THE EFFECTS OF COX-2 EXPRESSION IN PROSTATE CANCER CELLS:
MODULATION OF RESPONSE TO CYTOTOXIC AGENTS

By

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Running Title: Effects of COX-2 on cytotoxic response in prostate cancer

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ABBREVIATIONS
DMEM; Dulbecco’s Minimum Essential Medium; EAC: European Collection of cell cultures; FITC: fluorescein isothiocyanate; GSTpi: glutathione S-transferase π (pi);
IAP: Inhibitor of apoptosis protein; NAG-1; Nonsteroidal Anti-inflammatory Drug-Activated Gene; NSAIDS; non-steroidal anti-inflammatory drugs; NSCLC; non-small cell lung cancer; PI3-K: phosphatidylinositol 3-kinase; PIA: Proliferative inflammatory atrophy; PIN: prostatic intraepithelial neoplasia; PMSF;
phenylmethanesulfonyl fluoride; PSA: prostatic specific antigen; ROS: reactive oxygen species; RT-PCR : reverse transcription PCR; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand
ABSTRACT
Cyclooxygenase-2 (COX-2) has emerged as an exciting target for therapeutic intervention in the management of cancer. Immuno-histochemistry 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 co-incubation with the PI-3 kinase inhibitor wortmanin. Concomitant with reduced apoptotic response to cytotoxic agents, 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 PCR analysis. Celecoxib treatment resulted in highly significant increases in the mRNA expression of the smooth muscle component desmin, the detoxification enzyme GSTpi and Nonsteroidal-Anti-inflammatory response gene NAG-1 in the LNCaPCOX-2 cell line when compared with LNCaPneo cells. Significant decreases in survivin levels and increases in GST-pi and NAG-1 appeared to be COX-2-dependent effects as 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.
INTRODUCTION

In comparison with other cancers advanced prostate cancer is refractory to a large proportion of currently available chemotherapies. There is, therefore, a need to understand the underlying mechanisms that are responsible for the chemorefractory nature of this disease and to find better treatments and/or ways of circumventing this drug resistance.

There are currently a number of lines of evidence suggesting that COX-2 (cyclooxygenase-2) is a promising new target for cancer therapy – both at the level of chemoprevention and also to treat cancer. COX-2 has been reported to be elevated in a number of types of cancers, such as prostate (Yoshimura et al. 2000), breast (Davies et al. 2002), colorectal (Soslow et al. 2000), pancreatic (Tucker et al. 1999) and liver (Koga et al. 1999). Indeed, the production of prostaglandins due to the activity of COX-2 has been implicated in carcinogenic processes where chronic inflammation is indicated. Furthermore, epidemiological evidence indicating a significantly reduced incidence of colorectal adenomas in subjects who have taken NSAIDS over a prolonged period of time provides a rationale for using COX-2 inhibitors to treat cancer (Schreinemachers and Everson 1994). Recent evidence has shown that COX-2 overexpression may increase tumorigenic potential via prevention of cellular apoptosis (Tsujii and DuBois 1999). Moreover, the deletion of COX-2 in Apc knockout mice – which is representative of familial adenomatous polyposis – was consistent with a reduction in the number and size of colorectal adenomas and a restoration of apoptosis (Oshima et al. 1996).
A number of immuno-histochemical based studies have demonstrated higher expression of COX-2 in cancerous versus benign prostate tissues (Yoshimura et al. 2000; Gupta 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 which is the result of an imbalance between the proliferation rate and cell death. Indeed, over-expression 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 anti-apoptotic factors such as Bcl2 (Liu et al. 1998). Moreover, use of NSAIDs 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 to 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. Carboplatin, sulindac and VP-16 (etoposide) were obtained from Sigma Aldrich; celecoxib was obtained from Searle-Pharmacia, Skokie, Illinois, US (now Pfizer); Wortmanin was obtained from Calbiochem (Merck Biosciences, Beeston, Nottingham, UK); antibodies for p53, bcl-2, p27kip-1 and survivin were obtained from Autogen Bioclear (Calne, Wiltshire, UK); antibodies to AKT and phospho-AKT were obtained from Biosource International (Nivelles, Belgium); antibody to β-actin from Oncogene Research (distributed by CN Biosciences, Beeston, Nottingham, UK). All secondary antibodies conjugated to HRP were obtained from Sigma Aldrich.

