The Effects of Cyclooxygenase-2 Expression in Prostate Cancer Cells: Modulation of Response to Cytotoxic Agents

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ABSTRACT

Cyclooxygenase (COX)-2 has emerged as an exciting target for therapeutic intervention in the management of cancer. Immunohistochemistry studies have indicated higher expression of COX-2 in cancerous versus benign prostatic tissue. We have explored the role of COX-2 in prostate cancer in terms of attenuation of apoptosis and sensitivity to pharmacological agents, including COX-2 inhibitors. The human prostate cancer cell line LNCaP was stably transfected with COX-2 (LNCaPCOX-2) and compared with the empty vector control line (LNCaPneo). Chemosensitivity testing indicated no change in sensitivity to the cytotoxic effects of COX-2 inhibitors celecoxib or sulindac or VP16. However, LNCaPCOX-2 cells showed 3-fold resistance to carboplatin, which was partially reversed by coincubation with VP16. LNCaPCOX-2 cells expressed increased levels of survivin and Bcl-2 with enhanced activation of AKT. We also investigated the effects of celecoxib on expression levels of genes relevant to prostate cancer and drug resistance in our model system using quantitative polymerase chain reaction analysis. Celecoxib treatment resulted in highly significant increases in the mRNA expression of the smooth muscle component desmin, the detoxification enzyme glutathione S-transferase π (GSTπ), and nonsteroidal anti-inflammatory response gene (NAG-1) in the LNCaPCOX-2 cell line compared with LNCaPneo cells. Significant decreases in survivin levels and increases in GSTπ and NAG-1 appeared to be COX-2-dependent effects because they were more pronounced in LNCaPCOX-2 cells. Our findings indicate both COX-2-dependent and -independent mechanisms attributable to celecoxib and support its utility in the management of prostate cancer.
sus benign prostate tissues (Gupta et al., 2000; Yoshimura et al., 2000), and a more recent study by Wang et al. (2005) demonstrated incremental staining with higher Gleason scores. Prostate cancer is regarded as a slow growing malignancy that is the result of an imbalance between the proliferation rate and cell death. Indeed, overexpression of anti-apoptotic factors has been reported in human prostate cancer tissues (Tanji et al., 2000; Krajewska et al., 2003). In conjunction with these findings, reports in the literature have identified COX-2 inhibitors as inducers of apoptosis in prostate cancer cells with concomitant decreases in antiapoptotic factors such as Bcl-2 (Liu et al., 1998). Moreover, use of nonsteroidal anti-inflammatory drugs has been associated with a decrease in distant bony metastases with concomitant increases in survival in prostate cancer patients (Nguyen, 2004).

We have set out to clarify the role of COX-2 in human prostate cancer by transfecting the human prostate cancer line LNCaP with COX-2. The sensitivity of the transfected cells to various cytotoxic agents was compared with empty vector control LNCaPneo cells. The present study has addressed the role of COX-2 with respect to chemosensitivity to standard chemotherapeutic agents and has also attempted to elucidate the underlying mechanisms. Moreover, we have looked at the effects of the COX-2 inhibitor celecoxib in our COX-2-expressing prostate cancer model in terms of the mRNA levels of genes relevant to prostate carcinogenesis.

**Materials and Methods**

**Chemicals and Reagents.** All cell culture reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless stated otherwise. Caribopol, sulindac, and VP-16 (etoposide) were obtained from Sigma-Aldrich; celecoxib was obtained from Searle-Pharmacia (Skokie, IL; now Pfizer); wortmannin was obtained from Calbiochem (Merck Biosciences, Beeston, Nottingham, UK); antibodies for p53, Bcl-2, p27kip-1, and survivin were obtained from Autogen Bioclear (MerckBiosciences, Beeston, Nottingham, UK); antibodies for p53, and cDNA loading for COX-2 PCR was adjusted accordingly.

