Cyclooxygenase-2 Is Expressed in Neuroblastoma, and Nonsteroidal Anti-Inflammatory Drugs Induce Apoptosis and Inhibit Tumor Growth \textit{In vivo}

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Abstract

Neuroblastoma is the single most common and deadly tumor of childhood and is often associated with therapy resistance. Cyclooxygenases (COXs) catalyze the conversion of arachidonic acid to prostaglandins. COX-2 is up-regulated in several adult epithelial cancers and is linked to proliferation and resistance to apoptosis. We detected COX-2 expression in neuroblastoma primary tumors and cell lines but not in normal adrenal medulla from children. Treatment of neuroblastoma cells with nonsteroidal anti-inflammatory drugs, inhibitors of COX, induced caspase-dependent apoptosis via the intrinsic mitochondrial pathway. Treatment of established neuroblastoma xenografts in nude rats with the dual COX-1/COX-2 inhibitor diclofenac or the COX-2-specific inhibitor celecoxib significantly inhibited tumor growth \textit{in vivo} (P < 0.001). In \textit{vivo}, arachidonic acid and diclofenac synergistically induced neuroblastoma cell death. This effect was further pronounced when lipooxygenases were simultaneously inhibited. Proton magnetic resonance spectroscopy (\textit{1H MRS}) of neuroblastoma cells treated with COX inhibitors demonstrated accumulation of polyunsaturated fatty acids and depletion of choline compounds. Thus, \textit{1H MRS}, which can be performed with clinical magnetic resonance scanners, is likely to provide pharmacodynamic markers of neuroblastoma response to COX inhibition. Taken together, these data suggest the use of nonsteroidal anti-inflammatory drugs as a novel adjuvant therapy for children with neuroblastoma.

Introduction

Neuroblastoma is the most common neoplasia during infancy. The majority of these embryonal sympathetic nervous system tumors arise from primitive cells in the adrenal medulla. Patients > 1 year of age with metastatic disease and those with MYCN-amplified tumors have poor prognosis and often develop resistance to conventional therapy (1). Alternative treatments for these patients are therefore needed. Arachidonic acid (AA) is released from cellular phospholipids by phospholipase A2 and converted to prostaglandins by two cyclooxygenase (COX) enzymes, COX-1 and COX-2 (2). COX-1 is constitutively expressed in most tissues, whereas inflammatory stimuli, hormones and mitogens may induce COX-2 expression (2). COX-2 is overexpressed in a variety of adult cancers and has been implicated in resistance to apoptosis as well as induction of metastases and angiogenesis (3). We thus investigated the expression of COX-2 in neuroblastoma tumors and the therapeutic effect of nonsteroidal anti-inflammatory drugs (NSAIDs) against neuroblastoma cell lines \textit{in vitro} and xenografts \textit{in vivo}.

Materials and Methods

Human Tissue Samples. Twenty-eight neuroblastomas derived from children of different ages and all clinical stages, including different biological subsets (MYCN amplification, 7 of 28; 1p deletion, 9 of 26; Table 1) were analyzed. Three childhood ganglioneuromas and three samples of nonmalignant adrenal medulla (and cortex) from children were also included. All tissue samples were frozen at surgery and kept at −80°C until fixation.

Immunohistochemistry. Paraffin-embedded sections were incubated with a monoclonal mouse anti–COX-2 antibody (Zymed Laboratories Inc., South San Francisco, CA) overnight at 4°C. As a control for nonspecific background staining, sections were incubated with mouse IgG isotype control. Biotinylated antimouse IgG and streptavidin-horseradish peroxidase (HRP) complex were used as secondary antibodies (Zymed Laboratories Inc.). Reaction products were visualized with 3,3’-diaminobenzidine substrate chromogen system (DakoCytomation, Carpinteria, CA).

For identification of caspase-3 activity, sections of neuroblastoma xenografts were incubated overnight at 4°C with a polyclonal antibody specifically detecting cleaved caspase-3 (R&D Systems, Abingdon, United Kingdom). Sections were subsequently washed and incubated with secondary biotinylated antibody and streptavidin-HRP complex (Zymed Laboratories Inc.).

