Abstract. Background: The development of colon cancer is probably angiogenesis-dependent. Recently, sulindac sulfide was shown to possess anti-angiogenic activity. In the present work, the question of whether this activity reflects a specific interaction with angiogenesis or is secondary to the effect of sulindac sulfide on the survival of endothelial cells was addressed. Materials and Methods: Endothelial and normal mouse fibroblast cell lines were incubated with non-steroidal anti-inflammatory drugs (NSAIDs), arachidonic acid (AA) and prostaglandin E2 (PGE2). Cell viability (survival), PGE2 synthesis, cell cycle and apoptosis were measured. Western blotting and semi-quantitative RT-PCR multiplex methods verified the changes in the levels of pro-apoptotic proteins and their expressions, respectively. Results: Sulindac sulfide and celecoxib inhibited the survival of endothelial cells, whereas other NSAIDs were ineffective. In contrast to celecoxib, sulindac sulfide did not affect the survival of normal fibroblast cells. Both agents inhibited the production of PGE2 from AA and arrested the cell cycle in the S-phase. Moreover, sulindac sulfide activated caspases 3 and 8, decreased the levels of Bax and Bid proteins, caused cleavage of PARP and increased the expressions of the bax and caspase 3 genes. Conclusion: The results suggest that the anti-angiogenic activity of sulindac sulfide is secondary to the inhibition of endothelial cell survival resulting from cell cycle arrest and apoptosis.

Growing evidence supports the hypothesis that the development of colon cancer is angiogenesis-dependent. The microvessel density increases step-wise from normal mucosa to adenoma and carcinoma (1) and angiogenesis initiates the transition from low dysplasia adenoma to high dysplasia and cancer (2). A correlation exists between the microvessel count, histological grade and Dukes’ stage of human colon cancer (3), as well as between the tumor size and microvessel count (4).

To prove the above hypothesis, it is necessary to show that inhibition of angiogenesis arrests tumor growth and that chemopreventive agents possess anti-angiogenic properties. Indeed, bevacuzimab, a neutralizing antibody against vascular endothelial growth factor (VEGF), prolonged survival in patients with colorectal carcinoma (5) and sulindac, a chemopreventive agent (6), exerted anti-angiogenic activity (7, 8). However, the anti-angiogenic effect of sulindac sulfide, which is the most active metabolite of sulindac, seemed to be a secondary phenomenon as it was equally anti-angiogenic on growing and mature blood vessels, in contrast to the pro-angiogenic basic fibroblast growth factor (bFGF) (8). Moreover, sulindac sulfide induced three-fold stimulation of caspase 3 activity in the growing cells of the chorioallantoic membrane of chick embryo (8), suggesting that the anti-angiogenic activity could be secondary to the cytotoxic effect.

Therefore, the possibility that induction of apoptosis in endothelial cells by sulindac sulfide via inhibition of cell survival causes apparent anti-angiogenesis, and that inhibition of cyclooxygenase (COX) may contribute to the observed effect, were evaluated in the present study.

Materials and Methods

Cell cultures. The HMEC-1 human endothelial cell line was obtained from EMORY University (Atlanta, GA, USA) and was grown in MCDB 131 (Gibco, Warsaw, Poland) containing: 5% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamax, 100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin. Balb/3T3, a normal mouse fibroblast cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% heat-inactivated bovine serum, 2 mM glutamax, 100 units/ml penicillin, 100 µg/ml
streptomycin and 250 ng/ml amphotericin. The cells were cultured at 37°C in a humidified atmosphere including 5% CO2. The cells were incubated with the drugs (see below) for 24 and 48 h. Both floating and attached cells were harvested for subsequent analysis.

**Drugs.** The following drugs were studied: sulindac and its metabolites sulindac sulfide and sulindac sulfone (Sigma, Warsaw, Poland) were used at concentrations from 50 to 300 or 500 µM; indomethacin (Sigma), SC58125 (Cayman Chemicals, Warszaw, Poland) and celecoxib, valdecoxib and rofecoxib (Celon Pharma, Lomianki, Poland) were used at concentrations from 10 to 100 or 120 µM; arachidonic acid (AA) (Sigma) and prostaglandin E2 (PGE2) (Cayman Chemicals) were used at concentrations of 20 to 100 µM and 800 pg/ml, respectively. The drugs were dissolved in 100% dimethylsulfoxide (DMSO) and then diluted in the media for experiments. The final concentration of DMSO was maintained at 0.2%.

