Overexpression of Cox-2 in Human Osteosarcoma Cells Decreases Proliferation and Increases Apoptosis

Zheng Xu, Shilpa Choudhary, Olga Voznesensky, Meenal Mehrotra, Monica Woodard, Marc Hansen, Harvey Herschman, and Carol Pilbeam

Abstract

Overexpression of cyclooxygenase-2 (COX-2) is generally considered to promote tumorigenesis. To investigate a potential role of COX-2 in osteosarcoma, we overexpressed COX-2 in human osteosarcoma cells. Saos-2 cells deficient in COX-2 expression were retrovirally transduced or stably transfected with murine COX-2 cDNA. Functional expression of COX-2 was confirmed by Northern and Western analyses and prostaglandin production. Overexpression of COX-2 reduced cell numbers by 50% to 70% compared with controls. Decreased proliferation in COX-2-overexpressing cells was associated with cell cycle prolongation in G2-M. Apoptosis, measured by both Annexin V binding assay and terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining, was increased in cells overexpressing COX-2, and the increase was not reversed by treatment with NS-398, indicating that the effects were not mediated by prostaglandins. Retroviral COX-2 overexpression in two other human osteosarcoma cell lines, U2OS and TE85, also decreased cell viability. However, in the human colon carcinoma HCT-116 cell line, which is deficient in COX-2, retroviral overexpression of COX-2, at similar efficiency as in Saos-2 cells, increased resistance to apoptosis. Reactive oxygen species (ROS), measured by flow cytometry, were increased by COX-2 overexpression in Saos-2 cells but not in HCT-116 cells. Inhibition of peroxidase activity, but not of COX activity, blocked the ROS increase. Antioxidants blocked the increase in ROS and the increase in apoptosis due to COX-2 overexpression in Saos-2 cells. Our results suggest that (a) COX-2 overexpression in osteosarcoma cells may increase resistance to tumorigenesis by increasing ROS to levels that decrease cell viability and (b) the effects of COX-2 overexpression are cell type/tissue dependent. (Cancer Res 2006; 66(13): 6657-64)

Introduction

Cyclooxygenase (COX) is a rate-limiting enzyme in the conversion of arachidonic acid to prostanoids. There are two forms of COX: COX-1, which is constitutive, and COX-2, which is inducible by multiple factors including cytokines, hormones, and mitogens (1, 2). Both COX-1 and COX-2 convert arachidonic acid to prostaglandin G2 (PGG2) via a COX reaction and then reduce PGG2 to prostaglandin H2 (PGH2) in a peroxidase reaction. Most prostaglandin E2 (PGE2) produced in bone is the result of induction of COX-2 expression in osteoblasts (3).

A role for COX-2 in tumorigenesis was initially suggested by epidemiologic studies showing a 40% to 50% reduction in the incidence of colorectal cancer in individuals taking nonsteroidal anti-inflammatory drugs and by studies showing constitutive up-regulation of COX-2 in tumor cells (4, 5). Some studies have suggested that polymorphisms in the COX-2 gene (6) or chromosomal gain at the COX-2 locus (7) correlates with the risk for colorectal cancer or with survival of colorectal cancer patients. aberrant expression of COX-2 and the potential chemotherapeutic role of COX-2 selective nonsteroidal anti-inflammatory drugs have been shown in many other types of cancer, including breast and lung (8–11).

Several studies have shown COX-2 to be expressed in osteosarcoma cell lines (12, 13) and in osteosarcoma (14, 15). In this study, we examined endogenous expression of COX-2 and effects of overexpressing COX-2 on cell growth in Saos-2, U2OS, and TE85 human osteosarcoma cells. Overexpression of COX-2 decreased cell viability in the osteosarcoma cell lines but increased viability in a human colon carcinoma cell line, HCT-116. These differences correlated with differences in generation of reactive oxygen species (ROS). Our data suggest that, depending on cell/tissue type, COX-2 overexpression can have antitumorigenic effects mediated by increased ROS.

