Sulindac Sulfone Induces a Decrease of β-Catenin in HNSCC

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Abstract. *Background: The most common neoplasm arising in the upper gastrointestinal tract is head and neck squamous cell carcinoma (HNSCC). This is an aggressive epithelial malignancy. Many growth factors and cytokines have been discovered that are responsible for the growth and formation of tumours. Among these factors, β-catenin is considered to be the most important for reducing cell-cell adhesions in malignant tissue. The degradation of β-catenin triggers apoptosis by different routes. Sulindac sulfone has been shown to induce apoptosis in several different tumours. In the present study, we surveyed the concentration of β-catenin in an HNSCC line after incubation with different concentrations of sulindac sulfone. Materials and Methods: Immunohistochemical and Western blot analyses were performed after treatment of the UMSCC 11A cell line with different concentrations of sulindac sulfone (100, 200, 400, 600 and 800 μMol) for 48 hours. Results: At 100 μMol of sulindac sulfone, a decrease in β-catenin concentration of 5% was observed; increasing concentrations of sulindac sulfone resulted in >70% reduction in secreted β-catenin. Thus in conclusion, incubation with sulindac sulfone seemed to stop proliferation. With respect to the controls, there was no greater reduction in total protein. Conclusion: In this study, sulindac sulfone reduced levels of secreted β-catenin in the HNSCC cell line UM-SCC 11A after 48 hours of incubation. It is presumed that reduction of cell-cell adhesion, which is predominately affected by β-catenin, is an essential step in the progression from localized malignancy to stromal and vascular invasion and ultimately metastatic disease. The reduction in the level of mural expression of β-catenin has been associated with loss of differentiation in laryngeal carcinomas. Thus, prevention of intracellular β-catenin accumulation is regarded as an attractive target for chemopreventive agents.*

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The formation of tumors is accompanied by genetic changes. Tumour-forming cells appear to be less genetically stable than normal cells (1). The malignant tumor cells are characterized by their ability to cross tissue barriers and to invade local tissue. Epithelial tumour cells migrate by overcoming adherent cell cell contact and by creating a pathway through basement membrane and stroma (2). In recent decades, many growth factors and cytokines have been discovered that are responsible for the growth and formation of different tumors. Among these factors, β-catenin is considered to be the most important factor for reducing cell cell adhesion in malignant tissue (3). β-Catenin is a multifunctional protein. Appearing as a free cytosolic protein, it serves as a component of the cytoskeleton in a multi-protein complex at the plasma membrane where it binds E-cadherin to the actin cytoskeleton (2). The accumulation of β-catenin results in increased transcription of Tcf/Lef-regulated genes, including *cyclin D1* and *c-Myc*, and thus provides growth advantage to many tumours (4, 5). However, in normal tissue, cytosolic β-catenin is rapidly phosphorylated at multiple serine and theronine sites near the NH_2-terminal region of the protein by a multi-protein complex including adenomatosis polyposis coli (APC), glycogen synthase kinase 3-β (GSK-3β) and axin/conductin (6). Aberle *et al.* (7) reported that after obligatory phosphorylation, β-catenin is targeted for ubiquitination and subsequent degradation by proteosomes, and apoptosis of the cell is initiated. The second physiological way of initiating β-catenin-dependant apoptosis of the cell involves the activation of the caspase family of proteases. Common cleavage sites for caspase-3, -6 and -8 have been recognized in the β-catenin protein (8). Sulindac sulfone is an active metabolite of the prodrug sulindac. A non steroidal anti-inflammatory drug, it was initially developed to prevent polyp formation and promote regression of existing polyps in patients with familial adenomatous polyposis (FAP). Recently, sulindac sulfone has

been shown to induce apoptosis selectively in precancerous and cancerous tissue when applied to models of haematological malginancies and solid cancer such as colon, prostate, bladder, mammary and lung cancer (9, 10). Intracellularly, sulindac sulfone inhibits the cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE), which results in sustained increase of cGMP and activation of protein kinase G (PKG) (Figure 1). Interestingly, β-catenin is a substrate of PKG, thus, phosphorylation of β-catenin initiates the degradation of β-catenin *via*, proteosomes. The degradation of β-catenin thus triggers apoptosis by different routes.

In the present study, we surveyed the concentration of β-catenin in an HNSCC line after incubation with different concentrations of sulindac sulfone.

