
THE EFFECT OF NIMESULIDE, A SELECTIVE CYCLOOXYGENASE-2 INHIBITOR, ON ETS-1 AND ETS-2 EXPRESSION IN HEAD AND NECK CANCER CELL LINES

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Abstract: *Background.* The protooncogenes Ets-1 and Ets-2 are involved in carcinogenesis of different tumors. Nimesulide, a selective cyclooxygenase-2 (COX-2) inhibitor, has antiproliferative effects on tumor cells. The question arises whether nimesulide influences Ets-1 and Ets-2 synthesis in head and neck tumors.

Methods. Expression of Ets-1 and Ets-2 was analyzed in tumor tissues by immunohistochemistry. The influence of nimesulide and an extracellular signal-regulated kinase (ERK) inhibitor on cell proliferation of two head and neck cancer cell lines and Ets-1 and Ets-2 expression was determined by automated cell counting and Western blotting, respectively.

Results. Immunohistochemistry showed a high expression of Ets-1 and Ets-2 in tumor tissues. In both cell lines, Ets-1 and Ets-2 expression were reduced after 24 and 48 hours by nimesulide.

Conclusion. Both Ets-1 and Ets-2 are overexpressed in head and neck cancer specimens. Inhibition of Ets-1 and Ets-2 expression in head and neck cancer cell lines by nimesulide might explain the proapoptotic property of this COX-2 inhibitor. © 2005 Wiley Periodicals, Inc. *Head Neck* 27: 1068–1072, 2005

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Ets belongs to a family of transcription factors that controls the expression of genes that are critical for biologic processes, including cellular proliferation, differentiation, development, cellular transformation, and apoptosis.¹ Two isoforms, Ets-1 and Ets-2, are described, which are located on chromosome 11 and on chromosome 21, respectively.² Both factors are overexpressed in human malignant tissues (ie, carcinomas of the prostate,³ esophagus,⁴ and head and neck⁵). Interestingly, Ets-1 might be involved in angiogenesis, which is essential for tumor progression.⁶

Another protein that may be involved in carcinogenesis and angiogenesis is cyclooxygenase (COX). COX is an enzyme that catalyzes arachidonic acid to prostaglandins.⁷ The cyclooxygenase protein exists in two isoforms: COX-1 is mostly described as a housekeeping enzyme in

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various tissues. In contrast, COX-2 is inducible by inflammatory mediators, oncogenes, and carcinogens.⁸ COX-2 is overexpressed in various malignancies, including cancers of the liver,⁹ stomach,¹⁰ pancreas,¹¹ lung,¹² and head and neck,¹³ suggesting that COX-2 plays an important role in carcinogenesis.

Both isoforms of cyclooxygenase can be inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs). On the one hand, various studies could demonstrate that these drugs are able to inhibit proliferation by inducing apoptosis in cell lines of different malignancies by inhibition of the COX enzyme. On the other hand, it is recognized that the antiproliferative effects of selective COX-2 inhibitors can also occur by means of cyclooxygenase-independent mechanisms.¹⁴ Nevertheless, the mechanisms by which NSAIDs inhibit cell proliferation and induce apoptosis remain unclear.

Nimesulide, a selective COX-2 inhibitor, inhibits proliferation by means of induction of apoptosis and cell cycle arrest in adenocarcinoma cell lines.¹⁵ Nimesulide is well tolerated by adult, elderly, and pediatric patients in both clinical trials and large postmarketing surveillance studies.¹⁶

Recently, we showed in our laboratory that nimesulide was able to induce apoptosis in two head and neck squamous cell carcinomas (HNSCCs), SCC-9 and SCC-25.¹⁷

In this recent study, we further demonstrated that the selective COX-2 inhibitor nimesulide was able to downregulate the protooncogenes Ets-1 and Ets-2, which play a vital role in the inhibition of apoptosis.

MATERIALS AND METHODS

Nimesulide was generously provided by Helsinn Chemicals Dublin, Ireland Ltd. as a pure substance and dissolved in dimethylsulfoxide (DMSO). The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) inhibitor PD 98059 in solution was provided by Calbiochem (VWR International, Vienna, Austria).

