and this is supported by the finding that COX-2-sense cell clones grew somewhat faster than COX-2-deficient Caco-2 cell clones (data not shown).

In a recent study, transfection of human hepatocellular carcinoma cells with a COX-2 expression vector also resulted in an increased cell growth. These cells showed an enhanced serine/threonine protein kinase B (PKB) phosphorylation which correlated with COX-2 overexpression. Treatment of these cells with celecoxib inhibited PKB phosphorylation, but this effect was only partly reversible by addition of exogenous PGE_2. It was therefore concluded that celecoxib induced apoptosis is mediated by both COX-2-dependent and -independent mechanisms [30]. In addition, Narayanan *et al*. demonstrated, that celecoxib treatment of chemically induced prostate cancer in rats also resulted in an induction of a cell cycle block and apoptosis and this was associated with downregulation of COX-2 and nuclear factor kappa B, and with activation of peroxisome proliferator-activated receptor gamma. These data again strengthen the hypothesis that celecoxib exerts its anticarcinogenic effect partly through COX-2-independent mechanisms [31]. Clear evidence that COX-2 inhibition alone is not responsible for the anticarcinogenic effect of celecoxib has been obtained from *in vitro* studies with another COX-2-selective inhibitor, rofecoxib. This agent has no, or only a weak antiproliferative action in various cell lines [32,33]. It is concluded, therefore, that in addition to the COX-2-inhibiting effect other mechanisms also play an important role in the antiproliferative effect of celecoxib and that these mainly became apparent at high celecoxib concentrations [34]. This hypothesis is also supported by a clinical study of Steinbach *et al*. who have shown that high doses of celecoxib (400 mg twice daily) significantly reduce polyp formation and polyp size in patients with familial adenomatous polyposis, whereas a dose of 100 mg celecoxib twice daily (the recommended dose for the treatment of rheumatoid arthritis and osteoarthritis and sufficient for complete COX-2 inhibition) has only marginal effects [3].

Our study corroborates these prior findings. Using cell lines with controlled COX-2 expression we were unable to detect any differences between COX-2-expressing and COX-2-deficient Caco-2 cell clones in the ability of celecoxib to inhibit the cell cycle, to decrease the percentage of cells in the S and G_2/M phase, to downregulate cyclin A and cyclin B1 or to induce the cell cycle inhibitor proteins p21 and p27 (see Figs. 3, 6 and 7). Thus, we also come to the conclusion that COX-2-independent mechanisms play an important role in celecoxib induced antiproliferative effects.

Information on an underlying possible COX-2-independent mechanism has been obtained by Johnson *et al*. [35] who were able to show that celecoxib led to an increase in intracellular [Ca^{2+}] arising from an inhibition of the endoplasmatic reticulum Ca^{2+}-ATPase. This was associated with the induction of apoptosis. Interestingly, Ca^{2+}-ATPase inhibitory activity was seen with celecoxib, but not with other COX inhibitors tested, including aspirin, ibuprofen, naproxen, rofecoxib, DuP697 (5-bromo-2-(4-fluorophenyl)-3-(4-methyl-sulfonylphenyl)thiophene) and NS-398 (*N*-(2-cyclohexyloxy-4-nitro-phenyl)methane sulfonamide) [35]. Very recently, Jendrossek *et al*. found celecoxib to induce apoptosis by induction of the mitochondrial apoptosome complex without involvement of the death receptor pathway [36]. Furthermore, it has been shown that induction of apoptosis by celecoxib is also associated with an inhibition of 3-phosphoinositide-dependent kinase 1 (PKB1) and the PKB/Akt pathway in human colon and prostate cancer cell lines [24,30,37]. It is of interest that Kundu *et al*. were able to show that treatment of breast cancer cells with celecoxib increased the number of cells in G_0/G_1 and reduced the S phase fraction and that this effect was accompanied by an increase in ceramide concentration in the cells. The addition of C6-ceramide to these cells mimicked this effect of celecoxib, thus supporting the hypothesis that an enhanced ceramide concentration is associated with cell cycle arrest and growth inhibition [38].

The celecoxib concentrations for induction of apoptosis and cell cycle arrest used in the various studies, including the present one were higher than the IC_{50} required for COX-2-inhibition. Thus, the aforementioned pathways may also play a role in our Caco-2 cell clones because apoptosis in Caco-2 COX-2-sense and COX-2-antisense cells occurred only at rather high concentrations of celecoxib (60–100 μM). Interestingly, the concentrations required for downregulation of various cyclins and upregulation of cell cycle inhibitor proteins were somewhat lower (40 μM).