Materials and Methods

Cell culture. The UMSCC cell lines are well-described human HNSCC cell lines obtained from Dr. T. E. Carey (The University of Michigan, Ann Arbor, Michigan, USA). We used the HNSCC cell line UMSCC 11A. They are gained from laryngeal carcinomas and are capable of growth in conventional semi-solid media (11). Cell cultures were carried out in Falcon petri-dishes at 37˚C in a 5% CO_2 fully humidified atmosphere using Dulbecco's modified minimum essential medium (DMEM) (Fisher Scientific Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics [Life Technologies, Inc. (Gibco BRL), Gainthersburg, MD, USA]. Sulindac sulfone was dissolved in dimethylsulfide (DMS) and added to the cell culture medium.

β-Catenin-ELISA principle. Cell culture supernatants were collected in sterile test tubes and stored at –20˚C until used. Secreted β-catenin was measured in the supernatant of the cell lines using an ELISA technique (R&D Systems, Wiesbaden, Germany). The system uses a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against β-catenin. The specificity of anti-human β- catenin antibodies used in the ELISA kit was examined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. A volume of 100 μl of supernatant were used for each ELISA assay. After 8, 24, and 48 hours of incubation with 0, 100, 200, 400, 600 and 800 μMol sulindac sulfone, the expression of the β-catenin protein in the supernatants of the treated and untreated culture cells was analyzed. All analyses and calibrations were carried out in duplicate. The calibrations on each microtitre plate included β-catenin standards provided in the kit. Optical density was determined using a microplate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm and concentrations were reported in μg/ml. Controls were performed using DMS without sulindac sulfone during incubation.

Characterisation of the cell lines (immunohistochemistry). Immunohistochemical analysis was performed using a monoclonal mouse anti- human antibody directed against β-catenin (β-catenin: C19220; Transduction Laboratories, Lexington, KY, USA). Immunostaining was performed using the alkaline phosphatase-anti-alkaline phosphatase method (APAAP). Therefore the HNSCC cells were cultured on glass coverslips overnight before immunhistochemistry. When confluent, cells underwent a fixation with acetone and alcohol (2:1), were washed with PBS and received a microwave pre-treatment, which required boiling for 15 min at 600 W using citrate buffer for β-catenin. The following steps were performed by an automated staining system, DAKO TechMate 500 (DAKO, Hamburg, Germany). Sections were incubated with the primary antibody solution for 25 min at room temperature, using a working dilution of the antibody 1:300 for β-catenin. Slides were rinsed once in buffer (Buffer Kit, DAKO). Immunoreaction was demonstrated with the DAKO ChemMate Detection Kit (APAAP, Mouse, Code No. K 5000; DAKO) according to the specifications of the manufacturer. Sections were incubated with the chromogen alkaline phosphatase substrate (Neufuchsin; DAKO) for 20 min at room temperature. Finally, sections were counterstained by Mayer's-hematoxylin for 3 min, dehydrated in graded ethanol, and a coverslip applied. Negative controls used all reagents except the primary antibody. The results of the immunohistochemically obtained rates of expression were analysed semi-quantitatively. The stain intensity was noted as follows: strong reactivity >80% of the epithelium cells were positive; moderate reactivity 50-80% reactive; weak reactivity <50% reactive; and no positive cells.

Results

Incubation up to 48 hours with sulindac sulfone. After 48 hours of incubation with 100 μMol sulindac sulfone, the average level of β-catenin was 0.00166 μg/ml. The level of β-catenin after 8 hours was 0.00175 μg/ml. This is a decrease of 5% in growth rate. At 200 μMol of sulindac sulfone, average β-catenin was 0.00193 μg/ml after 8 hours and after 48 hours β-catenin had dropped to 0.00173 μg/ml, a 12% decrease in growth rate. At 400 μMol of sulindac sulfone, a decrease of 21% was observed (0.00149 μg/ml); at 600 μMol the decrease was 44% (0.00079 μg/ml) and incubation with 800 μMol resulted in a 73% reduction (0.00030 μg/ml). Both control cell cultures, 0 μMol sulindac sulfone and DMS with 0 μMol sulindac sulfone, revealed an increase of β-catenin of 9% and 4%, respectively (Figure 2 and 3).

The total protein concentration also decreased after incubation with sulindac sulfone. Incubation with sulindac sulfone seemed to stop proliferation; however, as compared to the controls, there was no greater reduction of the total protein. Overall, the decrease of the total protein was approximately 5 to 11% after 48 hours ($p>0.05$) (Figure 4).

Immunohistochemistry. At increasing concentrations of sulindac sulfone, intracellular β-catenin immunostaining decreased (Table I). The controls showed highest reactivity for intracellular β-catenin (Figure 5 A and B).