Chemicals. Diclofenac (Cayman Chemicals, Ann Arbor, MI) was dissolved in dimethyl sulfoxide and further diluted in medium (final dimethyl sulfoxide concentration, 0.1–0.7‰). Nor-dihydro-guaiaaretic acid [NDGA (10 μmol/L)], reduced glutathione (10 mmol/L), N-acetylcysteine (100 μmol/L), t-cycloserine (5 mmol/L), and α-tocopherol (100 μmol/L) were all from Sigma (Stockholm, Sweden).

Cell Lines. Neuroblastoma cell lines were grown in Eagle Minimal Essential Medium (SH-SY5Y) or RPMI 1640 [SK-N-BE(2), SK-N-SH, SK-N-AS, SK-N-FI, SK-N-DZ and IMR-32] medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin G, and 100 μg/mL streptomycin (Life Technologies, Inc., Stockholm, Sweden) at 37°C in a humidified 5% CO2 atmosphere.

Cytotoxicity Assay and Fluorescence-Activated Cell-Sorting Analysis. Cells were incubated with the indicated concentrations of drugs for 48 hours. Cell viability was assessed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma). The mitochondrial transmembrane potential was determined using tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Eugene, OR). After labeling (25 mmol/L TMRE, 30 minutes), cells were harvested, rinsed, resuspended in PBS, and analyzed on the FL2 channel on a FACSCalibur flow cytometer, using Cell Quest Software (Becton Dickinson, San Jose, CA). Quantification of apoptosis was performed by counting 4’,6-diamidino-2-phenylindole–stained nuclei using a fluorescence microscope. DNA content was assessed by fluorescence-activated cell-sorting analysis as described previously (4).

Western Blotting. Protein was extracted from cells in a buffer containing 25 mmol/L Tris (pH 7.8), 2 mmol/L EDTA, 20% glycerol, 0.1% Nonidet P-40, 1 mmol/L dithiothreitol, and protease inhibitors (Roche Diagnostic, Mannheim, Germany). Protein content was measured using Bradford reagent (Bio-Rad, Sundbyberg, Sweden). Equal quantities were separated by SDS-PAGE, transferred to

Received 5/21/04; revised 8/7/04; accepted 8/18/04.

Grant support: The Swedish Children’s Cancer Foundation, Swedish Cancer Society, Mary Beve’s Foundation, and The Cancer Society of Stockholm. This work was presented in part at the AACR 95th Annual Meeting in Orlando, Florida, March 27–31, 2004 and at the 11th Symposium of Advances in Neuroblastoma Research in Genova, Italy, June 16–19, 2004, where it was awarded the Audrey E. Evans Prize.

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[CANCER RESEARCH 64, 7210–7215, October 15, 2004]
nylon membranes (Millipore Inc., Sundbyberg, Sweden), and probed with antibodies against COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3, caspase-8, caspase-9, the BH3 interacting domain death agonist (BID; R&D Systems), and /H9252-actin (Sigma). Antimouse IgG or antirabbit IgG, conjugated with HRP (Pharmacia Biosciences, Uppsala, Sweden), served as secondary antibodies. Pierce Super Signal (Pierce, Rockford, IL) was used for detection.

Proton Magnetic Resonance Spectroscopy. Typically, 2 to 3 /H11003 10^7 cells were analyzed using 5-mm Shigemi tubes. Cells were washed twice with PBS, resuspended in PBS with 10% D2 O, and placed on ice until data acquisition. Samples were analyzed using a 500 MHz Bruker spectrometer at 25 °C. The residual H2 O signal at 4.75 ppm was suppressed by low-power presaturation. The acquisition parameters included the following: 90 ° pulse; repetition time, 1.5 seconds; 256 scans; 8k points; and spectral width of 5 KHz. After acquisition, spectra were Fourier transformed and phase corrected. Signal intensities were integrated using XWINNMR software (version 3.1; Bruker). Cell viability in the samples was assessed by trypan blue dye exclusion before proton magnetic resonance spectroscopy (1 H MRS).

Table 1  COX-2 expression in neuroblastoma, ganglioneuroma, and healthy adrenals

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Abbreviations: INSS, International Neuroblastoma Staging System; NB, neuroblastoma; NED, no evidence of disease; DOD, dead of disease; AWD, alive with disease; ND, not determined; DOC, dead of surgical complications; GN, ganglioneuroma; ADR, nonmalignant adrenal gland.

* Patient fulfilling clinicobiological criteria to obtain high-risk therapy.
† Multifocal primary.
‡ Negative for COX-2 expression in medullary cells, but positive for COX-2 expression in cortical cells.