Cell culture

Prostate cancer cell lines PC-3 and DU145 were obtained from the European Collection of Cell Cultures (ECACC). 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 which 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% foetal bovine serum (FBS; obtained from 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

24h after plating 2 x 10^5 LNCaP cells on 6cm dishes in DMEM with 5% charcoal stripped FBS-containing medium, cells were transfected with pBOSNeo or pBOSNeoCOX-2 vector, see Figure 1 (Kinoshita et al. 1999) using a standard calcium phosphate method. After 48h the transfected cells were trypsinated from the plate and replaced onto a 6cm culture dish in medium containing final concentration 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 which 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, Paisley, Scotland, UK) according to 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 Corporation (Southampton, UK) according to manufacturer’s instructions. Samples were then subject to the PCR reaction in a Touchgene thermal cycler (Technne; 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 and 15 mM MgCl₂ 10 mM dNTPs, 10 μM sense and anti-sense primers were obtained from Genosys Inc. (Sigma Aldrich, Poole, Dorset, UK). 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’-ACCCCACTGAAAAAGATGA-3’; anti-sense sense strand was 5’-ATCTTCAACCTCCATGATG-3’.

After an initial denaturation step of 94.0°C for 3 minutes, 35 cycles of PCR were performed: denature at 94.0°C for 45 seconds, anneal at 54.0°C for 45 seconds and chain extension at 72.0°C for 90 seconds. This gave rise to a 120 bp product which was separated on a 1.2% agarose gel and visualised 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 MTT (3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide) assay, using the method described by Mosmann (1983). Drugs were all dissolved in dimethyl sulphoxide (DMSO) as stock solutions apart from carboplatin which was dissolved in sterile 0.9% saline as a stock solution and stored at -20°C. All cells were plated at 3x10^4/ml in 96 well plates in a volume of 200 µL. Cells were allowed to grow and attach in the humidified incubator for 24 hours at 37°C and 5% CO₂, unless stated otherwise. Typically, eight different concentrations of drugs were used, with a 2-3 log fold range. Cells were incubated with drug for 72 hours (equivalent to 3 or 4 cell doublings). The assay was terminated when the cells were incubated with 0.05 mg/ml MTT for approximately 4 hours at 37°C. The medium was removed and the resulting formazan product was dissolved in 200µL DMSO. The absorbance of each well was read at 540 nm using an automated plate reader (Labsystems Multiskan RC) 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 - 40% confluence, allowed to attach for 2-3 hours and then treated with 100µM carboplatin for 48h. An Annexin V-FITC conjugated apoptosis detection kit incorporating propidium iodide (PI) was used as described by the manufacturer’s protocol (supplied by CN Biosciences, Beeston, UK). Samples were analysed by flow cytometry, using the FL1 (FITC) and FL3 (PI) lines and each reading was taken using 10,000 events.

Western blotting

Following seeding into tissue culture flasks cells were left to attach for 24h. Exponentially growing cells were treated with 100µM carboplatin for 72h 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 (1mM PMSF, 1mM NaVO₄, 2µg/ml aprotinin and 2µg/ml leupeptin as protease inhibitors, 150mM NaCl, in 50mM Tris buffer, 0.2% SDS, 1% NP-40, pH 7.5) and 50µg of protein/sample electrophoresed on SDS-PAGE gels with subsequent transfer blotting. Membranes were incubated overnight at 4°C with primary antibody i.e. to survivin, Bcl2, p53, p27kip2 AKT or phospho-AKT (ser 473). After washing, membranes were incubated with a secondary horseradish peroxidase (HRP) -linked appropriate species antibody preparation at room temperature for 1h with chemiluminescence used for visualization. Following the probing of each membrane with the primary antibody of choice, the membrane was
stripped and reprobed using an actin antibody (Autogen Bioclear, Calne, UK) to act as a loading control.