Discussion

In this study, sulindac sulfone decreased levels of β-catenin in the HNSCC cell line UM-SCC 11A. As a metabolite of sulindac, sulindac sulfone represents a new class of pro-

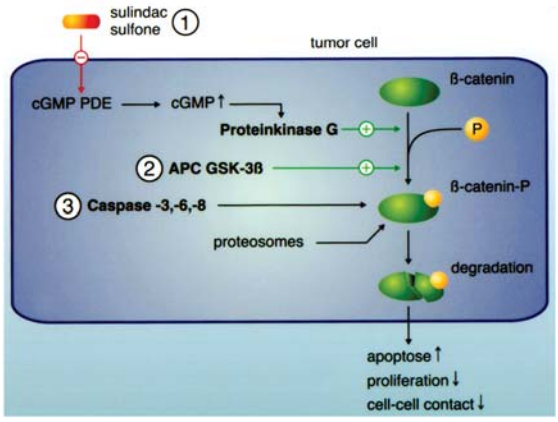

Figure 1. *Different mechanisms of β-catenin-dependent induction of apoptosis.*

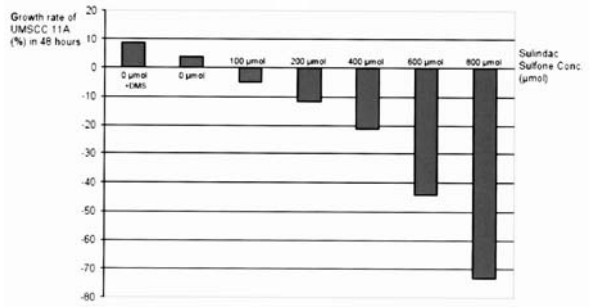

Figure 2. *Growth rate of the cells after incubation with different levels of sulindac sulfone.*

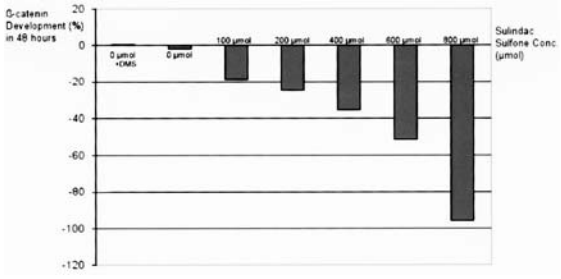

Figure 3. *Concentration of secreted β-catenin after incubation with different levels of sulindac sulfone.*

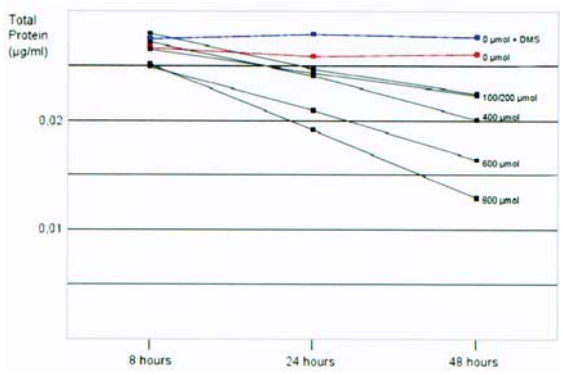

Figure 4. *Total protein concentration after incubation of cells with different levels of sulindac sulfone.*

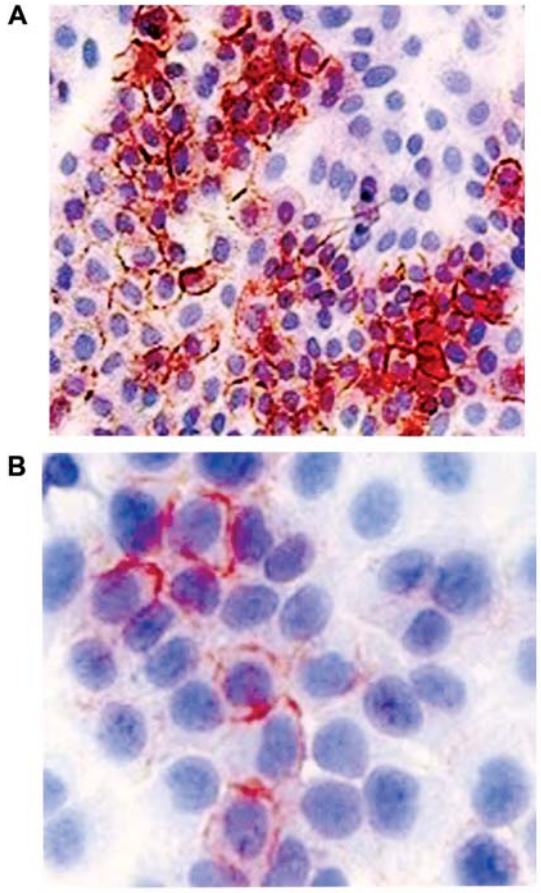

Figure 5. *Immunohistochemical reactivity for cells treated with different levels of sulindac sulfone. A, 0 µMol (×10); B, 600 µMol (×20).*

