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Apoptosis induced by sulindac sulfide in epithelial and mesenchymal cells from human abdominal neoplasms

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Abstract

We investigated whether the therapeutic action of sulindac, used for the treatment of familial adenomatous polyposis, desmoid tumors, and against colon cancer, could be mediated by its active metabolite, sulindac sulfide, in cell growth and apoptosis on cell lines derived from abdominal neoplasms. Sulindac sulfide actions on cell growth and apoptosis were evaluated in epithelial human colon tumor 8 (HCT8) cell line and mesenchymal cell lines (bovine bone endothelial (BBE) cell line, desmoid tumor-derived cells, human colorectal cancer-derived fibroblasts). Sulindac sulfide (0.1–60 μg/ml) induced a dose-dependent inhibition of cell proliferation of all cell lines tested. Apoptosis was induced at doses of 20 and 40 μg/ml, respectively, in BBE and HCT8 cells with no effect on desmoid tumor cells and colorectal cancer-derived fibroblasts. Since mesenchymal cells respond to clinically effective concentrations of the compound, its preferential action on the stromal compartment of intestinal polyps, desmoid tumors and colon cancer can be proposed, with consequent regression of the tumor.

Keywords: Sulindac sulfide; Colorectal cancer; Apoptosis

1. Introduction

Sulindac is a nonsteroidal anti-inflammatory drug (NSAID) that must be metabolized to its sulfide derivative to have anti-inflammatory activity (Duggan et al., 1977; Shen and Winter, 1977), blocking prostaglandin synthesis (Vane, 1977) through non-selective inhibition of the two cyclo-oxygenase isoenzymes cyclo-oxygenase 1 and cyclo-oxygenase 2 (Oshima et al., 1996). Since prostaglandins act as mitogens in several cell lines (Jimenez de Asua et al., 1975; Hubbard et al., 1988) and are elevated in many tumors including colon cancer (Rigas et al., 1993), the modulatory effect of sulindac on carcinogenesis has been associated with the inhibition of cyclo-oxygenase 1 and cyclo-oxygenase 2. However, some reports indicate that the anti-neoplastic activity of sulindac might be independent, at least in part, of prostaglandin metabolism, since its sulfone metabolite, inactive on cyclo-oxygenases (Duggan et al., 1977; Shen and Winter, 1977), exhibits anti-neoplastic activity in experimental mammary (Thompson et al., 1997) and colon cancer of rats (Charalambous and O'Brien, 1996).

On the basis of this observed activity, sulindac has been used as cancer preventive therapy in familial adenomatous polyposis patients, in which it reduces the number and size of polyps (Waddel et al., 1989; Giardiello et al., 1993; Tonelli and Valanzano, 1993).

In a previous paper we reported that patients with familial adenomatous polyposis (Spagnesi et al., 1994), who responded to sulindac therapy (200 mg/day × 60 days) with a dramatic decrease in the number of polyps, did not have reduced epithelial mucosal proliferation. It was later shown that sulindac sulfide reduces cell proliferation (Schnitzler et al., 1996) and induces apoptosis in cultures of epithelial human colon carcinoma cell lines (Piazza et al., 1995; Shiff et al., 1995; Goldberg et al., 1996).

In order to identify both possible additional cellular targets as well as their differential responses to sulindac, we evaluated the 'in vitro' effects of sulindac sulfide on cells derived from stromal (fibroblasts derived from colorectal cancer), and epithelial components of colon cancer tissue (human colon tumor 8 cell line, HCT8 cells) and on

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an endothelial cell line (bovine bone endothelial cell line, BBE cells) (Streeten et al., 1989) and on primary cultures of cells derived from desmoid tumors which are also sensitive to sulindac therapy (Waddel et al., 1983; Belliveau and Graham, 1984).

2. Materials and methods

2.1. Drug synthesis and characteristics

Sulindac sulfide was synthesized as follows: 57% HI (1.2 ml, 9.1 mmol) was added dropwise to a cool suspension of sulindac (800 mg, 2.2 mmol) in 12 ml of $CHCl_3$. The mixture was stirred for 2 h at 0°C and for 1 h at room temperature. The solvent was then removed in vacuo and the crude yellow product was purified by column chromatography (eluent: cyclohexane/ethylacetate 1:2). The reaction yield was 85%. The structure of the compound was confirmed by IR (infra red) and ^{1}H-NMR (hydrogen-nuclear magnetic resonance spectroscopy) and its purity was $> 98\%$.