Materials and Methods

Materials. Murine COX-2 cDNA was previously described (16). Human COX-1 and COX-2 cDNA, NS-398, and PGE2 were from Cayman Chemical (Ann Arbor, MI). cDNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained by PCR (Clontech, Palo Alto, CA). Culture media were from Life Technologies, Inc. (Grand Island, NY) unless otherwise specified. Dihydroethidium was from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture. Saos-2 cells from different laboratories were arbitrarily labeled strains 1 to 3. Saos-2 strain 2, U2OS, and TE85 cells were from the American Type Culture Collection (Manassas, VA). Saos-2 strain 1 was from Dr. Gloria Gronowicz (University of Connecticut Health Center, Farmington, CT). Saos-2 strain 3 was from Dr. Eva Y.-H.P. Lee (Department of Biological Chemistry, University of California, Irvine, CA; ref. 17). HCT-116 human colon carcinoma cells were a gift from Dr. D. Rosenberg (University of Connecticut Health Center).

Cells were cultured in a humidified atmosphere of 5% CO2 at 37°C. Saos-2 strains 1 and 2 were cultured in McCoy’s 5A modified medium with 10% FCS (Life Technologies). U2OS cells were cultured in similar media with 15% FCS. Saos-2 strain 3 and TE85 cells were cultured in DMEM/F12 medium with 10% FCS. HCT-116 cells were cultured in high-glucose DMEM with 10% FCS. All media contained 100 units/mL penicillin and 50 µg/mL streptomycin. Vehicle was 0.1% alcohol or 0.1% bovine serum albumin (BSA), as appropriate.
Reverse transcription-PCR. Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH) and converted to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamer (Invitrogen, Carlsbad, CA) following the recommended protocol of the company. Primers for human COX-2 were 5′-ATACTAGGCCCCTT CCTCTCTGTCCT-3′ (sense) and 5′-TACTTCTCTGACTGCGGGT-3′ (antisense). Primers for GAPDH were 5′-CATGATGCCATAGGTTCACC-3′ (sense) and 5′-TGAAGGTCTGTTGAAACGGATTGC-3′ (antisense). Reaction conditions were 94°C for 2 minutes followed by 30 cycles at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 45 seconds, followed by holding at 72°C for 5 minutes.

Cloning and stable transfection. COX-2 murine cDNA (17,195 bp) was amplified by PCR and subcloned into pCR 2.1 vector (Invitrogen). A silent mutation (C to T) replaced the ClaI site at 1,575 bp (QuiikChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA). The 1.9-kb HindIII/XbaI COX-2 fragment was subcloned into a Cla-BGH PA polylinker derivative of pBR322, provided by David Rowe (University of Connecticut Health Center). The ClaI fragment was subcloned into the ClaI site of pBCKS+ plasmid, containing 3.6 kb of the rat type 1 collagen (Col1A1) promoter with first exon and intron, provided by Dr. Rowe. The pOBCol3.6-COX-2 construct was verified by sequencing (University of Connecticut Health Center Molecular Core).

The pOBCol3.6-COX-2 construct was cotransfected with pSV-2 neo using Lipofectamine (Life Technologies, Inc., Rockville, MD) as previously described (18). Following transfection, cells underwent 2 weeks of selection with 400 μg/ml G418 (Life Technologies). Single colonies were expanded and maintained in medium with 200 μg/ml G418.

Cloning of viral construct, retrovirus production, and transduction. The ClaI fragment of murine COX-2 was excised from the Cla-BGH PA polylinker derivative of pBR322 as described above and subcloned into the ClaI site of pBluescript-KS+ plasmid, containing 3.6 kb of the rat type 1 collagen (Col1A1) promoter with first exon and intron, provided by Dr. Rowe. The pOBCol3.6-COX-2 construct was verified by sequencing (University of Connecticut Health Center Molecular Core).

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Cyclic AMP assay. Cells were plated at 5,000/cm² and grown for 6 days. Before extraction, cells were treated with 0.5 mmol/L isobutyl methyl xanthine for 20 minutes and then with PGE$_2$ (1 μmol/L) for 20 minutes. Cyclic AMP (cAMP) was measured in the cells according to EIA kit instructions (Cayman) and was normalized to total protein measured with a BCA assay kit (Pierce; n = 3 samples).