apoptotic drugs, the so-called selective apoptotic antineoplastic drugs (SAAND). Piazza *et al*. (9) reported that the mechanism of action of SAANDs is completely independent of cyclooxygenase (COX)-1 or COX-2 inhibition and p53 pathways. They showed that in proliferating HT-29 cells, sulindac sulfone was able to block G_1 cell cycle progression but also induced apoptosis. However, in non- proliferating cells, sulindac sulfone did not reduce cell growth. Piazza *et al*. (9) concluded that sulindac sulfone resulted in growth inhibition and apoptosis only in cells without cell cycle arrest. Lim *et al*. (12)

Table I. *Immunohistochemical score (IHS): Percentage grading of the immunostaining of β-catenin in UMSCC cell line after treatment with different concentrations of sulindac sulfone (n=100%).*

	Sulindac sulfone (μMol)					
IHS	0	100	200	400	600	800
0	7	5	17	20	14	17
I	16	36	22	33	35	52
II	14	28	61	47	51	31
III	63	31	0	0	0	0

0: No positive cells; I: weak reacitivity (<50% positive cells); II: moderate (50%-80% positive cells); III: strong reactivity (>80% positive cells).

showed sulindac sulfone induced inhibition of growth in human prostate cancer. Soriano *et al*. (13) reported chemopreventive effects in human lung cancer; several other types of solid tumor cells including bladder, cervical and hepatic were also reported to show apoptosis after treatment with sulindac sulfone (9, 14, 15). The authors also reported that the targeted enzyme cGMP PDE was overexpressed in these tumors. Franchi *et al*. (16) showed that in HNSCC, cGMP PDE was significantly increased. The present study showed a small reduction in total protein levels ($p>0.05$), which indirectly served to reflect the number of all incubated cells. This result showed that the proliferation rate of the treated UM-SCC 11A cells is possibly affected by increased cell death. Thus, as a therapeutic agent, sulindac sulfone inhibited cell growth in HNSCC. It is presumed that a reduction of cell-cell adhesion, which is predominately affected by β-catenin, is an essential step in the progression from localized malignancy to stromal and vascular invasion, and ultimately metastatic disease (17). Cytoplasmic accumulation of β-catenin is considered as an oncogene factor (3). Lopez-Gonzalez *et al*. reported that the reduction in the level of mural expression of β-catenin was associated with loss of differentiation in laryngeal carcinomas (18). Hirvikoski *et al*. reported that the percentage of tumours showing increased cytoplasmic reactivity for β-catenin increased with histological dedifferentiation in laryngeal cancer (19). Increased cytoplasmic β-catenin seems to be a pivotal feature of malignancy. Untreated cultured UM-SCC 11A cells showed increased reactivity against cytoplasmic β-catenin, however membranous reactivity was relatively weak (Figure 5A). After incubation with sulindac sulfone, cytosolic β-catenin expression significantly decreased. In previous studies, diminished cytoplasmic β-catenin concentration seemed to reflect stabilized proliferation and reduced invasiveness of cells. Accumulated cytosolic β-catenin translocates to the nucleus, where it regulates oncogenic transcription factors (20). Thus, prevention of β-catenin accumulation is regarded as an attractive mode of action for chemopreventive agents. In this study, increased levels of sulindac sulfone resulted in greater reductions of secreted β-catenin in the HNSCC cell line. It is possible that by treatment with sulindac sulfone, PKG is activated in these cells (Figure 1). Thompson *et al*. (21) reported that PKG can phosphorylate β-catenin *in vitro*. However, it remains possible that other protein kinases could also phosphorylate additional sites of the β-catenin protein, leading to ubiquitation and protozomal degradation. Fukuda *et al*. (22) reported that inhibition of caspase activity did not return β-catenin protein levels to control levels, indicating that caspases are not completely responsible for loss of β-catenin protein expression by sulindac sulfone. Further research is necessary to reveal the mechanisms of sulindac sulfone-induced apoptosis in different HNSCC cell lines. Regarding the clinical application of sulindac sulfone, it is difficult to directly compare our short-term, high-dose *in vitro* results with longer term human and animal studies: In this *in vitro* study the concentration of sulindac sulfone used was markedly higher than has been measured *in vivo* in the plasma of humans, which Davies *et al*. (23) reported to be approximately 50 μMol in human plasma.

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