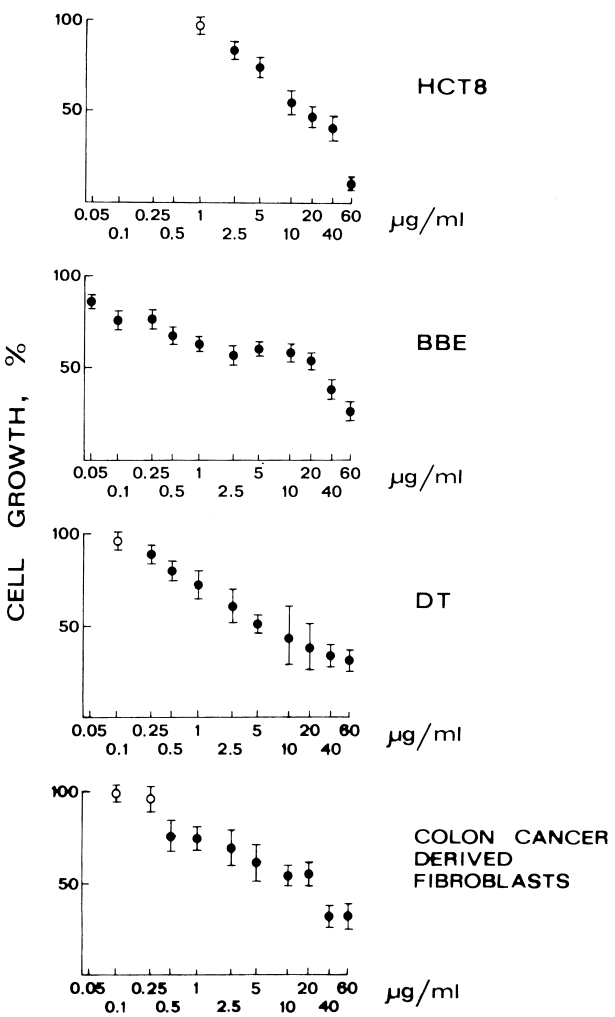

Fig. 1. Effect of sulindac sulfide on cell growth of HCT8 cells, BBE cells, desmoid tumors cells and colorectal cancer-derived fibroblasts. Results are expressed as means ± S.D. of three experiments in duplicate. The values significantly different ($P < 0.05$) from the control level (100%) are represented as a full circle (●). The open symbols (○) refer to values which do not differ from controls.

Table 1
Parameters of the dose–effect curves fitted on the data of Fig. 1 by means of logistic probit regression

Cell cultures	ED_{50} (μg/ml)[a]	Slope (±95% C.L.)
HCT8	2.9	−0.56 (−0.51, −0.60)
BBE	2.64	−0.17 (−0.13, −0.21)
Desmoid tumor cells	2.11	−0.32 (−0.26, −0.37)
Colon cancer fibroblasts	1.97	−0.33 (−0.27, −0.38)

[a] The data were calculated with ln (natural logarithm) concentration units.

2.2. Drug preparation

Stock solutions of sulindac sulfide were prepared at 20 mg/ml concentration in dimethyl sulfoxide (DMSO) and diluted with the appropriate media for cell culture testing. The final concentration of DMSO was kept at 0.1%. All drug dilutions were prepared fresh on the day of testing.