Cell growth and proliferation. Cells were counted with Coulter Counter (Coulter Corporation, Miami, FL). Cell proliferation was measured by 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining and flow cytometry. Cells were incubated with CFSE (Molecular Probes) at a concentration of 25 μmol/L in PBS for 10 minutes at 37°C. The reaction was stopped by adding FCS. Cells were replated at 48,000/well in six-well dishes and grown for 3 to 5 days. After preparation by trypsinization and washing, fluorescence intensity was measured on flow cytometry using excitation at 488 nm at the FL1 detection channel (FACSCalibur, Becton Dickinson, San Jose, CA) and analyzed with CellQuest software (Becton Dickinson).

Apoptosis. Apoptosis was measured using the Annexin V–FITC apoptosis detection kit from Calbiochem (San Diego, CA) or the Annexin V–allophycocyanin apoptosis detection kit from BD PharMingen (San Jose, CA). Cells were plated at 5,000/cm² in 100-mm dishes and cultured for 3 days. Both adherent and floating cells were collected and resuspended in 1× cold binding buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L CaCl₂, 1 mmol/L MgCl₂, 4% BSA] for analysis. Cells were also stained with propidium iodide to detect dead cells. Analysis was done on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson). Unstained cells were classified as “live”; cells stained for Annexin V only were “early apoptotic”; cells stained for both Annexin V and propidium iodide were “late apoptotic”; and cells stained for propidium iodide only were “dead.”

Apoptosis was also measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) with the alkaline phosphatase in situ cell death detection kit from Roche Molecular Biochemical (Mannheim, Germany). Nuclei were stained with DNA-specific fluorochrome, 4′,6-diamidino-2-phenylindole (DAPI). The ratio of TUNEL-positive to DAPI-stained cells was calculated for 2,000 cells per well and the average of three wells calculated. The experiment was done thrice to obtain the mean ± SE for n = 3.

Cell cycle analysis. Cells were plated at 5,000/cm² in 100-mm cell culture dishes and cultured for 4 days. Cells were trypsinized, fixed with 70% ethanol, and stored at –20°C. Cells were stained with 20 μg/ml propidium iodide solution in PBS containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A at room temperature and analyzed on FACSFlow cell flow cytometer (Becton Dickinson). After gating out cellular aggregates and debris, the cell cycle distribution of cells was analyzed using ModFit LT V3.0 software (Verity Software House, Inc., Topsham, ME).

Measurement of ROS. Flow cytometric measurement of ROS was done using dihydroethidium (20). Cells were stained with 10 μmol/L of dihydroethidium in phenol red–free medium for the last 30 minutes of culture, harvested in PBS, and analyzed at the FL-2 channel on a FACSCalibur flow cytometer (Becton Dickinson). For analysis, GFP populations were gated and equal numbers of events were considered for both Saos-2 and HCT-116 cells. Mean dihydroethidium fluorescent intensities were then obtained using CellQuest software. Cells were treated with detection dye and antioxidants for the last 30 minutes to 24 hours of culture. Vehicle for these experiments was water or DMSO (0.1%).

Statistical analysis. Statistical significance of differences was determined by ANOVA with post-hoc comparison by Bonferroni test (SigmaStat software, Jandel Scientific, San Rafael, CA).

Results

Endogenous COX-2 expression in Saos-2 cells. Saos-2 cells from three different laboratories were arbitrarily labeled strains 1, 2, and 3. To induce COX-2, cells were treated with interleukin-1 (IL-1; 10 ng/ml) or fresh FCS (10%; refs. 3, 21). In Saos-2 strain 2, COX-2 mRNA was easily detectable under both basal and
treatment conditions by reverse transcription-PCR (RT-PCR; Fig. 1A and B). Strains 1 and 3 had barely detectable COX-2 mRNA, with or without treatment. In two other cell lines derived from different osteosarcomas, TE85 and U2OS, COX-2 mRNA was inducible in one (U2OS) and undetectable in the other (TE85; Fig. 1C). Although COX-1 mRNA was constitutively expressed (Fig. 1D), PGE₂ production depended on COX-2 expression. Cumulative medium PGE₂ levels measured in untreated Saos-2 strain 2 cells cultured for 7 days in three experiments and normalized to the cell count at the end of culture were 108 ± 1.4, 146 ± 5.3, and 152 ± 23.0 pg/10⁵ cells. In contrast, PGE₂ was undetectable (<1 pg/10⁵ cells) in the media of Saos-2 strain 1 and strain 3 cells.