**MTT assay.** This assay relies on the ability of viable cells to reduce a tetrazolium salt (MTT) metabolically to a purple formazan product. The cells were grown in 96-well plates (1x10^4/200 µl/well). After incubation with the reagents, the medium was removed and the cells were treated with 50 µl of MTT for 4 h at 37°C. Next, 150 µl of solubilization solution (10% SDS) was added and the mixture incubated at 37°C overnight. The solubilized formazan product was spectrophotometrically quantified with the help of a microtiter plate reader, Power Wave XS (Bio-Tek, Winooski, VT, USA), at 570 nm wavelength.

**Prostaglandin E2 (PGE2) synthesis.** The cells were incubated in MCDB 131, with or without 20 µM or 100 µM of AA and celecoxib (30 and 100 µM) or sulindac sulfide (100 and 150 µM), for 24 h. The levels of PGE2 were determined using commercially available ELISA kits (Cayman Chemicals) according to the manufacturer’s protocol.

**Cell proliferation.** The cells were grown in 6-well plates (4x10^5/well). After incubation, both the adherent and floating cells were removed and counted using a Coulter® Z™ Series counter (Beckman, Fullerton, CA, USA).

**Flow cytometric analysis of cell cycle.** The cells (~1x10^6) were resuspended in 4 ml of 80% ethanol (~20°C) and incubated at ~20°C for 24 h, washed twice in phosphate-buffered saline (PBS), and stained with 50 µg/ml propidium iodide (PI) and 100 µg/ml RNase in 0.1% PBST solution (PBS buffer, Triton) for 30 min in the dark at 4°C. The samples were then measured using FACS Caliber flow cytometry (BD Biosciences, San Jose, CA, USA). The DNA histograms were analyzed using CellQuest software (BD Biosciences).

**Apoptosis measurements.** Apoptosis was measured, according to the manufacturer's instructions, using an annexin V-FITC kit (BD Biosciences). The cells were collected after treatment, washed twice with PBS and centrifuged to collect the cell pellet. The cell pellet was resuspended in ice-cold binding buffer. Next, annexin V-FITC and PI solutions were added to the cell suspension and mixed gently. The samples were then incubated for 15 min in the dark before being analyzed in the flow cytometer.

**Immunoblotting.** The cells were washed with cold PBS buffer and then the nuclear and cytoplasmic fractions were extracted using the NE-PER extraction kit (Pierce, Gdansk, Poland). The protein concentration in the samples was measured using the BCA protein assay kit (Bio-Rad, Warsaw, Poland). Samples containing 30 µg of protein with SDS-PAGE loading buffer were denatured and fractionated by 12% SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and probed with anti-human antibodies against: Bid (BD Biosciences), procaspase 3, pro-caspase 8, Bax and PARP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The signal was detected by a colorimetric method using the Opti-Amplified Immun-blot Assay kit (Bio-Rad).

**Semi-quantitative RT-PCR multiplex.** The mRNA levels of bax, caspase 3 and GAPDH were analyzed by RT-PCR using total RNA from HMEC-1 cells isolated using the Total RNA kit (Kucharczyk TE, Warsaw, Poland), as described by the manufacturer. One µg of total RNA was used in the reverse transcription reaction with RevertAid M-MuLV reverse transcriptase and oligo (d)18 primer (Fermentas, Vilnius, Lithuania). The resulting total cDNA was used as the template in the PCR multiplex to measure the bax and caspase-3 mRNA levels. The reactions were performed using the hAP02G-MPCR Amplification kit (Biosource International, Nivelles, Belgium). The GAPDH mRNA levels were used as internal controls. The amplified fragments were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

**Statistical analysis.** The data were analyzed by the one-way ANOVA test. To assess the significance of differences between the untreated group and treated groups, the Tukey post-hoc test was used and p values less than 0.05 (marked with asterisks on graphs) were considered to be statistically significant.