2.3. Cell cultures

Primary desmoid tumors cultures and colorectal cancer-derived fibroblasts were isolated from biopsies of human desmoid tumors ($n = 3$) or colon cancer ($n = 5$) specimens, as previously described (Tonelli et al., 1994; Picariello et al., 1997). Briefly, the specimens from desmoid tumors and colon cancer tissues were dissected into small fragments, digested with collagenase II (Sigma, Milan, Italy) (1 mg/ml in Hanks' balanced salt solution) for 2–3 h at 37°C in 5% CO_2 and 95% air or overnight, for desmoid tumors and for colon cancer specimens, respectively. Digested fragments were then mechanically dispersed in Hanks' balanced salt solution (Gibco, Grand Island, NY, USA). The cell suspension was centrifuged and the pellet was resuspended in the appropriate growth medium, Coon's modified Ham's F12 medium (Gibco) containing 10% fetal calf serum (Gibco), penicillin (100 IU/ml), and streptomycin (100 μg/ml). BBE cells, a clonal endothelial cell line from bovine bone, were cultured in the appropriate growth medium, Coon's modified Ham's F12 medium containing 10% Nu-Serum™ (Collaborative Research, Bedford, MA, USA) and 1% Ultroser-G™ (Sepracor, Villeneuve La Garenne, France), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Streeten et al., 1989). HCT8 cells, a stabilized cell line from human colon adenocarcinoma, were obtained from the American Type Cultures Collection (ATCC, Rockville, MD, USA) and were cultured in the appropriate growth medium, RPMI-1640 medium (Gibco), containing 1 mM pyruvic

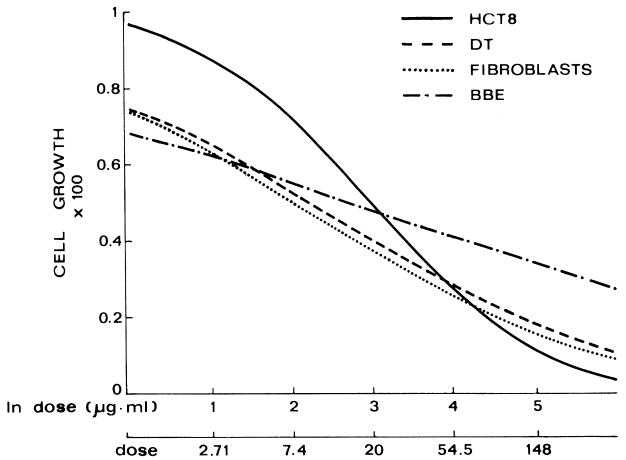

Fig. 2. Kinetics of cell growth for the different cell lines, calculated by GLIM4 logistic probit regression analysis. The curves describing the dose-related inhibition of growth were plotted using the original concentration values and their natural logarithmic (ln) transformation.

acid sodium salt, 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were cultured at 37°C in 5% CO_2 and 95% air until confluence, and then were detached with a trypsin/ethylenediaminetetraacetic acid (EDTA) solution for subculturing. All human samples examined in this study were collected with the approval of an Ethics Committee.

2.4. Cell growth

HCT8, BBE, desmoid tumor cells and colorectal cancer fibroblasts were plated in 6-well plates (PBI International, Milan Italy) at a density of 8×10^4 cell/well in growth medium. Appropriate amounts of drug solution were added directly to the growth medium the day after plating. Control cells were plated in growth medium supplemented with 0.1% DMSO. After 4 days (HCT8 cells) or 6 days (BBE, desmoid tumor cells and colon cancer fibroblasts) the cells were detached with trypsin/EDTA, and were counted with a Coulter counter. Cell growth was expressed as percent of control values. An aliquot of cells was tested for viability by the trypan-blue dye exclusion method.

2.5. Apoptosis

Apoptosis was evaluated with four different methods.

2.5.1. Colorimetric estimate of DNA fragmentation with the diphenylamine reaction

This was performed as previously described (Burton, 1956). Briefly, cells (5×10^6) were plated in growth medium; after 24 h the cells were stimulated with different concentrations of sulindac sulfide and the appropriate control was performed. After 24–48 h of incubation, the cells were detached with trypsin/EDTA, washed with phosphate-buffered saline (PBS) and resuspended with 500 μl of phenol red-free growth medium without fetal calf serum and 750 μl ice-cold lysis buffer (5 mM Tris, pH 8; 20 mM EDTA, pH 8; 0.5% Triton X-100). After a 15-min incubation, the lysis products were centrifuged 20 min at 10 000 $\times g$ to separate the intact chromatin of the pellet from fragmented DNA in the supernatant. The pellet was rinsed with TE buffer (10 mM Tris, pH 8; 1 mM EDTA, pH 8). Diphenylamine, which had been kept in the dark, was used as follows: 1.5 g diphenylamine/100 ml acetic acid; 1.5 ml sulfuric acid; 0.1 ml acetaldehyde/20 ml reagent. The reagent was freshly prepared for each experiment and 2 vols. of this solution were added to the supernatant and to the pellet. Acetaldehyde was prepared at a concentration of 16 mg/ml. The color was developed by heating the samples for 16–20 h in a 30°C water bath. Optical density was measured against ice-cold lysis buffer at a wavelength of 600 nm. The amount of fragmented DNA was expressed as percent of the supernatant absorbance relative to the total absorbance of the pellet and the supernatant. After 24 h of incubation with sulindac sulfide, DNA fragmentation was irrelevant. Therefore, all subsequent experiments were performed with 48 h of incubation.

