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Nimesulide and indomethacin induce apoptosis in head and neck cancer cells

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BACKGROUND: Non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit the enzyme cyclooxygenase (COX). There are two isoforms of the enzyme. Recent investigations indicate that both isoforms, COX-1 and COX-2, are involved in carcinogenesis.
METHODS: We investigated the effects of nimesulide, a COX-2 selective and indomethacin, a non-selective NSAID on the head and neck squamous cell carcinoma (HNSCC) cell lines SCC-9 and SCC-25. Effects on cell numbers and apoptosis were assayed by cell counting, immunofluorescence and fluorescence activated cell sorting (FACS). COX expression was examined by Western blotting.
RESULTS: The investigated cell lines express COX-1 and COX-2. Nimesulide and indomethacin induce apoptosis and cause a reduction of cell number. Incubation with NSAIDs upregulated COX-2 expression.
CONCLUSION: The results of our study on HNSCC cells together with data from different studies showing anticancer activity of NSAIDs suggest that COX inhibitors could play a role in HNSCC treatment and prevention.
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Keywords: apoptosis; cyclooxygenase; head and neck cancer; indomethacin; nimesulide

Introduction

Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid (AA) to prostaglandins (PG). There are two isoforms of COX. COX-1 is considered to be the constitutively expressed isoform of this enzyme found in most tissues. It mediates the synthesis of PGs required for physiological tissue homeostasis, and is involved in vascular homeostasis, platelet activity and renal function.

The COX-2 expression however is not found in unstimulated tissue. It is inducible by a variety of extracellular and intracellular stimuli like inflammatory factors, carcinogenes and growth factors (1–3). Multiple studies have shown an upregulation of COX-2 in transformed cells and malignant tissues of different localization (4–8).

More recently it has been demonstrated that both COX isoforms are inducible. In addition, elevated COX-1 expression has been reported for breast cancer, prostate carcinoma and cervical carcinoma (9–11).

The enzymatic activity of both COX-1 and COX-2 is inhibited by conventional non-steroidal anti-inflammatory drugs (NSAIDs). The newer specific or preferential COX-2-selective drugs have been developed in order to reduce side-effects related to the inhibition of physiologically important PG by COX-1.

Experimental and clinical studies could demonstrate a role of NSAIDs in the prevention of human gastrointestinal cancer (12, 13). Three independent studies have reported a 40–50% lower risk of colorectal cancer in people who are continuously taking aspirin or other NSAIDs (14–16). In addition it has been shown that NSAIDs induce apoptosis in cancer cells *in vitro* and it has been demonstrated that an overexpression of COX-2 inhibits apoptosis (17–19).

An upregulation of COX-2 has been also found in squamous cell carcinomas of the head and neck (20). Moreover a recent study shows the upregulation of COX-2 even in pre-malignant oral lesions (21). Furthermore it has been reported that NSAIDs increase the percentage of HNSCC cells in G0/G1 phase and cause a reduction of cell numbers (22, 23).

Inhibition of COX-2 represents a promising strategy to prevent or treat head and neck cancer (24, 25).

In this study we evaluated the effects of a conventional and a COX-2 selective NSAID on HNSCC cell lines. We examined cell growth and apoptotic cell death in cell lines that overexpress COX. In addition we determined the effects of indomethacin and nimesulide on the protein expression of COX-1 and -2.

Material and methods

Drugs and antibodies

We compared the selective COX-2 inhibitor nimesulide (Alexis Biochemicals, San Diego, CA, USA) and the non-selective COX inhibitor indomethacin (ICN Biomedicals Inc., Costa Mesa, CA, USA). Non-steroidal drugs were diluted in dimethylsulfoxide (DMSO; Sigma Aldrich,

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Vienna, Austria) and used in a concentration of 100 μM to 1 mM.