**Results**

The effects of sulindac, its metabolites, and other non-steroidal anti-inflammatory drugs (NSAIDs) on human endothelial (HMEC-1) and mouse fibroblast (Balb/c 3T3) cell lines in vitro. Among the tested NSAIDs incubated with endothelial cells, sulindac sulfide and celecoxib exerted the greatest inhibitory effects on survival, as measured by the inhibition of mitochondrial dehydrogenase (MTT assay, see Materials and Methods). The inhibitory effects were detected after 24-h incubation and increased with increasing incubation time. The strongest effect, approaching 100%, was observed after 48-h incubation with 120 µM of celecoxib and 150 µM of sulindac sulfide (ED50’s: 46 and 86 µM, respectively) (Figure 1).

The inhibitory effects of sulindac sulfide, celecoxib, sulindac sulfone and sulindac on HMEC-1 cell survival were dose-dependent and the correlation coefficients, which were estimated from the inhibitory dose-response curves, were 0.985, 0.945, 0.962 and 0.999, respectively. SC58125, at concentrations up to 100 µM, induced a small, but statistically significant, activity. In contrast, valdecoxib, rofecoxib and indomethacin had no significant effects on endothelial cells in vitro at concentrations up to 120 µM, following incubation for 24 or 48 h (data not shown).
The NSAIDs with the strongest activity against endothelial cells were also tested against normal mouse fibroblast cells (Balb/c 3T3) in vitro. The effects of sulindac sulfide (up to 300 μM) and sulindac (up to 500 μM) on the fibroblast cell line, after 48-h incubation, did not reach statistical significance. The effects of sulindac sulfone and celecoxib were significant at 500 μM and 25 μM, respectively. The strongest effect was observed with celecoxib, exceeding 90% at 125 μM (Figure 2).

The ratio of doses inducing inhibition of survival in 50% of cells (ED₅₀) was estimated from the dose-
response curve obtained for fibroblast and endothelial cells (ED_{50} Balb/c3T3/ED_{50} HMEC-1). In the case of celecoxib the ratio was 0.9, whereas in the case of sulindac sulfide (ED_{50} for Balb/c3T3 was extrapolated from the dose-response curve since up to 300 \mu M the effect of sulindac sulfide was not significant), it was 5.2. Any value above 1 indicated that the inhibitory effect of the tested substances was greater towards endothelial than normal fibroblast cells.

Inhibitory effects of sulindac sulfide and celecoxib towards endothelial cells and their interactions with arachidonic acid (AA) and prostaglandin E_2 (PGE_2). After 24 h of incubation, the endothelial cell basal production of PGE_2 was 66±6 pg/ml (n=3). The addition of AA increased the production, depending on the dose. The presence of celecoxib or sulindac sulfide inhibited AA-stimulated production of PGE_2 to the level of basal production (Figure 3). The presence of AA in the incubation medium significantly increased, while the presence of exogenous PGE_2 significantly decreased, the inhibitory effect of sulindac sulfide on survival of HMEC-1 cells (Figures 4 A and 4 B).

Effects of sulindac, sulindac sulfide, sulindac sulfone, celecoxib and SC58125 on the cell cycle and apoptosis in endothelial cells. The tested compounds that were incubated with endothelial cells for 24 h had exerted various effects on the cell cycle and apoptosis. In the control culture, the percent of cells in each phase was stable and the number of cells in the S- and G2/M-phases amounted to 50% of the total. Celecoxib and sulindac sulfide arrested the cell cycle in the S-phase at the expense of the G1- and G2/M-phases, while sulindac, sulindac sulfone and SC58125 arrested the cell cycle in the G1-phase (Figure 5 A). Additionally, sulindac sulfide and celecoxib induced the accumulation of a large number of dead cells in the sub-G1 peak. Sulindac sulfide and celecoxib provoked morphological changes associated with apoptosis in the HMEC-1 cells, which was confirmed by microscopy (Figure 5 B), and increased the number of apoptotic cells to 60.3% and 95.1%, respectively, as shown by flow cytometry (Figure 5 C).

Incubation of endothelial cells with sulindac sulfide resulted in a reduction of the levels of Bid and Bax proteins in the cytosolic fraction, as well as in reduction of the levels of pro-caspase 3 and, to a lesser degree, pro-caspase 8 (Figure 6 A). This was in contrast to the effect of sulindac sulfone. In addition, incubation of endothelial cells with
sulindac sulfide led to poly (ADP-ribose) polymerase (PARP) protein cleavage (Figure 6 A).