**Cell Culture.** Prostate cancer cell lines PC-3 and DU145 were obtained from the European Collection of Cell Cultures. LNCaP stable transfectants (Neo and COX-2) were developed as described below. Stock cells of LNCaP transfectants were treated every third passage with 0.35 mg/ml neomycin to select for cells that maintained the plasmid containing either the Neo or COX-2 plasmid. All prostate cancer cells were grown in RPMI 1640 medium, supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen, Paisley, UK) and 2 mM l-glutamine. Cells were grown in a humidified incubator with 5% CO₂ at 37°C. At each passage, cells were detached from the culture flask with porcine trypsin-EDTA solution.

**Gene Transfection.** Twenty-four hours after plating 2 × 10⁵ LNCaP cells on 6-cm dishes in Dulbecco’s modified Eagle’s medium with 5% charcoal-stripped fetal bovine serum-containing medium, cells were transfected with pBOSNeo or pBOSNeoCOX-2 vector (see Fig. 1) (Kinoshita et al., 1999) using a standard calcium phosphate method. After 48 h, the transfected cells were trypsinized from the plate and replaced onto a 6-cm culture dish in medium containing a final concentration of 0.8 mg/ml G418 (Geneticin; Invitrogen) to select stably transfected cells. Discrete colonies formed by 14 days after selection. Stable clones were continuously cultured in the presence of 0.8 mg/ml G418. Stock cells of LNCaP transfectants were treated every third passage with 0.35 mg/ml neomycin to select for cells that maintained the plasmid containing either the Neo or COX-2 plasmid.

**RT-PCR for COX-2 mRNA Expression.** Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The resulting RNA pellet was washed with 75% ethanol and dissolved in RNase-free water. Total RNA was converted to cDNA using ImProm-II reverse transcriptase kit from Promega (Southampton, UK) according to the manufacturer’s instructions. Samples were then subject to the PCR reaction in a Touchgene thermal cycler (Techne; supplied by Jencons PLS, East Grinstead, W. Sussex, UK). cDNA was amplified using Taq Polymerase (5 units/μl), buffer containing 500 mM KCl, 100 mM Tris-HCL, pH 9.0, 1% Triton X-100, 15 mM MgCl₂, 10 mM dNTPs, and 10 μM sense and antisense primers were obtained from Genosys Inc. (supplied by Sigma-Aldrich). To ensure equivalent loading of cDNA for PCR, total RNA was approximated by performing RT-PCR of the β₂-microglobulin housekeeping gene. The sense strand was 5’-ACCCACCT-GAAAGAATGA-3’, and the antisense strand was 5’-ATCT- TCAACCTCATTAG-3’. After an initial denaturation step of 94.0°C for 3 min, 35 cycles of PCR were performed: denaturing at 94.0°C for 45 s, annealing at 54.0°C for 45 s, and chain extension at 72.0°C for 90 s. This gave rise to a 120-bp product that was separated on a 1.2% agarose gel and visualized with ethidium bromide. The strength of the bands were used to approximate levels of total RNA, and cDNA loading for COX-2 PCR was adjusted accordingly.

**Chemosensitivity Testing.** Cell cytotoxicity assays were performed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, using the method described by Mosmann (1983). Drugs were all dissolved in dimethyl sulfoxide as stock solutions apart from carbo-platin, which was dissolved in sterile 0.9% saline as a stock solution and stored at −20°C. All cells were plated at 3 × 10⁴/ml in 96-well plates in a volume of 200 μl. Cells were allowed to grow and attach in the humidified incubator for 24 h at 37°C and 5% CO₂, unless stated otherwise. Typically, eight different concentrations of drugs

![Image description](https://example.com/image.png)
were used, with a 2- to 3-log-fold range. Cells were incubated with drug for 72 h (equivalent to three or four cell doublings). The assay was terminated when the cells were incubated with 0.05 mg/ml 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium for approximately 4 h at 37°C. The medium was removed, and the resulting formazan product was dissolved in 200 μl of dimethyl sulfoxide. The absorbance of each well was read at 540 nm using an automated plate reader (Labsystems Multiskan RC; Thermoelectron Corporation, Waltham, MA) with Genesis 3.05 software. A graph of cell viability against drug concentration was produced from the mean absorbance values by calculating the percentage growth of drug treated cells against control cells. From these graphical data, the IC₅₀ values were derived.