2.5.2. Immunofluorescence microscopy with the TUNEL assay

For the HCT8 cell line, colorectal cancer fibroblasts, desmoid tumor cells and BBE cells, 8×10^5 cells were incubated with different concentrations of sulindac sulfide (0, 20, 40, 60 μg/ml) for 48 h. DNA breaks were detected by nick-end labeling with the TUNEL (TdT-mediated

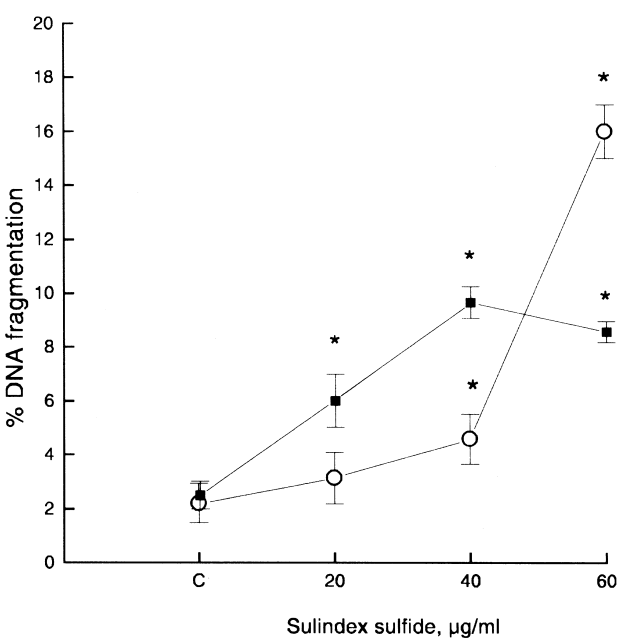

Fig. 3. Effect of sulindac sulfide on DNA fragmentation in HCT8 (○) and BBE cells (■). The amount of fragmented DNA was expressed as described in Section 2 and is the means ± S.D. from three separate experiments. $* P < 0.05$.

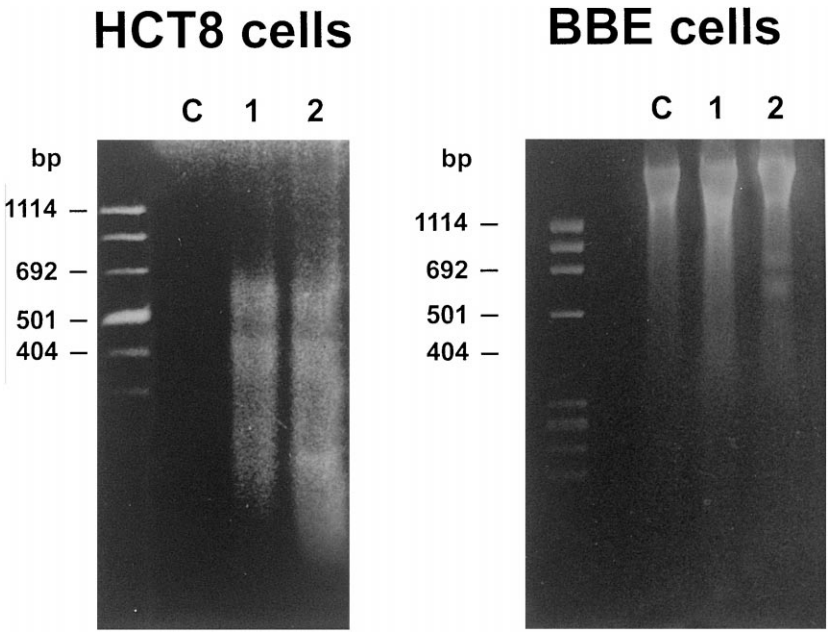

Fig. 4. Gel electrophoresis of genomic DNA from HCT8 and BBE cells, either untreated or treated with 40 and 60 μg/ml sulindac sulfide. (C, control; lane 1, 40 μg/ml sulindac sulfide; lane 2, 60 μg/ml sulindac sulfide. The position of DNA molecular weight standards is shown to the side of each panel.)

dUTP-biotin nick end labeling) method (Gavrieli et al., 1992) according to the manufacturer's instructions (TUNEL reaction, Boehringer Mannheim, Germany).