The reduction of the pro-caspase 3 level, which was induced by sulindac sulfide, was paralleled by increased transcription of the caspase 3 gene (Figure 6 B). Transcripts of the caspase 3 and bax genes were also increased by incubation of endothelial cells with sulindac sulfone (Figure 6 B).

**Discussion**

The major finding of this work was the explanation of the anti-angiogenic effect of sulindac sulfide, which is the most potent metabolite of sulindac, as a non-specific phenomenon, secondary to the antiproliferative and pro-apoptotic properties of this drug. Sulindac sulfide induced
Figure 5. A, DNA histograms showing cell cycle phase distribution of endothelial cells treated with sulindac, sulindac sulfone, sulindac sulfide, SC58125 and celecoxib (in concentrations as indicated) for 24 h and determined by FACS analysis, as described in Materials and Methods. Flow cytometric profiles of the DNA content upon PI staining from a representative experiment are shown. The percentages are given for each cell phase as mean±SD (n=6). B, Photomicrographs of the HMEC-1 cells after 24-h treatment with various compounds, as evaluated by Olympus B202 UV-visible microscope, magnification 15 x. Arrows indicate apoptotic cells. C, The percentage of apoptosis (population of apoptotic cells in the R3 and R5 areas), determined by FACS analysis, after 24-h treatment with various compounds in concentrations as indicated. The profiles are representative of six independent experiments. The results are expressed as the mean±SD, *p<0.05.
a three-fold stimulation of caspase 3 activities in the growing cells of the chick embryo chorioallantoic membrane (CAM) (8). Additionally, earlier observations (8) indicated that sulindac sulfide did not specifically interact with an angiogenic signaling cascade, since it exerted anti-angiogenic effects both in growing and mature blood vessels of CAM at the time when DNA synthesis in these cells had leveled off (9). Quite recently, Niederburger et al. (10) reported that celecoxib inhibited proliferation and induced apoptosis in human umbilical vein endothelial cells. These facts taken together suggest the possibility that sulindac sulfide may also inhibit the survival of endothelial cells. In the present work, the activity of sulindac sulfide was investigated and compared with other NSAIDs inhibitors of COX-1 and COX-2 enzymes. The preliminary experiments of Western blot analysis showed that the endothelial cells (HMEC-1) expressed both isoforms of the COX enzyme (results not shown).

Among the tested NSAIDs, incubated for 24 to 48 h with endothelial cells, celecoxib, sulindac and its metabolites, sulindac sulfide and sulindac sulfone, induced statistically significant dose- and time-dependent inhibition of HMEC-1 cell survival. The effects of celecoxib and sulindac sulfide were roughly six times greater than those of sulindac sulfone and sulindac. Sulindac sulfide inhibited endothelial cell survival at concentrations lower or comparable to those inducing an anti-angiogenic effect in the vessels of CAM of chick embryo (8). This supports the hypothesis that the anti-angiogenic effect of sulindac sulfide is secondary to its cytotoxicity towards endothelial cells.

Unexpectedly, valdecoxib, rofecoxib (COX-2 inhibitors) and indomethacin (COX-1 inhibitor), at concentrations up to 120 μM, did not induce significant inhibition of HMEC-1 cell survival when incubated with endothelial cells up to 48 h (results not shown). The results regarding valdecoxib and rofecoxib were surprising as these drugs are specific COX-2 inhibitors. However, they corroborated the data of Niederberger et al. (10), who found that both celecoxib and rofecoxib inhibited the production of prostanoids in endothelial cells, yet only celecoxib affected the proliferation of those cells.

Interestingly, sulindac sulfide was more than five times less cytotoxic than celecoxib to normal mouse fibroblasts. The ratio of cytotoxic ED50 for fibroblasts to the ED50 found for endothelial cells indicated that, in contrast to celecoxib, sulindac sulfide preferentially acted on endothelial cells.