**Annexin V Analysis for Apoptotic Response.** LNCaPneo and LNCaPCOX-2 cells were seeded into tissue culture flasks to give approximately 30 to 40% confluence, allowed to attach for 2 to 3 h, and then treated with 100 μM carboplatin for 48 h. An annexin V-fluorescein isothiocyanate-conjugated apoptosis detection kit incorporating propidium iodide was used as described by the manufacturer’s protocol (CN Biosciences). Samples were analyzed by flow cytometry, using the FL1 (fluorescein isothiocyanate) and FL3 (propidium iodide) lines, and each reading was taken using 10,000 events.

**Western Blotting.** After seeding into tissue culture flasks, cells were left to attach for 24 h. Exponentially growing cells were treated with 100 μM carboplatin for 72 h alongside untreated control cells. Whole-cell lysates were then obtained by trypsinizing the monolayer of adherent cells, combining them with the floating cell population and washing with PBS at 4°C. Cell pellets were then subjected to osmotic rupture in hypotonic detergent-based buffer (1 mM phenylmethanesulfonyl fluoride, 1 mM NaVO₄, 2 mM aprotinin, and 2 μg/ml leupeptin) to act as a loading control.

**Drug Treatment of LNCaP Cells for Quantitative PCR Analysis.** LNCaPneo and LNCaPCOX-2 cells were seeded into tissue culture flasks at sufficient density to give 40 to 50% confluence. Cultures were incubated under standard culture conditions for 24 h to allow cells to attach. Each cell line was treated with either a low dose of celecoxib at 100 nM or a high dose at 30 μM, without untreated control flasks being set up at the same time. The dose of 100 nM was estimated to be in the range for a COX-2-dependent dose, whereas the dose of 30 μM was selected to represent a subcytotoxic dose that was deemed to represent a concentration at which COX-2-independent effects may be seen. This dose was approximately an IC₅₀ concentration (i.e., the dose of drug to reduce cell viability by 20% relative to control cells). Cells were incubated for 48 h, and then the RNA was extracted as for the PCR methodology. Experiments were set up on three separate occasions to provide three biological replicates.

**RNA Extraction.** RNA was extracted from LNCaPneo and LNCaPCOX-2 cells using the TRIazol reagent as described above for the RT-PCR analysis. The quality of the resulting RNA was checked with an Agilent 2100 Bioanalyzer using an RNA NanoLabchip according to the manufacturer’s instructions (Agilent Technologies UK Ltd., Stockport, Cheshire, UK). The RNA was diluted with RNase free water and assessed for quality (260/280 nm absorbance ratio, approximately 2.00) and quantity using the Nanodrop spectrophotometer (supplied by Labtech International, Ringmer, East Sussex, UK).

**Real-Time Quantitative PCR.** cDNA was made using the ImPromII reverse transcription system (Promega). Real-time quantitative PCR was performed using the Stratagene QPCR MX3005 thermal cycler (Stratagene Europe, Amsterdam, The Netherlands). Reactions were performed in a 20-μl volume with 5 pmol primers and 4 mM MgCl₂ using the Brilliant SYBR Green QPCR Master Mix reagent (Stratagene). For surviving, the protocol was 10 min at 95°C for activating the hot start Taq polymerase, then 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C for 45 cycles. For desmin, the protocol was 94°C for 10 min, then 45 cycles of 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C. For glutathione S-transferase α (GSTα), the protocol was 95°C for 10 min, then 40 cycles of 95°C for 20 s and 60°C for 60 s. For SRPT2da, the protocol was 95°C for 10 min, then 35 cycles of 95°C for 60 s, 62°C for 2 min, and 72°C for 3 min. All analyses were set up in duplicate and were also repeated on at least two occasions. Relative expression of genes was normalized to that of actin and gene expression in each sample calculated as 2ΔΔCt.