** Fig. 1. COX-2 expression in neuroblastoma tumors and cell lines. A. Immunohistochemistry showing specific COX-2 expression in the cytoplasm of tumor cells in a neuroblastoma (top row; hematoxylin and eosin staining (left panel), COX-2 staining (middle and right panels); sample 3, Table 1), differentiated ganglion cells in a benign ganglioneuroma (middle row; sample 30, Table 1), and nonmalignant adrenal tissue showing absence of COX-2 expression in the medulla and weak expression in the cortex (bottom row; sample 32, Table 1). B. Western blotting detected a protein band of approximately 71 kDa corresponding to COX-2 in all neuroblastoma cell lines investigated. The blot was stripped and probed with β-actin to ensure equal protein loading.**
Xenografts and In vivo Administration of Nonsteroidal Anti-Inflammatory Drugs. The establishment of SH-SY5Y neuroblastoma xenografts was performed as described previously (4). Two independent therapeutic experiments were carried out. In the first experiment, male nude rats (HsdHan:Rnu; n = 19; Harlan, Horst, The Netherlands) were randomly assigned to receive 200 (n = 6) or 250 mg/liter (n = 6) diclofenac in drinking water or no treatment (n = 7), respectively. In the second experiment, nude rats (n = 12) were randomized to receive 10 days of treatment with celecoxib (10 mg once daily) administered through a gastric feeding tube (n = 6) or no treatment (n = 6). Treatment was started on the appearance of palpable tumors. The mean tumor volume at the start of treatment was 0.07 mL (95% confidence interval, 0.05–0.09 mL) in the first experiment and 0.39 mL (95% confidence interval, 0.38–0.40 mL) in the second experiment. Tumor volume was estimated as described previously (4). Tumor weight was recorded at autopsy, after which tumors were frozen in liquid nitrogen for immunohistochemistry. All animal experiments were approved by the regional ethics committee for animal research in accordance with the Animal Protection Law (SFS1988:534), the Animal Protection Regulation (SFS 1988:539), and the Regulation for the Swedish National Board for Laboratory Animals (SFS1988:541).

Statistical Analysis. The Mann-Whitney U test for two independent samples was used to test significance of observed differences between treatment groups, in vitro and in vivo. The mean change in tumor volume within the respective groups of animals was tested for significance using the Wilcoxon matched pairs test. All statistical tests were two-sided. P < 0.05 was considered significant.

Results and Discussion
Expression of Cyclooxygenase-2 in Neuroblastoma Tissues and Cell Lines. We analyzed neuroblastomas from different biological subsets and at all clinical stages for COX-2 expression (Table 1). Twenty-seven of 28 neuroblastoma samples (96%) showed specific expression of COX-2 protein in the cytoplasm of the tumor cells (Fig. 1A). No COX-2 expression was detected in the surrounding nonmalignant adrenal medulla tissues. One neuroblastoma sample, from a localized stage 1 tumor with favorable biology (triploid DNA without MYCN amplification; Table 1), did not express COX-2. Three ganglieneuromas were investigated and showed COX-2 staining in the tumor-derived differentiated ganglion cells but not in the surrounding benign stroma (Fig. 1A). COX-2 protein was not detected in nonmalignant adrenal medulla tissue (n = 3), whereas surrounding normal
The adrenal cortex showed a weak staining for COX-2 (Fig. 1A). Of interest, COX-2 is expressed in adult adrenal cortex, but not in the medulla, whereas adult malignant pheochromocytomas show enhanced COX-2 expression (5).

Seven neuroblastoma cell lines were investigated, and all showed expression of COX-2 protein (Fig. 1B).

**Treatment of Neuroblastoma with Nonsteroidal Anti-Inflammatory Drugs In vitro.** Treatment of neuroblastoma cell lines with the selective COX-2 inhibitor celecoxib or the dual COX-1/COX-2 inhibitor diclofenac resulted in dose-dependent inhibition of cell growth (Fig. 2A). The IC₅₀ ranged from 12.5 to 50 μmol/L for celecoxib and 100 to 600 μmol/L for diclofenac, respectively (Fig. 2A). Assessment of nuclear morphology demonstrated DNA fragmentation compatible with increased apoptosis in cells treated with diclofenac (Fig. 2B). Depolarization of the mitochondrial membrane potential was detected in six of seven neuroblastoma cell lines on treatment with diclofenac (Fig. 2C). Western blotting confirmed activation of caspase-9 and caspase-3, whereas no activation of caspase-8 or BID was observed (Fig. 2D). This suggests that the intrinsic apoptotic pathway is involved in NSAID-induced apoptosis of neuroblastoma cells.