**Overexpression of COX-2 in Saos-2 by retroviral transduction.** We used a bicistronic retroviral system to overexpress murine COX-2 in Saos-2 strain 1 cells (Supplementary Fig. S1A). A new transduction was done for each experiment to avoid phenotypic changes in response to COX-2. Based on GFP intensities, transduction efficiency was 40% to 60% in cells transduced with either empty vector or CMV-mCOX-2/eGFP (data not shown). Expression of COX-2 was confirmed by Northern and Western analyses of COX-2-overexpressing cells (13.9 ± 3.1%) than in CMV/eGFP–transduced cells (4.1 ± 0.8%; P < 0.05). Cell apoptosis was also examined by Annexin V binding via allopurinol staining and flow cytometry (Fig. 2D). The population for analysis was gated to exclude cells not expressing GFP. Compared with vector only–transduced cells, COX-2-transduced cells showed decreased cell survival (73.7 ± 3.1% compared with 84.7 ± 1.16%) and increased early apoptosis (21 ± 0.9% compared with 10.4 ± 1.2%).

**Overexpression of COX-2 in Saos-2 cells by stable transfection.** To examine a population of cells with more homogeneous overexpression of COX-2, we stably transfected Saos-2 strain 1 cells with the 3.6-kb rat ColIα1 promoter that drives expression of murine COX-2 and DNA conferring neomycin resistance (Neo). Three colonies expressing Neo were expanded, screened for COX-2 mRNA, and compared for cell growth (Supplementary Fig. S2A). Cell growth was inversely correlated with COX-2 mRNA expression. No COX-2 mRNA or protein was detectable in colony 1 (Supplementary Fig. S2A-C), and these cells, called SAOS1 Neo, were used as controls in further analyses. Colony 3, with the highest expression of COX-2, was called SAOS1 COX-2 and used for further studies. PGE₂ production was increased in SAOS1 COX-2 cells relative to SAOS1 Neo cells, following addition of arachidonic acid (10 μmol/L) for the last 15 minutes of culture, and this increase was abrogated by NS-398 (Supplementary Fig. S2D).

Cell number was decreased 50% in SAOS1 COX-2 cultures compared with SAOS1 Neo cultures plated at equal densities and cultured for 7 days (Fig. 3A). PGE₂ had no effect on cell number in SAOS1 Neo cultures and NS-398 had no effect on cell number in SAOS1 COX-2 cultures. To examine proliferation, cells were stained with CFSE and cultured for 5 to 7 days. CFSE irreversibly couples to cellular proteins (22). When cells divide, CFSE labeling is distributed equally between daughter cells, which are half as fluorescent as their parents. The peak CFSE fluorescence intensity on flow cytometry was shifted by COX-2 overexpression, indicating 1 to 1.5 fewer cycles of cell replication than in CMV/eGFP–transduced cells (Fig. 2B). PGE₂ had no effect on CMV/eGFP–transduced cells and NS-398 had no effect on COX-2-transduced cells (data not shown). Similar results were seen in a second experiment (data not shown). COX-2 expression was associated with a decrease in G₁-phase population compared with CMV/eGFP–transduced cells (Fig. 2C). There was a trend toward increasing G₂-M population in COX-2-overexpressing cells compared with cells transduced with CMV/eGFP.

To examine apoptosis, cells were cultured for 3 days and stained with TUNEL–alkaline phosphatase and DAPI (data not shown). The ratio of TUNEL–positive to DAPI–stained cells was greater in COX-2-overexpressing cells (13.9 ± 3.1%) than in CMV/eGFP–transduced cells (4.1 ± 0.8%; P < 0.05). Cell apoptosis was also examined by Annexin V binding via allopurinol staining and flow cytometry (Fig. 2D). The population for analysis was gated to exclude cells not expressing GFP. Compared with vector only–transduced cells, COX-2-transduced cells showed decreased cell survival (73.7 ± 3.1% compared with 84.7 ± 1.16%) and increased early apoptosis (21 ± 0.9% compared with 10.4 ± 1.2%).