**Primers.** Primers were designed using Primer 3 software and supplied by Invitrogen. These were for survivin, forward, ACCAGGTGGAAAGGGGA, and reverse, AAGCATGAGGCCTGAGGGA; for desmin, forward, CCAAAAGAAGCCAGGAC, and reverse, TGTATGAGCCCTCGAAGAC; for GSTα, forward, CATCCTCCTCTCATCACAACTATGA, and reverse, GTTCTGCCCTCGTGTCG; for NAG-1, forward, CTCCAGATTTCGAGATGC, and reverse, AGAGATAGGCAGGTTGAGG; and for β-actin, forward, GCATCCAC-GAAACTACCTTC, and reverse, CAGGAGGCAACTGTACCTTG.

**Statistical Analysis.** For comparison of all data obtained for LNCaPneo and LNCaPCOX-2, we used the SPSS 12.0.1 program (SPSS Inc., Chicago, IL). Comparison of means was carried out using the one-way ANOVA or equality of means test, as appropriate. Differences were considered significant if a p value of 0.05 or less was obtained.

**Results**

**COX-2 Transfection Renders LNCaP Cells Resistant to Carboplatin.** Characterization of the cell lines showed that the transfection of LNCaP cells with COX2 resulted in COX2 expression at the mRNA and protein levels (Fig. 1). This assessment was carried out frequently during the program of work to ensure that the expression levels were consistent. Figure 2A clearly shows that there are negligible differences in the sensitivity of LNCaPCOX-2 and LNCaPneo to the cytotoxic agent VP-16 or to the COX-2 inhibitors celecoxib or sulindac. However, LNCaPCOX-2 cells were shown to be over 3-fold resistant to carboplatin. The respective IC₅₀ values were 38.1 ± 2.7 versus 123.4 ± 15.4 μM for the LNCaPneo and LNCaPCOX-2 cells (p = 0.03). The resistance could in part be abolished in LNCaPCOX-2 cells by treatment with the phosphatidylinositol 3-kinase inhibitor wortmannin (Fig. 2B). The IC₅₀ value for carboplatin LNCaPCOX-2 cells in the presence of wortmannin was 77.7 ± 10.5 μM (p = 0.016). However, wortmannin had no effect on the cytotoxicity of carboplatin in LNCaPneo cells. We saw a similar effect with the alkylating agent melphalan where LNCaPCOX-2 cells showed a similar level of resistance, which was also reduced in the presence of wortmannin (data not shown).

**COX-2 Transfection Suppresses the Apoptotic Response to Carboplatin.** To test the hypothesis that suppression of the cytotoxicity of carboplatin was in part due to suppression of apoptosis, we looked for appearance of membrane phosphatidylserine in the drug-treated LNCaP cells. The annexin V data showed a clear difference in the apoptotic response for LNCaPneo versus LNCaPCOX-2 cells, with a weaker response seen for latter (Fig. 3). In addition, we saw
with a concomitant transactivation of p27kip1 (Fig. 4A). These treatments clearly induced a p53 response in LNCaPneo cells and increased constitutive expression of the IAP survivin and Bcl-2 in LNCaPCOX-2 versus LNCaPneo cells (Fig. 3).

COX-2-Expressing Cancer Cells Show Diminished p53 Response and Increased Phospho-AKT. Carboplatin treatment clearly induced a p53 response in LNCaPneo cells with a concomitant transactivation of p27kip1 (Fig. 4A). These findings are consistent with LNCaP cells being wild type for p53. A much weaker response was shown for LNCaPCOX-2 cells under the same conditions. Likewise, there was a reduction in the activation of AKT, as shown by diminished p-AKT in response to carboplatin in LNCaPneo cells, which was absent for LNCaPCOX-2 cells (Fig. 4B).

COX-2-Expressing Cancer Cells Show Significant Changes in Gene Expression Levels after Treatment with Celecoxib. As seen in Fig. 5, celecoxib treatment gave rise to changes in mRNA levels of particular genes. Genes that were up-regulated and of particular relevance to prostate cancer were desmin and GSTpI. NAG-1 is a downstream target of p53, and levels of this gene were shown to be significantly increased in LNCaPCOX-2 cells upon treatment with celecoxib. A negligible effect was seen for the LNCaPneo cells. The gene that showed greatest down-regulation in LNCaPCOX-2 cells was the IAP survivin, which was reduced by celecoxib treatment in a dose-dependent manner to similar levels seen for LNCaPneo cell line.