It might be argued that celecoxib concentrations up to 100 μM are higher than those found in plasma samples of patients or animals in anticancer therapy. However, tumour regression in patients or animals requires weeks of drug treatment, whereas in our cell culture experiments, antiproliferative effects were already observed a few hours after incubation with celecoxib. Furthermore, all treatments occurred in medium containing 10% FCS and celecoxib is known to have a high protein binding capacity. Therefore, we determined the free (non-protein bound) fraction of celecoxib in medium containing 10% FCS using LC/MS-MS. We found approximately 30% of the added celecoxib (0–100 μM) to be non-protein bound and hence to be available for affecting the cells (data not shown). Thus, the free and therefore effective celecoxib concentrations in our cellular systems are lower than the total concentrations

of drug in the medium. To finally answer the question of celecoxib concentrations needed, further experiments should be aimed at determination of intracellular concentrations both in the *in vitro* and *in vivo* experiments.

In conclusion, we have obtained additional evidence that the apoptosis-inducing effects of celecoxib only partly depend on COX-2 expression of the cells. On the other hand, COX-2-independent effects are of importance since celecoxib was able to induce apoptosis in COX-2-deficient hCOX-2-as cells and cell cycle arrest occurred independently of the cellular COX-2 expression.

Acknowledgments

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References

[1] Fosslien E. Molecular pathology of cyclooxygenase-2 in neoplasia. Ann Clin Lab Sci 2000;30(1):3–21.
[2] Giardiello FM, Spannhake EW, DuBois RN, Hylind LM, Robinson CR, Hubbard WC, Hamilton SR, Yang VW. Prostaglandin levels in human colorectal mucosa: effects of sulindac in patients with familial adenomatous polyposis. Dig Dis Sci 1998;43(2):311–6.
[3] Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. New Engl J Med 2000;342(26):1946–52.
[4] Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 1995;55(17):3785–9.
[5] Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–8.
[6] Liu XH, Kirschenbaum A, Yao S, Stearns ME, Holland JF, Claffey K, Levine AC. Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. Clin Exp Metastasis 1999;17(8):687–94.
[7] Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res 1998;58(2):362–6.
[8] Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83(3):493–501.
[9] Subbaramaiah K, Dannenberg AJ. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol Sci 2003;24(2):96–102.
[10] Zweifel BS, Davis TW, Ornberg RL, Masferrer JL. Direct evidence for a role of cyclooxygenase 2-derived prostaglandin E(2) in human head and neck xenograft tumors. Cancer Res 2002;62(22):6706–11.
[11] Reddy BS, Hirose Y, Lubet R, Steele V, Kelloff G, Paulson S, Seibert K, Rao CV. Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. Cancer Res 2000;60(2):293–7.
[12] Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB J 2001;15(14):2742–4.
[13] Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 2001;15(12):2057–72.
[14] Yamazaki R, Kusunoki N, Matsuzaki T, Hashimoto S, Kawai S. Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells. FEBS Lett 2002;531(2):278–84.
[15] McCracken JD, Wechter WJ, Liu Y, Chase RL, Kantoci D, Murray Jr ED, Quiggle DD, Mineyama Y. Antiproliferative effects of the enantiomers of flurbiprofen. J Clin Pharmacol 1996;36(6):540–5.
[16] Wechter WJ, Kantoci D, Murray Jr ED, Quiggle DD, Leipold DD, Gibson KM, McCracken JD. R-flurbiprofen chemoprevention and treatment of intestinal adenomas in the APC(Min)/+ mouse model: implications for prophylaxis and treatment of colon cancer. Cancer Res 1997;57(19):4316–24.
[17] Reddy BS, Kawamori T, Lubet RA, Steele VE, Kelloff GJ, Rao CV. Chemopreventive efficacy of sulindac sulfone against colon cancer depends on time of administration during carcinogenic process. Cancer Res 1999;59(14):3387–91.
[18] Stoner GD, Budd GT, Ganapathi R, DeYoung B, Kresty LA, Nitert M, Fryer B, Church JM, Provencher K, Pamukcu R, Piazza G, Hawk E, Kelloff G, Elson P, van Stolk RU. Sulindac sulfone induced regression of rectal polyps in patients with familial adenomatous polyposis. Adv Exp Med Biol 1999;470:45–53.
[19] Song X, Lin HP, Johnson AJ, Tseng PH, Yang YT, Kulp SK, Chen CS. Cyclooxygenase-2, player or spectator in cyclooxygenase-2 inhibitor-induced apoptosis in prostate cancer cells. J Natl Cancer Inst 2002;94(8):585–91.
[20] Bräutigam L, Vetter G, Tegeder I, Heinkele G, Geisslinger G. Determination of Celecoxib in human plasma and rat microdialysis samples by liquid chromatography tandem mass spectrometry. J Chromatogr Biomed Appl 2001;261(2):203–12.
[21] Li M, Wu X, Xu XC. Induction of apoptosis by cyclo-oxygenase-2 inhibitor NS398 through a cytochrome *c*-dependent pathway in esophageal cancer cells. Int J Cancer 2001;93(2):218–23.
[22] Li M, Wu X, Xu XC. Induction of apoptosis in colon cancer cells by cyclooxygenase-2 inhibitor NS398 through a cytochrome *c*-dependent pathway. Clin Cancer Res 2001;7(4):1010–6.
[23] Chang HC, Weng CF. Cyclooxygenase-2 level and culture conditions influence NS398-induced apoptosis and caspase activation in lung cancer cells. Oncol Rep 2001;8(6):1321–5.
[24] Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. J Biol Chem 2000;275(15):11397–403.
[25] Houchen CW, Sturmoski MA, Anant S, Breyer RM, Stenson WF. Prosurvival and antiapoptotic effects of PGE2 in radiation injury are mediated by EP2 receptor in intestine. Am J Physiol Gastrointest Liver Physiol 2003;284(3):G490–8.
[26] Sanchez T, Moreno JJ. Role of EP(1) and EP(4) PGE(2) subtype receptors in serum-induced 3T6 fibroblast cycle progression and proliferation. Am J Physiol Cell Physiol 2002;282(2):C280–8.
[27] Fujino T, Yuhki K, Yamada T, Hara A, Takahata O, Okada Y, Xiao CY, Ma H, Karibe H, Iwashima Y, Fukuzawa J, Hasebe N, Kikuchi K, Narumiya S, Ushikubi F. Effects of the prostanoids on the proliferation or hypertrophy of cultured murine aortic smooth muscle cells. Br J Pharmacol 2002;136(4):530–9.