Figure 1. Endogenous COX-2 and COX-1 mRNA expression in human osteosarcoma cell lines. Cells were plated at 5,000/cm² in six-well dishes and cultured for 7 days before treatment with COX-2 agonists. Cells were treated with FCS (20%) for the last 4 hours of culture (A) or with IL-1 (10 ng/mL) for the last 24 hours of culture (B). RT-PCR was done for COX-2 and the housekeeping gene GAPDH. C, RT-PCR for COX-2 mRNA in three different osteosarcoma cell lines treated with IL-1 (10 ng/mL) for the last 3 hours of culture. D, Northern blot analysis for COX-1 and GAPDH was done on RNA extracted from the experiment described in (C).
population compared with control cells. Addition of NS-398 to the COX-2-overexpressing cell cultures had no effect (data not shown).

Three experiments measuring apoptosis are summarized in Fig. 3D. Cells were cultured for 3 days, double stained with Annexin V-FITC and propidium iodide, and analyzed by flow cytometry. There was a significant decrease (from 85% to 44%) in cell survival in COX-2 cells compared with Neo cells, which is associated with significant increases in early and late apoptosis, as well as cell death. Addition of NS-398 or PGE2 had no effect (Fig. 3D).

**Ability of Saos-2 cells to respond to PGE2.** There was no effect of nonsteroidal anti-inflammatory drug or PGE2 on Saos-2 cells in the above experiments. The two PGE2 receptors most important in osteoblasts, EP2 and EP4, act via the cAMP/protein kinase A pathway (3). To confirm that cells could respond to PGE2, we measured cAMP production after treatment with PGE2 (1 μmol/L). PGE2 increased cAMP, normalized to total protein, from 0.7 ± 0.4 to 16.0 ± 2.1 pmol/mg protein (P < 0.01).

**Effects of COX-2 overexpression in other osteosarcoma cells and in HCT-116 cells.** Saos-2 cells lack p53 (23). Because COX-2 may exert some effects via p53 (24), we examined the effects of retroviral transduction of COX-2 in TE85 and U2OS cells, both of which have wild-type p53 (25, 26). Expression of murine COX-2 mRNA was confirmed by Northern blot analysis (data not shown). To examine the effects on cell number, cells were plated at 5,000/cm², cultured for 3 days, and the mean ± SE of six wells was calculated. For the TE85 line, cell number was significantly reduced (P < 0.01) from 1.6 ± 0.1 × 10⁵ cells/well in vector-transduced cells to 0.7 ± 0.4 × 10⁵ cells/well in COX-2-overexpressing cells. For the U2OS line, cell number was significantly reduced (P < 0.01) from 1.2 ± 0.6 × 10⁵ cells/well in vector-transduced cells to 0.2 ± 0.2 × 10⁵ cells/well in COX-2-overexpressing cells. Similar results for both lines were seen in a second independent experiment.

To examine cell viability, cells were stained with Annexin V-allophycocyanin and propidium iodide and analyzed by flow cytometry after gating for GFP expression. For the TE85 line, the mean of two measurements showed the same trend as seen in Saos-2 cells. Live cells dropped from 85.2 ± 3.0% in vector-transduced cells to 74.6 ± 1.8% in COX-2-overexpressing cells whereas apoptotic cells rose from 10.3 ± 2.5% to 15.6 ± 0.7%. For the U2OS line, the mean of three measurements showed a similar trend. Live cells dropped from 91.1 ± 3.8% in vector-transduced cells to 87.5 ± 2.7% in COX-2-overexpressing cells whereas apoptotic cells rose from 6.5 ± 1.7% to 12.4 ± 2.7%.