Discussion

There has been recent and substantial evidence from the literature that supports the use of nonsteroidal anti-inflammatory drugs, of which COX-2 inhibitors form a large component, for the use of cancer chemoprevention mainly but not exclusively in colon cancer (Giovannucci and Willett, 1994). In addition, COX-2 inhibitors have been considered as part of combination chemotherapy for the treatment of a variety of cancers (Dawson et al., 2003; Jagle et al., 2007).

In contrast to other studies (Soriano et al., 1999; Hashitani et al., 2003; Lin et al., 2005), the LNCaPCOX-2 model system failed to show increased sensitivity to celecoxib compared with the control LNCaPneo cells. Moreover, we failed to see a sensitization of the LNCaPCOX-2 cells to agents such as carboplatin if using a carboplatin/celecoxib combination (with isobologram analysis; data not shown). The reasons behind this are unclear but may relate to the cytostatic as well as cytotoxic nature of carboplatin. In addition, despite increased COX-2 expression in our model, this did not render cells more sensitive to the cytotoxic effects of celecoxib. This is in contrast to other studies that clearly showed a chemosensitizing effect in COX-2-expressing cells treated with a cytotoxic agent combined with a COX-2 inhibitor (Hashitani et al., 2003; Lin et al., 2005). Moreover, in contrast to our study, the latter studies were also able to demonstrate increased chemosensitivity of their COX-2-expressing cells to COX-2 inhibitors.

In non-COX-2-expressing cells, celecoxib has been shown to be cytotoxic (Patel et al., 2005) because LNCaP and PC-3 cells devoid of COX-2 expression treated with celecoxib (2.5–5.0 μM) showed a cell cycle blockade at G1. In the same study, it was shown that the PC-3 xenograft models (devoid of COX-2 expression) were reduced in volume after treatment with celecoxib at a dose corresponding to plasma concentrations in the range of 2 to 5 μM. Hence, the idea emerged that celecoxib possesses properties that are independent of its activity as a COX-2 inhibitors. Such COX-2-independent effects have been shown to include antiangiogenic activity and cell cycle arrest (G1 blockade) where levels of prostaglandin E2 were shown to be unaffected in xenograft models treated with celecoxib in the absence of COX-2 expression (Patel et al., 2005). In agreement with that particular study, we were able to demonstrate G1 cycle arrest in both LNCaPCOX-2 and -neo cells treated with celecoxib at 5 μM, and the blockade was of similar magnitude for both cell lines (data not shown).

The resistance of LNCaPCOX-2 cells to carboplatin coincided with a reduction in apoptosis. Moreover, our studies have identified the involvement of Akt- and COX-2-dependent signaling in the resistance of the LNCaPCOX-2 cells. The phosphatidylinositol-3-kinase/Akt axis is a pleiotropic apoptosis-suppressing pathway that is particularly involved in trophic factor deficiency-induced apoptosis through the differential regulation of pro- and antiapoptotic gene expression via their downstream targets glycogen synthase kinase-3 (Beurel and Jope, 2006) and FoxO (van der Horst and Burgers, 2007). We plan to investigate further the phosphatidylinositol-3-kinase/AKT axis and downstream effectors in our COX-2-expressing LNCaP model.

Although there is evidence in the literature for COX-2 inhibition giving rise to an enhanced TRAIL-induced apoptosis (Yamanaka et al., 2006), we were unable to see such an effect in the model used in the present study (data not shown). In support of our observations, Chen et al. (2001) showed that elevated AKT activity protected LNCaP cells...
from TRAIL-induced apoptosis. In our study, LNCaPCOX-2 showed increased constitutive active AKT expression versus LNCaPneo cells; however, both cell lines were resistant to TRAIL-induced apoptosis.