2.5.3. DNA gel electrophoresis

Internucleosomal fragmentation was evaluated as previously described (Wyllie, 1980). Genomic DNA was obtained from 1×10^7 HCT8 and BBE cells incubated with 0–20–40–60 μg/ml sulindac sulfide for 48 h. Briefly, cells were washed with PBS, resuspended in lysis buffer (5 mM Tris, pH 8; 20 mM EDTA, pH 8; 0.5% Triton X-100; 0.5 mg/ml proteinase-K) and incubated for 15 min on ice. After centrifugation at $10\,000 \times g$ the supernatant was digested with 0.5 mg/ml proteinase-K, heated for 1 h at 50°C, and digested with 0.25 mg/ml boiled RNAase IIA (Sigma) for 1 h at 37°C. DNA was extracted twice with 1 volume of phenol:chloroform (1:1) and centrifuged at 500 $\times g$ for 5 min at room temperature. One-tenth volume of 3 M sodium acetate (pH 5.2) and 2 vols. of absolute ethanol were added to the supernatant and incubated for 18 h at −20°C. After centrifugation at $10\,000 \times g$ for 20 min at 4°C, the pellet was resuspended in TE buffer and mixed with bromophenol blue. The sample was resolved on a 2% agarose gel stained with ethidium bromide, visualized by UV transillumination, and photographed using Polaroid 667 film.

2.5.4. Ultrastructural studies

The morphological characteristics of the apoptotic process were evaluated by transmission electron microscopy (TEM). Cells were plated in the appropriate growth medium for 24 h. After 48 h of incubation with sulindac sulfide, the cells were detached with trypsin/EDTA, centrifuged and the pellet was fixed at room temperature in 4% cold glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and fixed at 4°C with 1% OsO_4 in 0.1 M phosphate buffer pH 7.4. The samples were then dehydrated in serial acetone, passed through propylene oxide and embedded in Epon

Table 2
Sulindac sulfide effect on DNA fragmentation in HCT8 cells, colorectal cancer-derived fibroblasts, desmoid tumor and BBE cells

Sulindex sulfide (μg/ml)	HCT8 cells	BBE cells	Desmoid tumor cells	Fibroblasts
0	2.0 ± 0.5	0	5.81 ± 1.2	0
20	2.4 ± 0.9	10.61 ± 2.34	3.6 ± 2.0	0
40	47.4 ± 5.6	32 ± 3.20	6.5 ± 2.25	0
60	69.4 ± 4.5	39.96 ± 5.60	7.25 ± 2.4	19.7 ± 2.2

Results are expressed as percentages of fluorescent cells relative to the total cells counted on the glass slides. The values are expressed as the means ± S.D. of four to six measurements as described in Section 2.

812. Semi-thin sections (1–2 μm) were cut and stained with toluidine blue-sodium tetraborate. Ultrathin sections were also obtained from the same specimens, stained with uranyl acetate and alkaline bismuth subnitrate, and then examined with a Siemens Elmiskop electron microscope at 180 kV.

2.6. Statistical analysis

The results were expressed as means ± standard deviation (S.D.). The statistical significance of differences was analyzed with a one-way analysis of variance. Significance was adjusted for multiple comparison of means. The