Goat polyclonal anti-human COX-1 and COX-2 antibodies, the control peptides COX-1 and -2 and the horseradish peroxidase-conjugated secondary antibody, anti-goat IgG-HRP, were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

The M30 CytoDeath used for immunofluorescence and flow cytometry was obtained from Roche Molecular Biochemical, Mannheim, Germany. As secondary antibody we used Alexa Fluor 488 rabbit anti-mouse IgG from Molecular Probes (Eugene, OR, USA).

Cell culture

The larynx derived squamous cancer cell line JP-PA, human immortalized keratinozytes HaCat and two tongue derived cancer cell lines, SCC-25 and SCC-9 were studied. JP-PA and HaCat cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% HEPES, 1% glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. SCC-9 and SCC-25 were grown in RPMI 1640 medium containing 10% FCS and 100 U/ml penicillin and 100 μl/ml streptomycin (all reagents from Life Technologies Ltd., Paisley, Scotland). All cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2.

Cell counting

Cells were plated at 1×10^6 cells per 25 cm^2 flask. After 24 h medium with or without NSAIDs was added. After incubation for 72 h cells were washed twice and harvested by trypsinization [Trypsin/ethylenediaminetetraacetic acid (EDTA); Life Technologies Ltd., Paisley, Scotland]. Cells were counted in the Buerker-Tuerk-chamber and viability was tested by trypan-blue exclusion (Sigma Aldrich, Hampshire, UK). For all cultures counted the viability was more than 90%.

Western blotting

Subconfluent cell monolayers were washed twice with cold phosphate-buffered saline (PBS), frozen with liquid nitrogen and lysed with lysis buffer, consisting of 1% Nonident P40, 0,1%SDS, 150 mM NaCl, 50 mM TRIS/pH 7.4, 10 mM EDTA, 10 mM p-nitrophenolphosphate, 250 U/l aprotinin, 40 μg/ml leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium flouride and 40 mM β-glycerophosphate.

Cell lysates were centrifuged at 20 000 ***g*** for 20 min at 4°C. The supernatant was used for electrophoresis. Protein concentration was determined using Micro BCA from Pierce (Rockford, IL, USA). Protein from JP-PA, SCC-9, SCC-25, HaCat, COX-1 and COX-2 Control-peptide and protein of SCC-9 and SCC-25 after incubation with 1, 0.3 and 0.1 mmol indomethacin or nimesulide, per lane were separated by 8% SDS-Page and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany).

The blots were blocked in 0.1% Tween Tris-bufferd saline (TBS-Tween) containing 5% bovine serum albuminin (BSA) at 4°C overnight. Membranes were incubated with primary antibodies in 1% BSA in TBS-Tween: anti-COX-1 and anti-COX-2, 1:1000 for 90 min at room temperature. The secondary antibody, anti-goat IgG-HRP, was diluted 1:2000 and incubated 2 h at room temperature. As a control for protein loading tubulin Ab-4 diluted 1:2000 (Neo Markers, Fermont, CA, USA) was used. Immunoreactive protein was detected using the ECL chemiluminescence kit (Amersham Lifescience, Bucks, UK), followed by exposure to Kodak X-ray film.

Indirect immunofluorescence

The JP-PA, SCC-9 and SCC-25 cells grown on glass slides were treated for 48 h with nimesulide, indomethacin and DMSO under standard conditions as described above. Cells were fixed in methanol at −20°C for 30 min, washed twice with PBS and blocked with 1% BSA for 10 min. Afterwards the cell monolayers were incubated with M30 CytoDeath antibody for 1 h. The secondary antibody, anti-mouse-Ig-fluorescein (10 μg/ml), was added for 30 min. The result was examined under a Olympus BH.2 epifluorescence microscope and recorded on Kodak P1600x-color reversal film.

Fluorescence activated cell sorting (FACS)

The JP-PA, SCC-9 and SCC-25 were treated with indomethacin, nimesulide and DMSO.