In comparison with nonmalignant nervous tissue, neuroblastoma cells contain increased levels of AA (6), the main substrate for eicosanoid biosynthesis catalyzed by COX and lipoxygenases [LOXs (refs. 2 and 3; Fig. 2E)]. Simultaneous treatment with diclofenac and the pan-LOX inhibitor NDGA synergistically inhibited the survival of SH-SY5Y neuroblastoma cells (Fig. 2F). To further determine the biological effects of AA and AA metabolites in neuroblastoma, SH-SY5Y cells were supplied with exogenous AA in the absence or presence of COX/LOX inhibitors. Whereas AA alone modestly stimulated neuroblastoma cell proliferation (P < 0.001), concomitant inhibition of COX inhibited cell survival in a synergistic fashion (P < 0.001; Fig. 2F). Furthermore, simultaneous inhibition of COX and LOX pathways in the presence of AA inhibited cell survival more potently than all other combinations (P < 0.001; Fig. 2F). This suggests that forced accumulation of AA by abrogation of its downstream metabolism is highly toxic to neuroblastoma cells.

¹H MRS, which allows clinical monitoring of tumor biochemistry, is particularly useful for analysis of intracellular lipids (7), including polyunsaturated fatty acids (PUFAs), of which AA is the most abundant in vivo (8). Moreover, ¹H MRS typically shows increased content of PUFAs and methylene groups of mobile lipids in cancer cells undergoing therapy-induced cell death (7, 9). We therefore investigated the possibility of monitoring the levels of PUFAs with ¹H MRS in neuroblastoma cells treated with NSAIDs. A pronounced increase in the signal intensity of mobile lipids and, specifically, PUFAs was observed in SH-SY5Y cells treated with diclofenac (Fig. 2G). Thus, in neuroblastoma cells, NSAIDs induce accumulation of lipids and, in particular, PUFAs that could be directly involved in the cytotoxicity of NSAIDs. In view of these and the above-mentioned findings, it is of interest to note that AA-dependent tumor cell death involves...
induction of mitochondrial permeability transition (10). Several anti-
cancer therapies, including cisplatin, irradiation, and retinoids, all
used in the treatment of neuroblastoma, may induce intracellular release of AA (11, 12). Our findings raise the possibility that children
with neuroblastoma may benefit from NSAID-mediated inhibition of AA metabolism in combination with therapies that increase the intra-
cellular concentration of AA.

AA may stimulate sphingomyelinase to convert sphingomyelin to
ceramide, a potent inducer of apoptosis (13). However, inhibitors of ceramidase (reduced glutathione and N-acetylcysteine) failed to pre-
vent the cytotoxic effect of NSAIDs in neuroblastoma cells, as did
1-cycloserine, an inhibitor of ceramide de novo synthesis (ref. 14; data
not shown). PUFAs such as AA are potential targets of lipid peroxi-
dation by free radicals. However, radical scavengers (α-tocopherol and N-acetylcysteine) did not inhibit diclofenac-induced cytotoxicity
to neuroblastoma cells (data not shown).

**Nonsteroidal Anti-Inflammatory Drugs Effectively Inhibit Neu-
roblastoma Growth In vivo.** To investigate the effects of NSAIDs
on neuroblastoma growth in vivo, we treated nude rats carrying
SH-SY5Y xenografts with diclofenac. Tumor growth was signifi-
cantly inhibited after 2 days of diclofenac treatment (200 mg/L, P = 0.042; 250 mg/L, P = 0.024) compared with untreated controls.
Treatment with the lower dose of diclofenac completely inhibited tumor growth for the first 9 days, whereas untreated control tumors grew exponentially (Fig. 3A). At the higher dose level, tumor growth was completely inhibited throughout the treatment period (Fig. 3A).

Tumor weight at autopsy was significantly reduced in animals treated with diclofenac, irrespective of the dose, compared with untreated tumors (P = 0.009, both groups; Fig. 3B).