Drug treatment of LNCaP Cells for quantitative-PCR analysis

LNCaP-neo and LNCaP-COX-2 cells were seeded into tissue culture flasks at sufficient density to give 40-50% confluence. Cultures were incubated under standard culture conditions for 24h to allow cells to attach. Each cell line was treated with either a low dose of celecoxib at 100nM or a high dose at 30µM, with untreated control flasks being set up at the same time. The dose of 100nM was estimated to be in the range for a COX-2 dependent dose, whereas the dose of 30µM was selected to represent a sub-cytotoxic dose that was deemed to represent a concentration at which COX-2 independent effects may be seen. This dose was approximately an IC$_{20}$ concentration (i.e. the dose of drug to reduce cell viability by 20% relative to control cells). Cells were incubated for 48h and then the RNA extracted as for the PCR methodology. Experiments were set up on three separate occasions to provide 3 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 Bioanalyser 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 (260:280nm absorbance ratio approx. 2.00) and quantity using the

**Real-Time Quantitative PCR**

cDNA was made using the ImPromII reverse transcription system (Promega, Southampton, UK). Real-time quantitative PCR (Q-PCR) was performed using the Stratagene QPCRMX3005P thermal cycler (Stratagene Europe, Amsterdam, the Netherlands). Reactions were performed in a 20µl volume with 5 pmol primers and 4mM MgCl₂ using the Brilliant SYBR Green QPCR Master Mix reagent (Stratagene). For survivin the protocol was 10min 95°C for activating the hot start Taq polymerase, then 20s at 95°C, 20s at 60°C, 20s at 72°C for 45 cycles. For desmin the protocol was 94°C for 10min, then 45 cycles of 30s at 94°C, 60s at 60°C, 60s at 72°C. For GST-pi the protocol was 95°C 10min then 40 cycles of 95°C for 20s and 60°C for 60s. For SRP72kDa the protocol was 95°C 10min, then 35 cycles of 95°C for 60s, 62°C for 2min, 72°C for 3min. All analyses were set up in duplicate and were also repeated on at least 2 occasions. Relative expression of genes was normalized to that of actin and gene expression in each sample calculated as $2^{\Delta\Delta C_t}$.

**Primers**

Primer sequences were designed using Primer 3 software and supplied by Invitrogen (Paisley, UK). These were for survivin: forward: ACCAGGTGAGAAGTGAGGGA; reverse: AACAGTAGAGGAGCCAGGGA; for desmin: forward: CCAACAAGAACAACGACG; reverse: TGGTATGGACCTCAGAACC; for GST-pi: forward: CATCTCCCTCATCTACACCAACTATG; reverse:
GTCTTGCTCCCTGCTTCTG; for NAG-1: forward:
CTCCAGATTCCGAGAGTTGC; reverse: AGAGATACGCAGGTGCAGGT

For β actin forward: GCATCCACGAAACTACCTTC; reverse:
CAGGAGGAGCAATGATCTTG.

Statistical Analysis

For comparison of all data obtained for LNCaPneo with LNCaPCOX-2 we used the
SPSS 12.0.1 program. 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.