After incubation for 72 h adherent and floating cells were harvested, washed twice with cold PBS and fixed in methanol at −20°C. After 30 min cells were washed with PBS containing 0.1% Tween 20 twice and blocked with incubating buffer, PBS with Tween 20 and 1% BSA, for 10 min. Then cells were incubated with M30 CytoDeath antibody diluted in incubating buffer for 60 min. After washing, cells were incubated with the secondary anti-mouse-IgG-fluorescein antibody for 30 min. The stained cells were analyzed in a Coulter Epics XL MCL FACSscan equipped with the multicycle advanced version software.

Hoechst staining

To test the apoptosis inducing effect of both NSAIDs we analyzed the morphological criteria of apoptosis. SCC-9 and SCC-25 were grown on glass slides and treated with 1 mM indomethacin and nimesulide for 1, 6, 12, 24, 48 and 72 h.

Slides were washed with PBS, dried and then fixed in methanol:aceton (1:1) for 5 min. Cells were stained with 8 μg Hoechst dye 33 258 (a DNA-specific flourochrome bis-benzimide trihydrochloride) diluted in PBS for 10 min, protected from light. After washing cells twice with PBS the results were examined under a fluorescent microscope and recorded on Kodak P1600x-color reversal film.

Statistical analysis

After testing the presuppositions showing a normal distribution of values in the samples an adequate *a posteriori* test was performed (unpaired student's *t*-test) using the Graph Pad statistical software package (GraghPad Instad 2.0, San Diego, CA, USA).