Another regulator of apoptosis that may participate in cancer is survivin. The expression of this member of the IAP family is well known to be controlled by Akt (Papapetropoulos et al., 2000). Survivin is expressed in most human tumors, whereas in normal differentiated tissues, it is more or less undetectable. Krajewska et al. (2003) showed that elevated IAPs were a common feature of prostate cancers including prostatic intraepithelial neoplasia, but they showed no relationship with Gleason-grade or prostatic-specific antigen levels. A report by Krysan et al. (2004a) showed that survivin levels correlated positively with COX-2 expression levels in nonsmall cell lung cancer cells, both in vitro and in vivo. The underlying mechanism was shown to be COX-2-modulated survivin ubiquitination and stabilization. A further study by the same group (Krysan et al., 2004b) showed that inhibition of survivin expression by small interfering RNA enhanced apoptosis in nonsmall cell lung cancer cells in the presence of COX-2. We now show evidence to support those findings in our prostate cancer model. The levels of survivin both at the mRNA and protein levels were shown to be significantly increased in the presence of COX-2. Moreover, we show for the first time that treatment with the selective COX-2 inhib-
Desmin is a smooth muscle differentiation marker along with laminin and vimentin, whose transcriptional regulation is controlled by Akt (Jiang et al., 1999). Study of these markers is highly relevant to the problem of prostate cancer because they are reported to be lost or significantly decreased in hormone-induced prostate cancer (Wong and Tam, 2002). In dysplastic sites, there were clear areas of negligible or negative desmin immunostaining, particularly in poorly differentiated tumor regions. In the same study, normal control prostate lobes showed strong levels of desmin expression (Wong and Tam, 2002). Our observation of increased desmin expression in COX-2-expressing LNCaP cells, and its reversal after celecoxib treatment (at nontoxic concentrations), could point to a reversal of the dedifferentiated phenotype seen for prostate cancer.

In the precancerous setting, chronic inflammation of the prostate is considered a risk factor for prostate cancer (Lehrer et al., 2005). Sun et al. (2001) demonstrated that exogenous prostate-specific antigen itself is able to induce reactive oxygen species (ROS) in PC-3 and DU145 cell lines. Furthermore, a prooxidant state as a result of imbalance in the favor of ROS versus antioxidant processes has been reported for both the precancerous pathology described as high-grade prostatic intraepithelial neoplasia and prostate cancer (Nelson et al., 2002). These findings provide a rationale for using COX-2 inhibitors in the chemoprevention of prostate cancer. The GST group of enzymes forms a significant component of cellular antioxidant defense mechanisms. In the case of prostate cancer, it is the GSTpi form that has particular relevance. Human prostate cancer is characterized by an early and profoundly reduced level of GSTpi. The build-up of ROS due to inflammatory processes associated with early stages of prostate carcinogenesis may be protected by the activity of GSTpi. Immunohistochemical studies have shown that areas of proliferative inflammatory atrophy, associated with chronic inflammatory prostatic disease, also show increased levels of Bcl-2, COX-2, and GSTpi as well as decreased apoptosis (Zha et al., 2001; Nelson et al., 2002). Conversely a decrease, or even absence of GSTpi due to promoter methylation, is often seen in prostate cancer (Crochitto et al., 2004). In the present study, we saw extremely low levels of GSTpi mRNA in the LNCaPCOX-2 model versus LNCaPneo cells. After treatment with celecoxib, the increase in GSTpi levels in COX-2-expressing cells was highly significant. These effects point to a COX-2-dependent effect as the increments seen for LNCaPneo under similar conditions were virtually negligible. The reduction in GST in carboplatin-resistant cells is counterintuitive because it has been established for some time that increases in components of the glutathione pathway in anticancer drugs resistance (for review, see Townsend and Tew, 2003). However, one should consider anticancer drug resistance as a potentially multifactorial phenomenon. In the model, we describe that it is clear that there is a shift in the balance between apoptotic and antiapoptotic factors that seems pivotal in mediating the response to cytotoxic insult.

In conclusion, we show data that indicates a role for celecoxib as both an inducer of apoptosis and an agent that can correct inappropriate gene expression levels associated with malignant progression in human prostate cancer. We provide evidence to support the use of celecoxib in the management of
prostate cancer in both the adjuvant and chemoprevention settings.

References


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