To examine the effects of COX-2 overexpression in another type of tissue, we used the human colon carcinoma HCT-116 cell line, which is also deficient in COX-2 expression (27). Expression of murine COX-2 in retrovirally transduced HCT-116 cells was confirmed by Northern blot analysis using a murine cDNA (Fig. 4A). COX-2 activity was confirmed in HCT-116 cells transduced with CMV-COX-2/eGFP by measurement of medium PGE2 after brief addition of arachidonic acid (Fig. 4B).

To compare the transduction efficiencies in retrovirally transduced Saos-2 and HCT-116 cell lines, we calculated the percentages of cell population expressing GFP on flow cytometry. In Saos-2 cells, the transduction efficiency was 60 ± 2% and 56 ± 2% for CMV/eGFP and CMV-COX-2/eGFP, respectively. In HCT-116 cells, the transduction efficiency was 66 ± 4% and 64 ± 3% for CMV/eGFP and CMV-COX-2/eGFP, respectively. The results are the mean ± SE of three independent transduction experiments for each cell line. Hence, transduction efficiencies were similar for both cell lines and both constructs.

Cell counts for CMV-COX-2/eGFP– and CMV/eGFP–transduced HCT-116 cells were compared in five separate experiments. An example is shown in Fig. 4C. In no experiment was cell count reduced in the COX-2-overexpressing cells. To examine cell viability, cells were stained with Annexin V-allophycocyanin and propidium iodide and analyzed on flow cytometry after gating for GFP expression (Fig. 4D). Data from seven independent...
experiments were pooled. COX-2 overexpression resulted in an increase in live cells from 86.3 ± 2.0% to 93.2 ± 1.1% and a decrease in apoptotic cells from 11.6 ± 1.8% to 6.2 ± 1.1%. The results are the opposite of those seen in osteosarcoma cells.

Production of ROS and effects of antioxidants. Because COX-2 expression can increase ROS (28) and high levels of ROS can induce cell death (29), we measured ROS generation in Saos-2 strain 1 cells. There was a significant increase (60%) in ROS production in CMV/COX-2/eGFP–transduced cells compared with CMV/eGFP cells (Fig. 5A). Addition for the last 2 hours of culture of the COX inhibitor NS-398 (1 μmol/L) had no effect on ROS production (Fig. 5A). Addition of sodium cyanide (0.2 mol/L), an inhibitor of the peroxidase activity of COX (28), blocked the increased ROS production in COX-2-overexpressing cells (Fig. 5A). Treatment for the last 6 hours of culture with a combination of N-acetyl-L-cysteine (NAC; 5 mmol/L) and ebselen (50 μmol/L), pharmacologic analogues of the intracellular antioxidants glutathione and glutathione peroxidase (30), respectively, completely inhibited the increased ROS production in CMV/COX-2/eGFP–transduced cells (Fig. 5A). No effect of NAC + ebselen was observed on ROS in vector only--transduced Saos-2 cells.
In contrast to Saos-2 cells, overexpression of COX-2 in HCT-116 cells caused a small (22%), but significant, decrease in ROS production (Fig. 5B). Treatment with NS-398 or sodium cyanide had no effect on ROS production (Fig. 5B). Treatment with NAC plus ebselen for the last 6 hours of culture decreased ROS production by 35% in vector only–transduced cells but did not further decrease ROS production in COX-2-transduced HCT-116 cells (Fig. 5B).

To examine apoptosis, cells were treated with NAC + ebselen for the last 24 hours of culture. This treatment completely blocked the increased apoptosis seen in COX-2-overexpressing Saos-2 cells (Table 1). There was no effect of NAC + ebselen on vector only–transduced Saos-2 cells. In contrast, the decreased apoptosis seen in COX-2-overexpressing HCT-116 cells was unaffected by treatment with NAC + ebselen (Table 1). There was a trend for NAC + ebselen to decrease apoptosis in vector only–transduced HCT-116 cells, which was consistent with the decreased ROS seen in these cells when treated with antioxidants (Fig. 5B). Overexpression of COX-2 did not decrease apoptosis further in antioxidant-treated HCT-116 cells.