Characterisation 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 LNCaP-COX-2 and LNCaP-neo to the cytotoxic agent VP-16 or to the COX-2 inhibitors celecoxib or sulindac. However, LNCaP-COX-2 cells were shown to be over three-fold resistant to carboplatin. The respective IC_{50} values were 38.1 ± 2.7 µM versus 123.4 ± 15.4 µM for the LNCaP-neo and LNCaP-COX-2 cells (p = 0.03). The resistance could in part be abolished in LNCaP-COX-2 cells by treatment with the PI3-kinase inhibitor wortmannin (Figure 2B). The IC_{50} value for carboplatin LNCaP-COX-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 LNCaP-neo cells. We saw a similar effect with the alkylating agent melphalan where LNCaP-COX-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

In order 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 phosphatidyl serine in the drug-treated LNCaP cells. The annexin V data showed a clear difference in the apoptotic response for LNCaP-neo versus LNCaP-COX-2 cells, with a weaker response seen for latter (Fig. 3). In addition, we saw increased
constitutive expression of the IAP survivin and Bcl2 in LNCaP COX-2 versus LNCaPneo cells (Figure 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 p27
\(^{kipl}\) Figure 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, Figure 4B.

**COX-2 expressing cancer cells show significant changes in gene expression levels following treatment with celecoxib**

As seen in Figure 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 glutathione-S-transferase-pi. 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 non-steroidal 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 et al. 1994). In addition, COX-2 inhibitors have been considered as part of combination chemotherapy for the treatment of a variety of cancers (Javle et al. 2007; Dawson et al. 2007).

In contrast to other studies (Lin et al. 2005; Soriano et al. 1999; Hashitani et al. 2003), 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, in spite of 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 which 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. 2003). 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) as LNCaP and PC-3 cells devoid of COX-2 expression treated with celecoxib (2.5-5.0µM) 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 following treatment with celecoxib at a dose corresponding to plasma concentrations in the range of 2-5µM. 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 anti-angiogenic 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 5µM 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 signalling in the resistance of the LNCaPCOX-2 cells. The PI3K/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 anti-apoptotic gene expression via their downstream targets glycogen synthase kinase-3 (Beurel and Jope 2006) and FoxO (van der Horst and Burgering 2007). We plan to investigate further the PI3K/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 tumours 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 PIN, but they showed no relationship with Gleason grade or PSA levels. A report by Krysan et al. (2004A) showed that survivin levels correlated positively with COX-2 expression levels in non-small lung cancer (NSCLC) cells, both in vitro and in vivo. The underlying mechanism was shown to be COX-2 modulated survivin ubiquitination and stabilisation. A further study by the same group (Krysan et al. 2004B) showed that inhibition of survivin expression by siRNA enhanced apoptosis in NSCLC 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 level 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 inhibitor celecoxib can give rise to significantly reduced survivin levels, as demonstrated by qPCR analysis. In addition to these findings, we have found that treatment of newly diagnosed prostate cancer with celecoxib in the neo-adjuvant (pre-operative) setting was associated with reduced survivin mRNA expression (Sooriakumaran et al. manuscript in preparation).
NAG1 is a downstream target of p53 and has been shown to mediate induction of apoptosis in human colorectal cancer cells (Okazaki et al. 2006). As its name suggests, study of this gene is highly relevant to the setting of celecoxib treatment in prostate cancer. In spite of the wild type p53 status LNCaP cells it appears that NAG1 was only induced in COX-2 expressing cells in the present study. We decided to examine the NAG-1 gene as work in our laboratory has implicated dysregulation of this gene as a consequence of resistance to platinum anticancer agents in head and neck cancer and also in ovarian cancer cell line models (unpublished data).

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 as 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 immuno-staining, particularly in poorly differentiated tumour 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 following celecoxib treatment (at non-toxic concentrations) could point to a reversal of the dedifferentiated phenotype seen for prostate cancer.