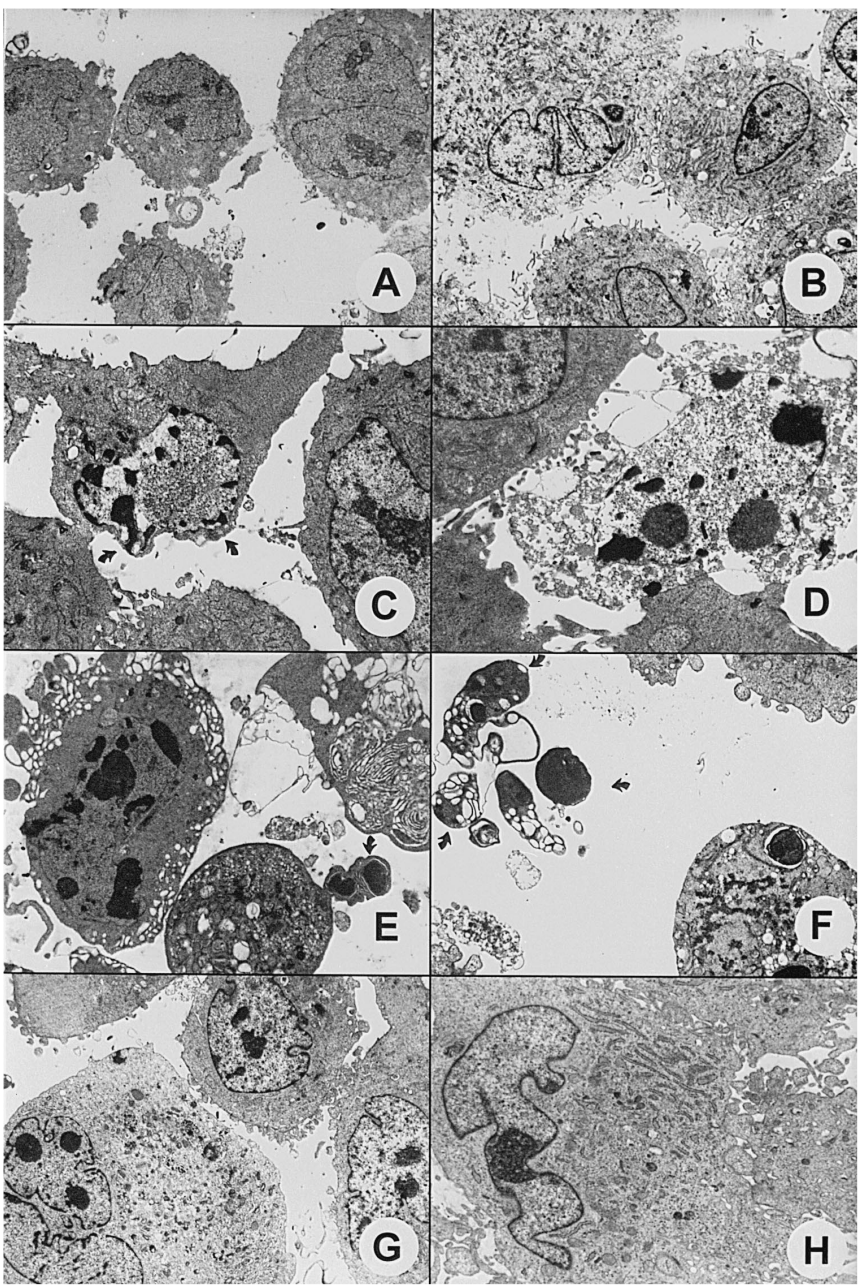

Fig. 5. TEM. (A) HCT8 cells (×5000) and (B) BBE cells (×5000): untreated cells had normal morphology. (C) HCT8 cells cultured in the presence of 60 μg/ml sulindac sulfide: a cell (arrows) shows typical features of apoptosis, namely chromatin compaction, cytoplasmic contraction and reduced size (×8000). (D) HCT8 cells cultured in the presence of 60 μg/ml sulindac sulfide: a cell at a more advanced stage of apoptosis shows extensive nuclear fragmentation (×8000). (E) BBE cells treated with 60 μg/ml sulindac sulfide: endothelial cells show vacuolated cytoplasm, nuclear fragmentation and surface blebbing (×6000). The formation of typical apoptotic bodies at the cell periphery is also shown (arrow). (F) BBE cells treated with 60 μg/ml sulindac sulfide at a more advanced stage of apoptosis: several apoptotic bodies are visible among the cells (arrows) (×6000). (G) Fibroblastic cells cultured in the presence of 60 μg/ml of sulindac sulfide: fibroblasts have a normal aspect (×8000). (H) Desmoid tumor cells cultured in the presence of 60 μg/ml of sulindac sulfide: a morphologically viable cell is visible (×8000).

dose–effect curves were analyzed with logistic probit regression analysis using the GLIM4 program for PC, update 8, of the Royal Statistical Society, London 1992 (Courtesy of A. Biggeri).