Immunohistochemistry. For immunohistochemical analysis, archival specimens of squamous cellular cancer of the tonsils with adjacent normal mucosa were used. Immunohistochemical analysis of protein expression in tissues was performed using established protocols.¹⁸ Dewaxed and dehydrated slides were microwaved for 4 × 5 minutes in 1 mM EDTA, pH 8.0, buffer. To reduce background signals, the slides were treated with

5% bovine serum albumin (BSA) (Sigma-Aldrich, Munich, Germany)/tris-buffered saline (TBS) for 30 minutes. After the blocking step, the slides were incubated with rabbit polyclonal antibodies to Ets-1 and Ets-2 (1:200, Santa Cruz Biotech, Santa Cruz, CA) or rabbit preimmune isotype control serum (1:1000, Vector Laboratories, Burlingame, CA) overnight at room temperature. The next day all slides were washed three times for 5 minutes each with TBS. Samples were incubated with a multilink (1:250, Dako, DK Burlingame, CA) for 1 hour at room temperature, washed, and incubated with alkaline phosphatase-conjugated streptavidine-AP/10% human serum (1:250, Dako, Glostrup, DK) for 1 hour at room temperature. Finally, visualization was achieved with fast red (Sigma-Aldrich, Munich, Germany) and counterstained with hemalum. The samples were then dehydrated and mounted.

Cell Lines and Culture. The tumor cell lines SCC-9 and SCC-25, both from HNSCCs of the tongue, were obtained from the American Type Culture Collection (ATCC). Cell lines were cultured in RPMI medium containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μL/mL streptomycin (all reagents from Life Technologies Ltd, Paisley, Scotland). Both cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂. Cells in exponential growth were treated with 600 μM nimesulide, 10 μM PD 98059, or with both agents for 24 and 48 hours because of the rationale that transcription factors like the Ets family should be regulated in a relatively short time. Cells were maintained in standard medium, harvested, and plated at equal densities of 5 × 10⁵ cells in 10-cm tissue culture dishes. Cells were exposed to nimesulide or DMSO controls after 24 hours after seeding.

Western Blot Analysis. Subconfluent cell monolayers were washed two times with cold PBS, frozen with liquid nitrogen, and lysed with lysis buffer, consisting of 1% Nonident P40, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris/pH 7.4, 10 mM EDTA, 10 mM *p*-nitrophenylphosphate, 250 U/L aprotinin, 40 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 40 mM β-glycerolphosphate. Cell lysates were centrifuged at maximum speed for 20 minutes at 4°C. The supernatant was used for electrophoresis. Protein concentration was determined using Micro BCA from Pierce (Rockford,

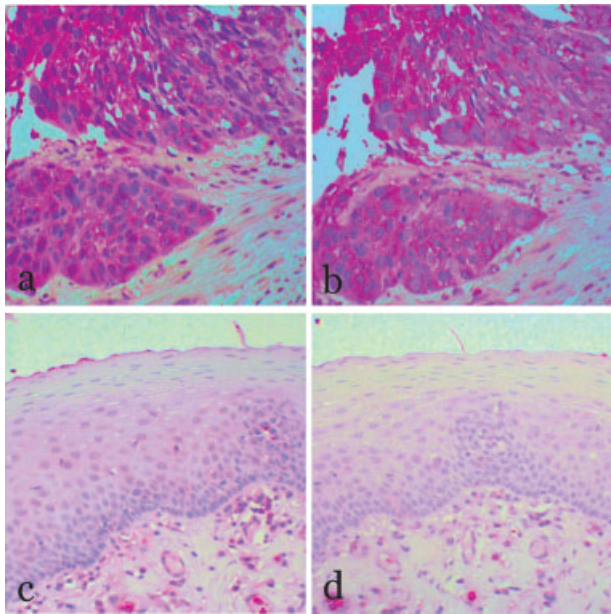


FIGURE 1. Ets-1 and Ets-2 immunohistochemistry on HNSCC: cytoplasmic expression pattern of Ets-1 (a) and Ets-2 (b) in squamous cell carcinomas of the head and neck and corresponding expression patterns of Ets-1 (c) and Ets-2 (d) in normal mucosa. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IL). Twenty micrograms protein from SCC-9 and SCC-25 and 5 μ g Ets control-peptide per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroblotted to nitrocellulose membranes (Schleicher & Schnell, Dassel, Germany). The transferred proteins were blocked in 0.1% Tween Tris-buffered saline (TBS-Tween) containing 5% BSA at 4°C overnight. Membranes were incubated with primary antibodies in 1% BSA in TBS-Tween: anti-Ets-1 (1:1000), anti-Ets-2 (1:500), and anti-actin (1:10000) for one and a half hours at room temperature. Anti-Ets-1 antibody and anti-Ets-2 antibody were purchased from Santa Cruz Biotechnology, Inc. The secondary antibody, anti-rabbit immunoglobulin G (IgG)-HRP, was diluted 1:2000 and incubated 2 hours at room temperature. Immunoreactive protein was detected using the ECL chemiluminescence kit (Amersham Lifescience, Little Calfront, UK), followed by exposure to Kodak X-ray film (PerkinElmer Life Sciences, Vienna, Austria).