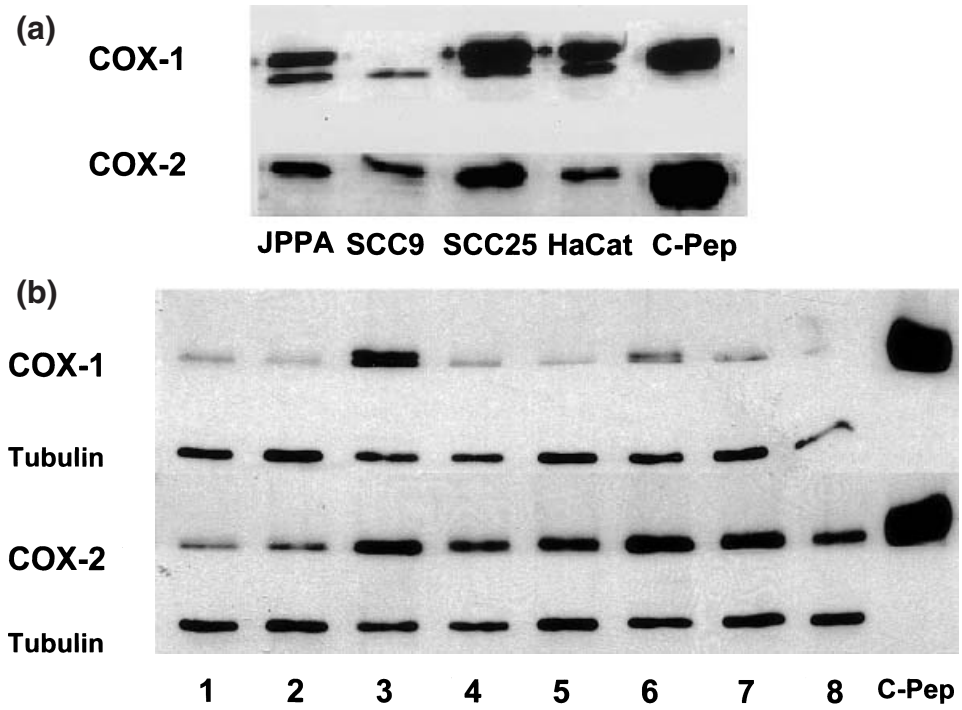

Figure 1 Western blot analysis of COX-1 and -2 expression in squamous carcinoma cell lines of the head and neck. (a) 5 μg protein of the cell lysate samples of untreated JP-PA cells, SCC-9 cells, SCC-25 cells, HaCat cells and 0.125 μg COX-1 and -2 controlpeptide (c-pep) were separated by 8% SDS-Page gel. All cell lines express COX-1 and -2. (b) COX-1 and -2 expression of SCC-25 cells (2.5 μg) after incubation with indomethacin and nimesulide for 48 h. Lane 1, untreated SCC-25, control. Lane 2, negative control, medium supplemented with an equivalent of DMSO. Lane 3, SCC-25 treated with 1 mM indomethacin. Lane 4, SCC-25 treated with 0.3 mM indomethacin. Lane 5, SCC-25 treated with 0.1 mM indomethacin. Lane 6, SCC-25 treated with 1 mM nimesulide. Lane 7, SCC-25 treated with 0.3 mM nimesulide. Lane 8, SCC-25 treated with 0.1 mM nimesulide. COX-1 and -2 upregulation is visible in the treated cells (Lane 3–8).

Results

COX expression of HNSCC cell lines

The presence of COX-1 as well as COX-2 protein is demonstrated in protein extracts prepared from SCC-9 and SCC-25, JP-PA and HaCat. All cell lines express COX-1 and COX-2 protein (Fig. 1). The anti-COX-1 antibody detected a 70 kDa doublet, which has been reported previously and is presumably because of differential glycosylation. The 72 kDa COX-2 protein was also detected without any treatment in all investigated cell lines, which means COX-2 expression is constitutive in SCC-9, SCC-25, JP-PA and HaCat. After incubation of SCC-9 and SCC-25 with indomethacin or nimesulide for 48 h we could see an upregulation of COX-1 and -2 in Western blot experiments.

Effects of NSAIDs on cell growth

As all investigated HNSCC cell lines express COX-1 and COX-2 we tested whether selective and non-selective COX inhibitors have different effects on cell number. Therefore we treated JPPA, SCC 9 and SCC 25 with 0.1, 0.3 and 1 mM indomethacin or nimesulide. After incubating the cells for 72 h we counted the viable cells in a hemocytometer. Viability was always over 90 percent. In the absence of NSAIDs, there was a four to eightfold increase in total cell number after 72 h. Treatment with nimesulide or indomethacin was associated with a statistically significant concentration dependent decrease in cell number, which was comparable for both substances. At a concentration of 1 mM indomethacin the cell count was reduced to approximately 30 percent of the originally plated cells after 72 h (10^6 cells were plated) (Fig. 2).

Apoptosis of HNSCC cells induced by NSAIDs

As we found reduction of total cell number we were interested whether this effect might at least in part be caused by induction of apoptosis. We investigated the effects of indomethacin and nimesulide on apoptosis in SCC-9 and SCC-25 by watching morphological cell changes and by using an antibody that binds to the caspase cleaved epitope of cytokeratin 18, a marker for early apoptosis, that is not detectable in non-apoptotic cells. for FACS and for indirect immunofluorescence detection.

Morphological evidence of apoptosis

After staining the nucleus of the cells grown on glass slides, it was possible to observe the hallmarks of apoptosis by fluorescence (Fig. 3): Nuclear changes, condensation of chromatin, its compaction along the periphery of the nucleus and fragmentation of the nucleus (18). We examined the apoptotic cell changes after 1, 6, 12, 24, 48 and 72 h of incubation with indomethacin and nimesulide (1 mM). After 1 and 6 h