Discussion

Although COX-2 expression has been implicated in the progression of multiple tumor types, it is not known if COX-2 plays a role in the development and progression of osteosarcoma. Studies have shown COX-2 to be expressed in osteosarcoma cells (12, 13, 31). We found COX-2 mRNA expression to be highly variable in U2OS, TE85, and Saos-2 cell lines, ranging from easily measurable constitutive and inducible expression to undetectable constitutive or inducible expression. Similar variability was seen in the three Saos-2 strains, which were derived originally from the same tumor. We do not know if any of the cell lines we studied are currently capable of causing osteosarcoma \textit{in vivo}, but Saos-2 strain 3, which had little detectable COX-2 expression, was previously shown to cause osteosarcoma when injected into nude mice (32). These data suggest that COX-2 overexpression is not a general requirement for growth of osteosarcoma cells in culture.

Osteosarcomas are thought to arise from cells of the osteoblastic lineage and human osteosarcoma cell lines are often used as models to study human osteoblastic function (33). In support of this assumption, osteoblasts have been shown to be the targets for transformation into osteosarcomas in mice overexpressing c-fos (34). In normal osteoblasts, COX-2 expression is generally low under basal conditions but highly inducible by many agents and responsible for most acute PG production in bone (3, 13). Prostaglandins produced by COX-2 enhance the differentiation of osteoblastic precursors into mature bone-forming osteoblasts (3). In primary calvarial osteoblastic cell cultures, endogenous and exogenous PGE2 decrease cell proliferation (35). These effects of PGE2 to increase differentiation and decrease proliferation in normal osteoblasts suggest that normally expressed COX-2 might be antitumorigenic for osteoblasts. Although PGE2 increased CAMP in Saos-2 cells in this study, there was no effect on cell number or proliferation. It is possible that the loss of the ability to respond to antiproliferative effects of PGE2 could be important for the tumorigenic phenotype.

Many studies have reported increased cell growth and decreased apoptosis in cells with spontaneous up-regulation of COX-2 and in cells engineered to overexpress COX-2 (36–40). In contrast, overexpression of COX-2 in Saos-2 cells reduced cell number by 50% to 70%, decreased proliferation with cell cycle prolongation in

### Table 1. Effect of antioxidants (NAC + ebselen) on apoptosis in Saos-2 and HCT-116 cells retrovirally transduced with mCOX-2 or vector only

<table>
<thead>
<tr>
<th>Cell type/treatment</th>
<th>Live (%)</th>
<th>Apoptotic (%)</th>
<th>Dead (%)</th>
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<tr>
<td><strong>(A) Saos-2 cells</strong></td>
<td></td>
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<tr>
<td>Vector</td>
<td>89.0 ± 2.2</td>
<td>7.7 ± 1.2</td>
<td>3.3 ± 1.3</td>
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<td>Vector + antioxidants</td>
<td>87.1 ± 1.9</td>
<td>9.2 ± 1.7</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>mCOX-2</td>
<td>78.2 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.6 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>mCOX-2 + antioxidants</td>
<td>89.8 ± 2.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.6 ± 1.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.6 ± 1.0</td>
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<tr>
<td><strong>(B) HCT-116 cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vector</td>
<td>86.5 ± 1.2</td>
<td>12.2 ± 0.7</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>Vector + antioxidants</td>
<td>90.4 ± 1.2</td>
<td>9.2 ± 0.9</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>mCOX-2</td>
<td>95.4 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>mCOX-2 + antioxidants</td>
<td>95.8 ± 1.7</td>
<td>4.1 ± 1.7 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

NOTE: Data are mean ± SE from three experiments.

<sup>a</sup>P < 0.05, significant effect of COX-2 overexpression.

<sup>b</sup>P < 0.01, significant effect of COX-2 overexpression.