3. Results

3.1. Effect of sulindac sulfide on cell growth

The effect of sulindac sulfide on cell growth of colorectal cancer-derived fibroblasts, desmoid tumors, HCT8 and BBE cells was evaluated as explained in detail in Section 2. Sulindac sulfide, in the dose range 0.1–60 μg/ml, dose-dependently inhibited cell proliferation in all cell lines (Fig. 1). The effective dose 50 (ED_{50}) calculated with logistic regression of the probit transformation of the data varied from a minimum of 7.1 μg/ml for colon cancer fibroblasts to 18.2 μg/ml for HCT8 cells (Table 1). Inspection of the curves plotted with the logistic regression parameters showed some interesting differences (Fig. 2). At low concentrations, the effect on HCT8 cells was minimal, whereas the other cell lines already showed significant growth inhibition. At concentrations near the ED_{50} these differences were not apparent, due to the higher value of the slope of the curve calculated for HCT8 cells (Table 1). The lack of growth inhibition of HCT8 cells at low concentrations might be relevant, since these effects were observed at concentrations similar to the peak concentration of sulindac sulfide in humans 4 h after the administration of an effective pharmacological dose (about 8 μg/ml) (Ravis et al., 1993).

3.2. Effect of sulindac sulfide on apoptosis

Sulindac sulfide induced a measurable and dose-dependent DNA fragmentation in BBE and HCT8 cells as assessed with the diphenylamine reaction method (Fig. 3). DNA fragmentation was induced by 20, 40 μg/ml in BBE cells and by 40, 60 μg/ml of sulindac sulfide in HCT8 cells. The drug induced DNA fragmentation at lower doses in BBE than in HCT8 cells.

DNA gel electrophoresis analysis of the two cell types gave similar results. HCT8 and BBE cells developed a nucleosomal ladder pattern of DNA degradation, with 200 bp multiple bands, characteristic of apoptosis after exposure to 40 and 60 μg/ml sulindac sulfide (Fig. 4). The results obtained with the TUNEL fluorescent immunoassay confirmed the data described above (Table 2). BBE cells showed an increase of fluorescent cells at 20 μg/ml sulindac sulfide whereas HCT8 cells showed an increase of fluorescent cells at 40 μg/ml or higher doses of the compound. No dose-related effect was observed in either desmoid tumors cells or colon cancer fibroblasts. The ultrastructural study demonstrated that HCT8 and BBE control cells had normal morphology. In particular, in both cell types, the nucleus appeared round in shape, showed a dispersed chromatin with irregular clumps tending to adhere to the inner layer of the nuclear membrane (Fig. 5A and B). None of the cells examined revealed morphological features of cells undergoing programmed cell death. Conversely, when HCT8 and BBE cells were cultured in the presence of 60 μg/ml sulindac sulfide, ultrastructural changes typical of apoptosis were frequently observed in the treated cells. In particular, these cells showed a sharply-defined condensation of chromatin in crescent caps against the nuclear membrane, condensation and vacuolization of the cytoplasm (Fig. 5C–E). Decrease in cell size was followed by nuclear fragmentation into membrane-bound masses ultimately forming apoptotic bodies (Fig. 5E and F). On the contrary, desmoid and fibroblastic cells cultured in the presence of 60 μg/ml sulindac sulfide kept a normal morphology (Fig. 5G and H).

4. Discussion

Long-term administration of NSAIDs is known to reduce the incidence of, and mortality due to, colorectal cancer (Giovannucci et al., 1994). Sulindac in particular has been shown to be an effective treatment for patients with familial adenomatous polyposis, where it reduces dramatically the number and size of polyps (Waddel et al., 1989; Giardiello et al., 1993; Tonelli and Valanzano, 1993).