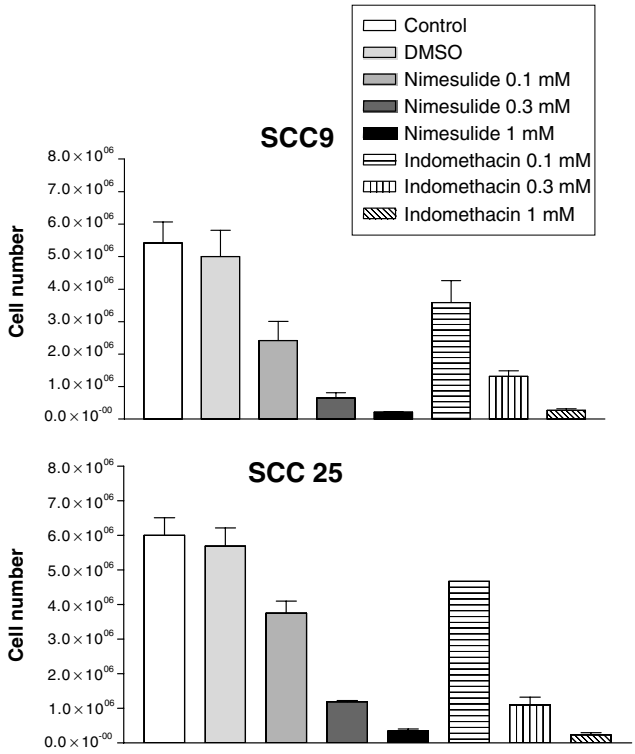

Figure 2 Effect of nimesulide and indomethacin on cell count of SCC-9 cells and SCC-25 cells. Both cell lines were incubated for 72 h with 0.1, 0.3 and 1 mM indomethacin or 0.1, 0.3 and 1 mM nimesulide as described in Materials and methods. Each Value represents the mean (±SEM) of three independent experiments. Indomethacin and nimesulide induce a dose dependent reduction in cell number.

there was no marked difference to the untreated cells. (Data not shown). After 12 h treated cells showed signs of apoptosis by nuclear changes. These changes remained constant over a period of 60 h.

FACS analysis of apoptotic cells

We examined the effect of different concentrations of nimesulide and indomethacin on apoptosis in SCC-9 and SCC-25 after incubation for 72 h using the caspase cleaved cytokeratin detecting antibody (M30 CytoDeath). Approximately 30% of the treated cells were apoptotic in all cell lines assayed after incubation with nimesulide (1 mM) or indomethacin (1 mM) (Fig. 4).

Indirect immunoflourescence

In addition to FACS, M30 CytoDeath was used for immunofluorescence microscopy. The antibody stains the cytoskeleton (cytokeratin 18) in contrast to the nuclear staining of Hoechst dye. We treated the cells grown on glass slides with 1 mM nimesulide or indomethacin for 48 h before we fixed them in alcohol and stained the cells with M30. Using the fluorescence microscope we could detect a high number of stained treated cells in each cell line (Fig. 5). It was possible to detect apoptotic cells at a very early phase up to late phase of apoptosis. Granular structures were observed in the late phase of apoptosis.