<sup>c</sup>P < 0.01, significant effect of antioxidants.
The present study, we found that overexpression of COX-2 increased drug–insensitive peroxidase activity of the COX-1 enzyme (47). COX catalysis is a free radical–mediated auto-oxidation process, and in osteoblasts, COX-2 overexpression decreased ROS and decreased apoptosis, and the concentration of individual ROS species (29). The ability of ROS, under certain conditions, to function as antitumorigenic agents and, under other conditions, to increase cell proliferation and survival and also to induce DNA damage leading to genetic lesions that initiate tumorigenicity (29) might explain some of the conflicting reports on effects of COX-2 overexpression. For example, overexpression of COX-2 inhibited cell growth in vitro but was sufficient to induce mammary gland tumorigenesis in vivo (41, 49). In addition, there have been contradictory results with forced overexpression of COX-2 in epithelial skin cells. In one case, COX-2 overexpression sensitized the skin for carcinogenesis (50), and in another case, it suppressed tumor development (51).

Some studies have reported inhibition of cell growth by forced overexpression of COX-2 in human breast epithelial cells and mesangial cells (41–43). One factor involved in the differing effects of COX-2 overexpression may be the cell or tissue type. Using the same retroviral protocol as for the osteosarcoma cells, we overexpressed COX-2 in the human colon carcinoma HCT-116 cell line, which is deficient in COX-2 expression (27). In contrast to its effects in osteosarcoma cells, COX-2 overexpression decreased apoptosis and did not decrease cell number in HCT-116 cells.

To examine the role of prostaglandins in the effects of COX-2 overexpression, we used 1 μmol/L of NS-398, a dose sufficient to inhibit PG production (44) but insufficient to inhibit the prostaglandin-independent effects of nonsteroidal anti-inflammatory drugs on cell growth and apoptosis (4, 45). Effects of COX-2 overexpression on Saos-2 cells were not reversed by treatment with NS-398 nor were they mimicked by added PGE2. Several other studies reporting inhibition of cell growth with COX-2 overexpression also found the effects to be independent of prostaglandins, as in our study (41, 42). One possible explanation is that the effects are secondary to the peroxidase activity of COX-2, which can operate independently of the COX activity even when the COX site is occupied by nonsteroidal anti-inflammatory drugs (46). COX catalysis is a free radical–mediated auto-oxidation process, during which numerous reactive species (either from the COX or peroxidase site) are generated (28). In a study of overexpression of COX-1, it was shown that nuclear factor-κB activation was regulated by ROS produced by the nonsteroidal anti-inflammatory drug–insensitive peroxidase activity of the COX-1 enzyme (47). In the present study, we found that overexpression of COX-2 increased apoptosis secondary to production of ROS, from the peroxidase site, in Saos-2 cells and inhibition of ROS production by antioxidants blocked apoptosis. On the other hand, overexpression of COX-2 in HCT-116 cells decreased ROS and decreased apoptosis, suggesting that increased ROS induces apoptosis in both cell types but that the role of COX-2 in generating ROS or in inducing endogenous antioxidant activity is cell/tissue type dependent.

Although our data suggest that overexpression of COX-2 in osteosarcoma cells decreases cell viability via increased ROS and may therefore inhibit tumor progression, increased ROS can also induce DNA damage and thus initiate neoplastic transformation (29, 48). The mechanisms by which cells respond to ROS depend on the molecular background of cell and tissues, the location of ROS production, the reducing activity of intracellular antioxidants, and the concentration of individual ROS species (29). The ability of ROS, under certain conditions, to function as antitumorigenic agents and, under other conditions, to increase cell proliferation and survival and also to induce DNA damage leading to genetic lesions that initiate tumorigenicity (29) might explain some of the conflicting reports on effects of COX-2 overexpression. For example, overexpression of COX-2 inhibited cell growth in vitro but was sufficient to induce mammary gland tumorigenesis in vivo (41, 49). In addition, there have been contradictory results with forced overexpression of COX-2 in epithelial skin cells. In one case, COX-2 overexpression sensitized the skin for carcinogenesis (50), and in another case, it suppressed tumor development (51).

Our in vitro studies suggest that neither prostaglandins nor ROS associated with the overexpression of COX-2 will promote tumorigenesis via cell-autonomous effects on proliferation and apoptosis in osteosarcoma cells. The in vitro effects on tumorigenesis of COX-2 overexpression in osteoblasts will depend on whether or not chronically elevated ROS will increase antioxidant activity, to the extent that cells become resistant to ROS, and on the ability of increased ROS to initiate neoplastic transformation in osteoblasts.

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