The mechanism of action of sulindac as an inhibitor of colon carcinogenesis is still not clarified. Sulindac, through its active metabolite sulindac sulfide, which is formed by the intestinal flora (Stang et al., 1985), blocks prostaglandin production through non-selective inhibition of cyclo-oxygenase 1 and cyclo-oxygenase 2. A high level of prostaglandins has been suggested to facilitate the growth of several tumor cells (Jimenez de Asua et al., 1975; Hubbard et al., 1988; Rigas et al., 1993) and cyclo-oxygenase 2 overexpression has been found to be associated with colon cancer (Boolbol et al., 1996). Accordingly, selective inhibitors of cyclo-oxygenase 2 have recently been proposed as potential drugs for the prevention of colon tumors (Oshima et al., 1996). It is also of interest that overexpression of cyclo-oxygenase 2 is associated with suppression of apoptosis in min +/− mice (Boolbol et al., 1996) and with altered adhesion properties of colon adenocarcinoma cell lines (Du Bois et al., 1996).

However, other data point to alternative mechanisms of action of sulindac. Sulindac sulfone, which does not modify prostaglandin metabolism, is an effective inhibitor of experimental mammary carcinogenesis (Thompson et al., 1997), and colon carcinoma cell lines which do not express cyclo-oxygenases respond to incubation with sulindac sulfide (Hanif et al., 1996). Therefore, sulindac sulfide seems to reduce cell growth and to induce apoptosis through mechanisms independent of cyclo-oxygenase inhibition.

An effect of sulindac sulfide on cyclins resulting in accumulation of cells in G_0/G_1 has been described in colon cancer cell lines (Goldberg et al., 1996).

Whatever its mechanism of action, sulindac has been shown to be an effective chemopreventive agent in familial adenomatous polyposis patients. We previously demonstrated an inhibitory effect of sulindac on colon polyps without effect on epithelial mucosal cell proliferation (Spagnesi et al., 1994).

The results of the present study demonstrated that sulindac sulfide can inhibit the growth of several cell lines which are important in colon carcinogenesis, such as epithelial colon cancer cells (HCT8) and mesenchymal cells from colorectal cancer. The compound is also effective on endothelial cells (BBE). However, after incubation with sulindac sulfide 'in vitro', the dose–response curves were different for each cell line, with BBE cells and fibroblast cell lines being more sensitive than HCT8 cells. The possibility that the decrease in cell number by sulindac sulfide is caused by a G_1/G_0 phase arrest cannot be ruled out, even though the lack of flow cytometry analysis does not allow any final conclusion. However, for HCT8 and BBE cells, the reduction of cell number is certainly related to apoptosis, in fact the phenomenon is evident after 48 h incubation with the drug.

However, since growth conditions and drug concentrations were essentially similar in all experiments, it is likely that the 'in vitro' cell growth variations express different responses of different cell types to sulindac sulfide, that might occur 'in vivo' as well. It is relevant that the serum concentration of sulindac sulfide after the administration of 200–300 mg daily of sulindac, is about 8 μg/ml, which is in the range of the concentrations used 'in vitro'.

In our 'in vitro' experiments with doses of 2–8 μg/ml we obtained a 40% inhibition of cell growth of fibroblasts and endothelial cells with a marginal effect on epithelial cells. If a similar differential effect was present 'in vivo' as well, this would explain the shrinking of polyps in familial adenomatous polyposis patients, and the lack of effect on epithelial mucosal cell proliferation reported to follow sulindac administration (Spagnesi et al., 1994). However, direct 'in vivo' confirmation of the different growth kinetics of the different cell types would be necessary for the validation of this hypothesis. In our experiments, we were able to induce apoptosis with sulindac sulfide in HCT8 and BBE cells also. The induction of apoptosis in colon cancer cell lines exposed to sulindac sulfide was described before (Piazza et al., 1995; Shiff et al., 1995; Goldberg et al., 1996; Hanif et al., 1996), but the apoptosis-inducing effect of sulindac sulfide on endothelial cells had never been described. This effect is very important considering the role of the microvasculature in tumor growth.

Clinical data also revealed an inhibitory effect of sulindac on desmoid tumor development (Waddel et al., 1983; Belliveau and Graham, 1984). Our data showed that sulindac sulfide does not induce apoptosis in primary cultures of desmoid tumors-derived cells at clinically significant concentrations.

In conclusion, a clear growth inhibition of epithelial and mesenchymal cells derived from abdominal neoplasms is observed at concentrations in the range of clinically attainable levels. Growth inhibition can also explain the efficacy of sulindac in familial adenomatous polyposis and its therapeutic activity in the treatment of desmoid tumors. On the basis of these data, the inhibition of cell growth and the induction of apoptosis could explain the anti-cancer effect of sulindac.

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