[28] Hansen-Petrik MB, McEntee MF, Jull B, Shi H, Zemel MB, Whelan J. Prostaglandin E(2) protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in Apc(Min/+) mice. Cancer Res 2002;62(2):403–8.

[29] Kawamori T, Rao CV, Seibert K, Reddy BS. Chemopreventive activity of Celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res 1998;58:409–12.

[30] Leng J, Han C, Demetris AJ, Michalopoulos GK, Wu T. Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: evidence for Akt inhibition in celecoxib-induced apoptosis. Hepatology 2003;38(3):756–68.

[31] Narayanan BA, Condon MS, Bosland MC, Narayanan NK, Reddy BS. Suppression of *N*-methyl-*N*-nitrosourea/testosterone-induced rat prostate cancer growth by celecoxib: effects on cyclooxygenase-2, cell cycle regulation, and apoptosis mechanism(s). Clin Cancer Res 2003;9(9):3503–13.

[32] Patti R, Gumired K, Reddanna P, Sutton LN, Phillips PC, Reddy CD. Overexpression of cyclooxygenase-2 (COX-2) in human primitive neuroectodermal tumors: effect of celecoxib and rofecoxib. Cancer Lett 2002;180(1):13–21.

[33] Waskewich C, Blumenthal RD, Li H, Stein R, Goldenberg DM, Burton J. Celecoxib exhibits the greatest potency amongst cyclooxygenase (COX) inhibitors for growth inhibition of COX-2-negative hematopoietic and epithelial cell lines. Cancer Res 2002;62(7):2029–33.

[34] Hawk ET, Viner JL, Dannenberg A, DuBois RN. COX-2 in cancer—a player that's defining the rules. J Natl Cancer Inst 2002;94(8):545–6.

[35] Johnson AJ, Hsu AL, Lin HP, Song X, Chen CS. The cyclo-oxygenase-2 inhibitor celecoxib perturbs intracellular calcium by inhibiting endoplasmic reticulum Ca^{2+}-ATPases: a plausible link with its anti-tumour effect and cardiovascular risks. Biochem J 2002;366(Pt 3): 831–7.

[36] Jendrossek V, Handrick R, Belka C. Celecoxib activates a novel mitochondrial apoptosis signaling pathway. FASEB J 2003;17(11): 1547–9.

[37] Arico S, Pattingre S, Bauvy C, Gane P, Barbat A, Codogno P, Ogier-Denis E. Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. J Biol Chem 2002;277(31):27613–21.

[38] Kundu N, Smyth MJ, Samsel L, Fulton AM. Cyclooxygenase inhibitors block cell growth. Breast Cancer Res Treat 2002;76(1):57–64.