RESULTS

Ets-1 and Ets-2 Expression in Squamous Cell Carcinomas of the Head and Neck. Immunohistochemical analyses revealed that squamous cell carcinoma

of the head and neck had high cytoplasmic expression pattern of Ets-1 (Figure 1a) as well as Ets-2 (Figure 1b).

Ets-1 and Ets-2 Expression in Squamous Cell Carcinoma Cell Lines of the Head and Neck after Exposure to Nimesulide Was Determined by Western Blotting. SCC-9 and SCC-25 cell lines showed a significant decreased protein expression of Ets-1 and Ets-2 after 24 and 48 hours treatment with nimesulide compared with control (Figure 2a, b).

Ets-1 and Ets-2 Expression in Squamous Cell Carcinoma Cell Lines of the Head and Neck after Treatment with the ERK-Kinase Inhibitor PD 98059 as well as Nimesulide in Combination with PD 98059 Analyzed by Western Blotting. Western blot analysis showed that the expression of Ets-1 and

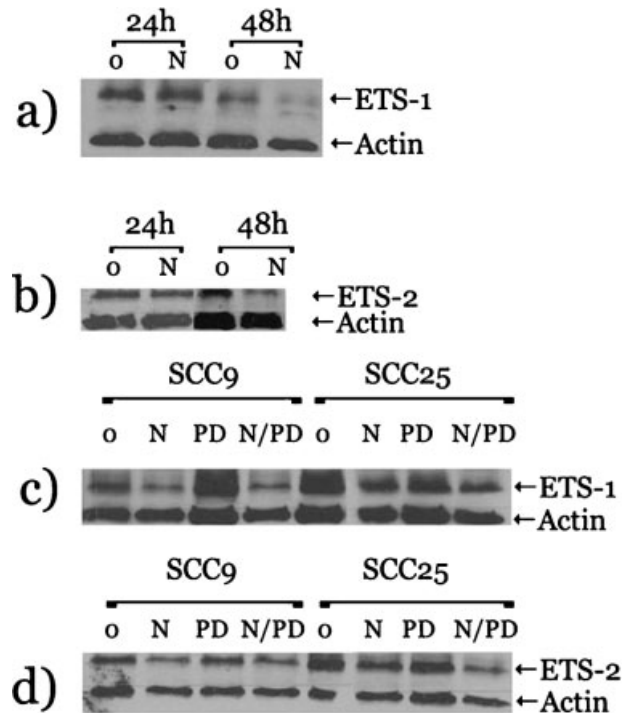


FIGURE 2. Both SCC cell lines show a significant decreased protein expression of Ets-1 (a) and Ets-2 (b) after 24 and 48 hours of treatment with nimesulide compared to control. (Figure 1 depicts exemplary cell line SCC25.) (c,d) Expression levels of Ets-1 (c) and Ets-2 (d) in SCC9 and SCC25 cell lines are not changed by treatment with PD 98059 (PD) compared control (O). Expression levels of Ets-1 or Ets-2 when treated with nimesulide in combination with PD 98059 (N/PD) are similar to those after nimesulide treatment alone (N), suggesting that PD 98059 does not contribute to a significant alteration in the reduction of Ets-1 (c,d). Equal protein loading was confirmed by reprobing with anti-actin antibody.

Ets-2 in the squamous cell carcinoma cell lines was not effected after incubation with the ERK-kinase inhibitor PD 98059 (Figure 2c, d) compared with control.

In the last series of experiments, the ability of both agents on Ets-1 and Ets-2 expression was investigated. SCC-9 and SCC-25 cell lines showed a reduction of Ets-1 and Ets-2 expression after treatment with both agents. Treatment with both agents did not alter the expression of Ets-1 and Ets-2 compared with nimesulide alone, suggesting that PD 98059 does not contribute to a significant alteration in the reduction of Ets-1 (Figure 2c, d).