Immunohistochemistry of tumors from diclofenac-treated animals demonstrated elevated expression of cleaved caspase-3 as compared with untreated tumors, indicating that diclofenac induces apoptosis of neuroblastoma in vivo (Fig. 3C).

Treatment with celecoxib significantly inhibited xenograft growth from day 4 and throughout the experiment, compared with controls (P = 0.001; Fig. 3D). Celecoxib-treated tumors were significantly smaller at autopsy compared with tumors from untreated rats (P < 0.001; Fig. 3E). No weight loss or other signs of toxicity were observed in rats treated with celecoxib or diclofenac (data not shown).

COX-2 expression has been associated with the production of angiogenic factors, which can be blocked by NSAIDs (2, 3). It is therefore possible that the potent inhibition of neuroblastoma growth in vivo in response to NSAIDs involves inhibition of angiogenesis, in addition to direct induction of tumor cell apoptosis. Moreover, it cannot be excluded that targets of NSAIDs other than COX-2 are involved in their effects against neuroblastoma. COX-independent mechanisms of NSAID-mediated apoptosis (e.g., inhibition of iκB kinase β activity or modulation of the peroxisome proliferator-activated receptor γ) have been described (15–17).

Choline compounds are characteristically elevated in malignant cells and tumors, as detected by 1H MRS (18). Neuroblastoma cells and tumors responding to chemotherapy are characterized by a reduc-
tion of the choline 1H MRS signal and an increase in mobile lipid
resonances (19). Here we observed a pronounced decrease in the signal intensity of the 1H MRS choline resonance in neuroblastoma cells treated with diclofenac (Fig. 2G). Interestingly, 1H MRS of extracts from COX-expressing breast cancer cells treated with the NSAID indomethacin showed a reduction in phosphocholine in indomethacin-sensitive cells (20).

In conclusion, COX-2 is expressed in neuroblastoma, and NSAIDs induce apoptosis and inhibit growth of neuroblastoma cells in vitro and xenografts in vivo. Because NSAIDs are clinically available and well tolerated, trials to evaluate their efficacy as an adjuvant therapy in children with neuroblastoma are warranted. Moreover, we have shown that 1H MRS provides biochemical markers for the response of neuroblastoma cells to NSAIDs. Because 1H MRS is clinically avail-
able through the use of conventional magnetic resonance scanners, this could provide an early noninvasive means of evaluating response to NSAIDs in children with neuroblastoma.

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**Fig. 3.** NSAIDs inhibit growth of established neuroblastoma xenografts in vivo. A. Diclofenac prevents the growth of SH-SY5Y xenografts in vivo. Diclofenac prevented a significant increase in tumor volume for 9 days (200 mg/L, n = 6 tumors; P = 0.22) or for the entire period (250 mg/L, n = 7 tumors; P = 1.0), respectively. Control tumors increased significantly in volume (n = 7 tumors; P = 0.013). Bars, SE. Statistical test, Wilcoxon matched pairs test. --- Untreated. --- 200 mg/L diclofenac; --- 250 mg/L diclofenac. B. Tumor weights of SH-SY5Y xenografts from rats treated with diclofenac and from untreated controls. Only tumors that were palpable at the start of treatment are shown. Tumor weight at autopsy was significantly reduced in animals receiving diclofenac, in the two doses, compared with untreated controls (P = 0.009, Mann-Whitney U test). C. Immunohistochemistry showing isotype IgG control (left panel), activated caspase-3 in diclofenac-treated xenografts (middle panel), compared with untreated SH-SY5Y xenografts (right panel), respectively. D. Celecoxib inhibits the in vivo growth of neuroblastoma xenografts. SH-SY5Y xenografts in rats treated with celecoxib (n = 6) grew significantly slower from day 4 and throughout the experiment, compared with untreated controls (n = 6; P = 0.001, Mann-Whitney U test). Bars, SE. --- Celecoxib; --- Control. E. Effects of celecoxib on tumor weight. Only xenografts that were palpable at start of treatment are shown. Tumors from animals treated with celecoxib (n = 9 tumors) were significantly smaller at autopsy compared with tumors from untreated rats (n = 8 tumors; P < 0.001, Mann-Whitney U test).
Cyclooxygenase-2 Is Expressed in Neuroblastoma, and Nonsteroidal Anti-Inflammatory Drugs Induce Apoptosis and Inhibit Tumor Growth In vivo

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