The second important finding of the present work was the demonstration that the effect of sulindac sulfide on survival of HMEC-1 cells may be, at least in part, related to the inhibition of COX. Several lines of evidence substantiate this point. First, spontaneous production of PGE2 was detected in the studied endothelial cells. Second, the addition of AA increased the production of PGE2, over basal synthesis, depending on the concentration used. Third, sulindac sulfide as well as celecoxib significantly inhibited PGE2 production by the endothelial cells, while, finally, co-incubation of PGE2

Figure 6. A, Immunoblotting analysis of the level of pro-apoptotic proteins, pro-caspases 8 and 3, Bid and Bax following 24-h incubation of endothelial cells with sulindac (S), sulindac sulfone (SN) and sulindac sulfide (SD), at concentrations as indicated. Immunoblotting analysis of PARP protein following incubation of the cells (24 h) with SD at a concentration of 150 μM. The results were quantified by scanning densitometer. M, weight marker [kDa]. B, Analysis of caspase 3 and bax mRNA expression by semi-quantitative RT-PCR method after 24-h incubation of endothelial (HMEC-1) cells with DMSO (control), SN and SD at concentrations as indicated. M, marker [bp]; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
with sulindac sulfide significantly attenuated the effect of that agent on endothelial cell survival.

When endothelial cells were co-incubated with AA and sulindac sulfide, the survival inhibition of HMEC-1 cells was significantly enhanced, which was in contrast to the effect of PGE2. Therefore, it is postulated that the cytotoxic mechanism of sulindac sulfide on endothelial cells is, in part, due to the reduction of PGE2 synthesis resulting from the inhibition of COX by sulindac sulfide in these cells. This view is supported by the following: there is a basal synthesis of PGE2. Therefore, it is postulated that the cytotoxicity and AA could be non-enzymatically oxidized to fatty acids (11), which are probably toxic to the cell. Thus, the presence of COX-2 in non-malignant endothelial cells may be of importance for cell survival, by scavenging AA and in the production of PGE2 that protects the cell.

However, this view is not supported by the observation that rofecoxib, although it inhibited prostaglandin formation in endothelial cells, did not affect its survival (10, present results). Furthermore, co-incubation of AA with celecoxib did not increase cytotoxicity, unlike sulindac sulfide. It is, therefore, likely that the effect of sulindac sulfide is due to COX-dependent and COX-independent mechanisms, the latter related to antiproliferative and pro-apoptotic activities.

Sulindac sulfide, similarly to celecoxib, arrested the cell cycle in the S-phase at the expense of the G1- and G2/M-phases and, at the same time, increased the sub-G1-peak representing apoptotic cells. This was evidenced by morphological changes of the cells and flow cytometric measurements after annexin V staining. These results are in close agreement with those of Niederberger et al. (10). In contrast to the effects of sulindac sulfide and celecoxib, SC58125 (specific inhibitor of COX-2), at concentrations up to 100 µM, showed no effect on cell cycle progression and did not induce apoptosis to a significant extent. Sulindac and sulindac sulfone arrested the cell cycle in the G1-phase and their effects on apoptosis were negligible. Thus, the strong antiproliferative and pro-apoptotic properties of sulindac sulfide may be the primary explanation for its anti-angiogenic activity. In contrast to the expectations, the anti-angiogenic activity of sulindac sulfide, similarly to the anti-angiogenic activities of various cytostatics (12), seems to be a secondary phenomenon (13).

The effects of caspases were investigated first to evaluate the pathway of sulindac sulfide-induced apoptosis. The detected increase in activity of caspase 8 may indicate the involvement of death receptors in the apoptotic pathway. A decrease in the level of Bid was observed in the cytosolic fractions, which was probably connected with the fragmentation of this protein by active caspase 8. The cleavage of Bid (tBid) may lead to changes in the cytosolic level of Bax, and a two-fold reduction of the level of this protein supports such a conclusion. This suggests a cellular redistribution of Bax from a cytosolic to a membrane-bound form. The activation of caspase 8 and translocation of tBid and Bax to the membrane may enhance the release of cytochrome c from the mitochondria and, consequently, may lead to the activation of caspase 3. Caspase 3 can cleave numerous proteins involved in the cell structure, signaling and repair, which is essential for DNA fragmentation (14). The increased transcript levels of the caspase 3 and bax genes in HMEC-1 cells corroborated this view after 24-h incubation with sulindac sulfide. Finally, the activation of caspase 3 by sulindac sulfide led to the specific cleavage of PARP protein into 85 kDa fragments, which indicates irreversible apoptosis of the endothelial cells. In conclusion, it is postulated that sulindac sulfide (the most active metabolite of sulindac) activates both the extracellular and mitochondrial pathways of apoptosis in endothelial cells. Such activity, combined with induction of apoptosis in colon carcinoma cells (15), may explain, at least in part, the way in which sulindac induces regression of adenomatous colorectal polyps in humans.

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