DISCUSSION

Nimesulide has been shown to exert antiproliferative effects on a variety of tumor cell lines, including colon cancer cell lines.⁷ Recently, we showed in our laboratory that nimesulide was able to induce apoptosis in head and neck cancer cell lines SCC-9 and SCC-25.¹⁷

In this study, we investigated the effect of nimesulide on the expression of two transcription factors, protooncogenes Ets-1 and Ets-2, in HNSCC cell lines.

Ets-1 is produced in different carcinomas, but it is also expressed in endothelial cells during physiologic angiogenesis, as well as during tumor angiogenesis.⁶ Depending on the tumor type, Ets-1 expression is either increased or exclusively found in invasive higher grade tumors. It could be shown that elevated Ets-1 expression in malignancies, including breast and ovary carcinomas, correlated with poorer prognosis.^{19–21} A recent study demonstrated that this transcription factor is also involved in the development and invasion of malignant melanoma.²² Ets-2, the second member of the Ets family of transcription factors that we investigated in the course of this study, is overexpressed in various cancerous tissues, including blood cancer, breast cancer, cancer of the prostate, and esophageal cancer.^{3,4} Upregulation of these two transcription factors correlates well with the grade of invasiveness and metastasis.²³ Using immunohistochemistry, we showed, in accordance with Pande et al,²⁴ that Ets-1 is upregulated in head and neck cancer specimens. Furthermore, as far as we know, for the first time we demonstrated upregulation of the protooncogene Ets-2 in head and neck cancer tissue.

Ets-1 and Ets-2 expression can be modulated by a variety of factors. This synthesis is induced

by hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), or tumor necrosis factor- α (TNF- α) by means of activation of the MEK1/ERK1/2 pathway.^{25–27} We investigated this kind of pathway with PD, an ERK-kinase inhibitor, but surprisingly this substance did not affect the expression of Ets-1 and Ets-2 in the cell lines used. Therefore, we deduce that the activation of Ets-1 and Ets-2 in our cell lines is independent of the ERK-kinase pathway.

In the past few years, it was shown that NSAIDs have an antiproliferative effect in neoplastic cells of different tissues. Furthermore, NSAIDs were able to induce apoptosis in different malignancies, like breast, squamous cell carcinoma of the skin, and colon adenocarcinoma cells.^{28–30} In mice hepatoma, nimesulide, a selective COX-2 inhibitor, inhibited tumor growth and induced apoptosis by inhibiting COX-2 and prostaglandin E₂ (PGE₂) expression.³¹ Different selective COX-2 inhibitors (eg, celecoxib, NS-398, and nimesulide) were tested, and all substances inhibited proliferation and acted proapoptosis in colon adenocarcinoma cells.³⁰ As we demonstrated recently in our laboratory, nimesulide induced apoptosis in head and neck cancer cell lines.¹⁷

In this study, we demonstrated that the selective COX-2 inhibitor nimesulide is also antiproliferative against HNSCC cell lines, possibly because of the reduction of the protooncogenes Ets-1 and Ets-2.

However, in a recent publication, it was shown that Ets-1 and Ets-2 cooperate in transcription with the AP-1 transcription factor, the product of the protooncogene families fos and jun.³² HER-2/neu, a transmembrane receptor, is known to stimulate COX-2 transcription by means of the RAS-RAF-MAP-Kinase pathway in HER-2/neu-positive breast cancer cells.³³ The inductive effects of HER-2/neu were mediated by enhanced binding of AP-1 to the cyclic adenosine monophosphate response element of the COX-2 promoter.³³ HER-2/neu is also expressed in cancer of the head and neck.³⁴ Taking these findings together, a possible link among HER-2/neu, COX-2 regulation, and Ets expression seems clearly possible and might warrant further studies.

In summary, we showed for the first time that Ets-2 is upregulated in HNSCC cell lines. Furthermore, we observed a significant reduction of Ets-1 and Ets-2 expression in head and neck cancer cell lines caused by nimesulide. Furthermore, we demonstrated that Ets-1 and Ets-2 expression is not regulated by means of the MEK-ERK sig-

naling pathway, but further studies are warranted to explain the exact and manifold effects of Cox-2 inhibitors like nimesulide in head and neck cancer cell lines.

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