In the pre-cancerous 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 PSA itself is able to induce reactive oxygen species (ROS) in PC-3 and DU145 cell lines. Furthermore, a pro-oxidant state as a result of imbalance in the
favour of ROS versus anti-oxidant processes has been reported for both the pre-
cancerous pathology described as high-grade PIN (HGPIN) and prostate cancer
(Nelson et al. 2003). These findings provide a rationale for using COX-2 inhibitors in
the chemoprevention of prostate cancer. The glutathione-S-transferase (GST) group of
enzymes form a significant component of cellular anti-oxidant defence mechanisms.
In the case of prostate cancer, it is GST-pi (π) form that has particular relevance.
Human prostate cancer is characterized by an early and profoundly reduced level of
GST-pi. The build up of ROS due to inflammatory processes associated with early
stages of prostate carcinogenesis may be protected by the activity of GST-pi. 2004).
Immunohistochemical studies have shown that areas of proliferative inflammatory
atrophy (PIA), associated with chronic inflammatory prostatic disease, also show
increased levels of Bcl2, COX-2 and GST-pi as well as decreased apoptosis (Zha et
al. 2001; Nelson et al. 2003). Conversely a decrease, or even absence of GST-pi due
to promoter methylation, is often seen in prostate cancer (Crocitto et al. 2004). In the
present study we saw extremely low levels of GST-pi mRNA in the LNCaPCOX-2
model versus LNCaPneo cells. Following treatment with celecoxib, the increase in
GST-pi 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 counter-intuitive as it has been established for some time that *increases* in
components of the glutathione pathway in anticancer drugs resistance (reviewed by
Townsend and Tew 2003). However, one should consider anticancer drug resistance
as a potentially multifactorial phenomenon. In the model we describe it is clear that
there is a shift in the balance between apoptotic and anti-apoptotic factors which
appears 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


FOOTNOTES:

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LEGENDS FOR FIGURES

FIGURE 1
Left hand side: Construction of plasmid for stable transfection of LNCaP cells with COX-2. Right hand side: A: Agarose gel electrophoresis of PCR products to show increased mRNA in COX-2 transfected cells, with equal loading of both LNCaPneo and LNCaPCOX-2 cells as shown by levels of the housekeeping gene β-2 microglobulin. B: Western blotting to show expression of COX-2 protein in LNCaPCOX-2 cells versus LNCaPneo cells.

FIGURE 2
A LNCaPneo (solid black), LNCaPCOX-2 (shaded); Sensitivity of prostate cancer cell lines to therapeutic agents. Data shown are representative of the mean of >3 repeat experiments, with the standard deviation being shown by error bars. B LNCaPneo (square pattern), LNCaPCOX-2 (diagonal line). Sensitivity of prostate cancer cell lines to carboplatin in the absence or presence of wortmannin. * indicates a statistical difference at a level of p = 0.03 ; ! p = 0.016.

FIGURE 3
Left hand side: Annexin V data obtained for LNCaP cell lines subjected to 100µM carboplatin for 48h. Data shown are typical of repeat experiments performed. Right hand side: western immuno-blotting of untreated LNCaP cell lines for anti-apoptotic factors.
FIGURE 4
A: Western immunoblotting for the detection of p53 and p27kip-1 following treatment with carboplatin for 48h.
B: 1= LNCaPneo control untreated; 2= LNCaPneo 100µM carboplatin 48h; 3= LNCaPCOX-2 control untreated; 4= LNCaPCOX-2 100µM carboplatin 48h.

FIGURE 5
Quantitative PCR data obtained for LNCaPneo and LNCaPCOX-2. Control untreated cells solid black bar; low dose celecoxib horizontal bars; high dose celecoxib diagonal bars. All assays were carried out at least 5 times and levels of statistical significance calculated using SPSS with p values of 0.05 and below being considered significant.
FIGURE 1
FIGURE 3
**FIGURE 4**

(A) Western blots of LNCaPneo and LNCaP COX-2 cell lines under untreated control and carboplatin 100μM conditions. The blots show the expression levels of p53, P27kip-1, and actin proteins.

(B) Western blots showing the phosphorylation status of AKT (P-AKT) and total AKT (AKT) in different samples (1-4). The blots indicate the phosphorylation levels under various conditions.
FIGURE 5