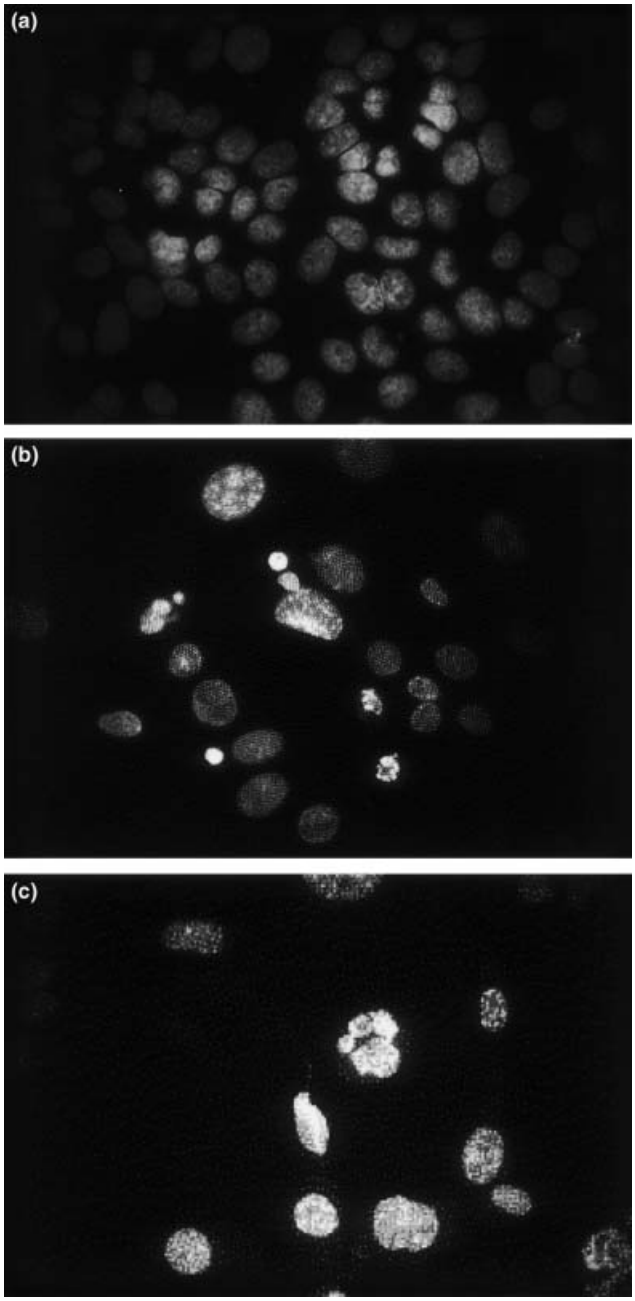

Figure 3 Morphological evidence of apoptosis. Immunofluorescence detection of apoptotic cells by nuclear staining (Hoechst Dye). (a) Untreated SCC-25 cells, control (medium + DMSO). (b) SCC25 cells treated with 1 mM indomethacin for 24 h. (c) SCC-25 cells treated with 1 mM nimesulide for 24 h. Nuclear changes like condensation of the chromatin and fragmentation of the nucleus can be seen in the treated cells (b) and (c). (Original magnification ×400).

Discussion

In this study we demonstrate anti-proliferative and apoptosis inducing effects of the selective COX-2 inhibitor nimesulide in comparison to the non-selective COX inhibitor indomethacin on HNSCC cell lines.

Elevated PG levels in tumors suggest that there might be an important link between prostaglandin synthase (COX) and tumor growth. Aside from accelerating cell

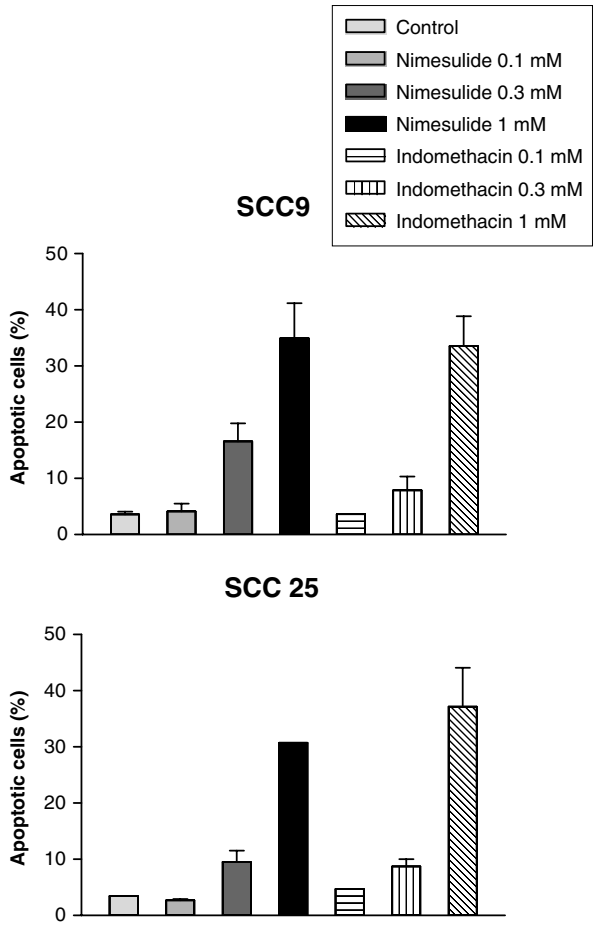

Figure 4 Apoptosis inducing effect of nimesulide and indomethacin on SCC-9 cells and SCC-25 cells. Both cell lines were incubated with 0.1, 0.3 and 1 mM nimesulide or 0.1, 0.3 and 1 mM indomethacin for 72 h. Apoptotic cells were quantified using M30 CytoDeath-Facs analysis. Data are expressed as the mean percentage of apoptotic cells. Nimesulide and indomethacin show an evident concentration dependent apoptosis inducing effect.

proliferation, enhanced synthesis of PG is thought to contribute to tumor growth by promoting angiogenesis and by inhibiting immune surveillance (26–28). Elevated levels of PGE_2 have been demonstrated in several types of tumors including human colon carcinomas, human breast cancer and SCC of the head and neck (28–31). The increase in PG may be explained by increased COX expression. For SCC of the head and neck an overexpression of COX-2 is reported, whereas COX-2 was not found in normal mucosa (20).

The results of our present study show that nimesulide and indomethacin exert very similar effects on COX expressing cell lines. Our cell lines investigated express COX-2 at high levels and both NSAIDs cause a concentration dependent decrease in cell counts. At a concentration of 1 mM nimesulide or indomethacin the cell number was reduced to approximately 30 percent of the originally plated cell count after 72 h. Our cell count data are in agreement with the experiments of Smith et al. (12). This group reported anti-proliferative effects of a COX-2 selective NSAID, NS-398, and indomethacin on colon carcinoma cell lines. NS-398 was found to inhibit proliferation independent of COX-2 expression. In contrast to the results obtained with NS-398, studies using the COX-2 inhibitors SC 58125 and meloxicam suggest that changes in cellular proliferation are COX-2 dependent (32, 33).

The mechanism of the anti-proliferative activity of NSAIDs is not yet clear. The fact that the dose of COX inhibitors required to inhibit tumorgenesis is far greater than required for inhibition of COX-2 activity, suggests the existence of additional COX-2 independent mechanisms of action for these agents.

In a recent study Yuan et al. demonstrated a possible COX-2 independent pathway accessible for NSAIDs (34). This group showed that NSAIDs, in addition to their inhibitory effect on COX, are able to reduce PG production by suppressing cytosolic phospholipase A_2 ($cPLA_2$), an enzyme of the AA metabolism upstream of COX responsible for AA release.

The drastic decrease in cell number induced by nimesulide and indomethacin might not only be because of the anti-proliferative activity of COX inhibitors, but might at least in part be caused by induction of cell death, in particular apoptotic cell death. It is reported that COX-2 overexpression is responsible for the inhibition of apoptosis in tumors (18). In COX-2 expressing colon carcinoma cell lines it has been demonstrated that COX-2 selective as well as non-selective NSAIDs induce apoptosis.

In our experiments we found induction of apoptosis in cells treated with nimesulide and indomethacin. As indomethacin and nimesulide showed similar results in cell count experiments as well as in apoptosis assays they seem to have a similar anti-tumor potential on HNSCC. We could further show in Western blots that both substances cause an upregulation of COX-1 and COX-2. There may be a feedback loop that upregulates COX expression when inhibited. Surprisingly incubation with the COX-2 selective inhibitor nimesulide caused also an upregulation of COX-1. This could mean that COX-2 selective inhibitors also have an influence on the enzyme COX-1.

Recently it has been reported that COX-1 could by itself play a role in carcinogenesis. Moreover an overexpression of COX-1 was found in human breast cancer, prostate cancer and cervical cancer (9–11). It has been demonstrated that COX-1 expression can be induced by VEGF, AA and tobacco carcinogen *in vitro* (35–37).

For head and neck cancer treatment with NSAIDs was published by William R. Panje in 1981. He demonstrated seven cases where the usually recommended dosage of indomethacin (75–100 mg/day) caused stabilization or even regression of head and neck cancer (38). Unfortunately follow-up studies have not been performed. Eight years later Tanaka et al. demonstrated that indomethacin prevents chemically induced squamous cell carcinoma of the tongue in rats (39).

Recently published studies concentrate on combining COX inhibitors and cytostatic drugs. It has been demonstrated that NSAIDs enhance the cytotoxic effect

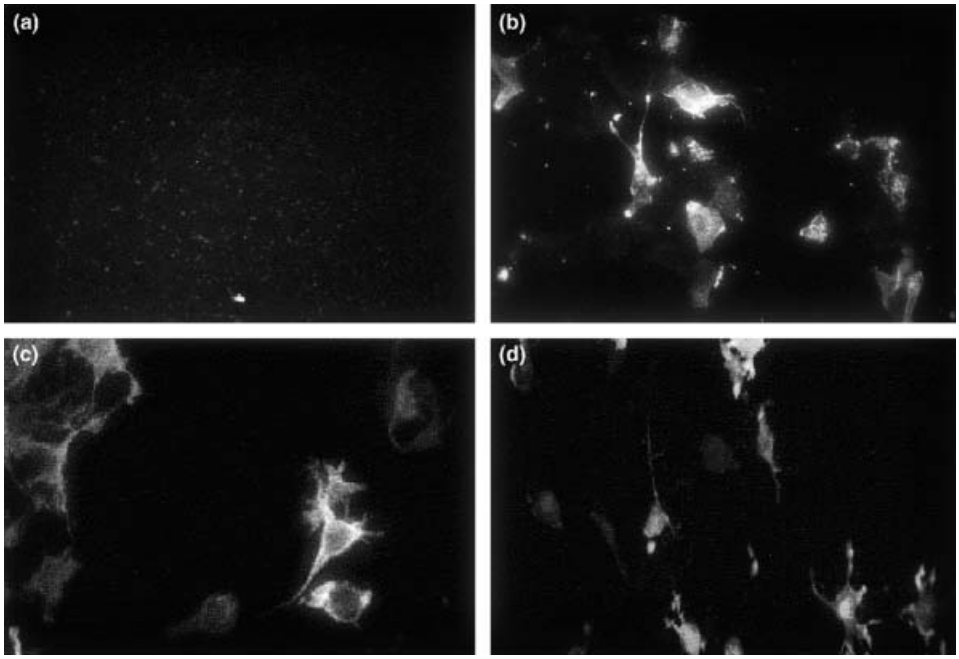

Figure 5 Immunofluorescence detection of apoptotic cells using CytoDeath M30 antibody after incubation with indomethacin or nimesulide for 48 h. (a) SCC-9 cells treated with 1 mM nimesulide (magnification ×40). The bright spots show apoptotic cells. (b) SCC-9 cells treated with 1 mM indomethacin (magnification ×400). (c) SCC-25 cells treated with 1 mM nimesulide (magnification ×400). (d) SCC-25 cells treated with 1 mM indomethacin (magnification ×400). (b–d) Cells in different phases of apoptosis can be detected. Granular structures are characteristic for cells in the late phase of apoptosis.

(19, 40). In addition it has been shown in a dog animal model that the combination of cisplatin and piroxicam, a non-selective COX inhibitor, induced remission more often than cisplatin alone (41, 42).

Currently we are initiating clinical investigations on the effects of a selective COX-2 inhibitor on HNSCC *in vivo*. We want to demonstrate an apoptotic inducing effect of nimesulide, which was obvious on HNSCC cell lines, *in vivo*. The anti-cancer activity of NSAIDs shown in several different studies together with the promising results of our study on HNSCC cells suggest that COX inhibitors could play a role in head and neck cancer treatment and prevention.

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