A Gene Expression Profiling Approach Assessing Celecoxib in a Randomized Controlled Trial in Prostate Cancer

P. SOORIAKUMARAN1,2, P. MACANAS-PIRARD3, G. BUCCA1, A. HENDERSON1,2, S.E.M. LANGLEY1,2, R.W. LAING2, C.P. SMITH1, E.E. LAING1 and H.M. COLEY1

1Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey GU2 7XH; 2St. Luke’s Cancer Centre, Royal Surrey County Hospital, Guildford, Surrey GU2 7XX, U.K.

Abstract. Background: We performed a pilot study, looking at the COX-2 inhibitor celecoxib, on newly diagnosed prostate cancer patients in the neo-adjuvant setting using DNA microarray analysis. Patients and Methods: This was a single-blinded, randomized controlled phase II presurgical (radical prostatectomy) 28-day trial of celecoxib versus no drug in patients with localized T1-2 N0 M0 prostate cancer. cDNA microarray analysis was carried out on prostate cancer biopsies taken from freshly obtained radical prostatectomy samples. Results were confirmed by qPCR analysis of a selection of genes. Results: Multiple genes were differentially expressed in response to celecoxib treatment. Statistical analysis of microarray data indicated 24 genes were up-regulated and 4 genes down-regulated as a consequence of celecoxib treatment. Gene changes e.g. survivin, SRP72kDa, were associated with promoting apoptotic cell death, enhancement of antioxidant processes and tumour suppressor function (p73 and cyclin B1 up-regulation). Conclusion: Celecoxib at 400 mg b.i.d. for 4 weeks perioperatively gave rise to changes in gene expression in prostate cancer tissue consistent with enhancement of apoptosis and tumour suppressor function. Given the short time interval for the duration of this study, the data are encouraging and provide a good rationale for conducting further trials of celecoxib in prostate cancer.

Prostate cancer is the commonest non-dermatological cancer in men in Western countries (1) and novel strategies of management are badly needed. Cyclooxygenase-2 (COX-2) is intimately involved in the development and progression of a variety of malignancies, including prostate cancer. COX-2 has been shown to interact with angiogenic, apoptotic, proliferative, invasive, metastatic and other pathways involved in cancer evolution (2). Chronic inflammation and oxidative stress are key players in COX-2-dependent carcinogenesis. COX-2 has been shown to be an independent predictor of prostate cancer progression following radical prostatectomy (3). Moreover, the outcome of radiotherapy in prostate cancer patients has been shown to be associated with COX-2 expression, whereby high levels were indicative of treatment failure (4). Celecoxib is a selective COX-2 inhibitor (coxib) that appears to have an anticancer effect in numerous in vivo and in vitro models. Coxibs, most notably celecoxib, also exert their anti-cancer effects via COX-2-independent mechanisms including interference with Akt (signal transduction), NF-κB (inflammatory mediator of tumorigenesis) and other mediators of cancer development and progression. The end result of both COX-2-dependent and COX-2-independent actions is an inhibition of cancer at multiple stages (2). Presently, the superior anticancer and cardiovascular safety profiles of celecoxib make it the coxib of choice for clinical trials.

The purpose of this study was to investigate the effects of celecoxib in early prostate cancer. Patients were randomized to 400 mg celecoxib twice daily (b.i.d.) versus no drug for 4 weeks prior to radical prostatectomy (RP). We initially assessed the effects of the treatment on markers of proliferation, apoptosis and angiogenesis using paraffin-embedded tissue taken from the RP specimens from the patients in this clinical trial (5). We then went on to assess the effects of celecoxib on gene expression profiling of prostate cancer by subjecting samples from drug-treated and control patients to DNA microarray analysis. Our overall aim was, therefore, to conduct a pilot study investigating the effects of celecoxib on the gene expression profiling (GEP) of tumour biopsies taken from patients with localized prostate cancer.

Patients and Methods

Patient selection. The study was a single-blinded, randomized, controlled phase II pre-surgical trial of celecoxib versus no drug for 28 days prior to radical prostatectomy (RP) for patients with localized (T1-2 N0 M0) prostate cancer. We used a 2:1 block
randomization, hence the difference in numbers of controls and treated patients. The power calculation was based on this and the use of a 2:1 randomization schedule meant we had greater numbers of treated patients and thus needed fewer patients in the study to reach the desired power. Ethical approval was obtained prior to starting the study and all patients recruited to the trial gave their informed, signed consent. Patients who had decided to undergo RP as their management option were eligible. Patients taking NSAIDs or other coxibs were excluded, as were those with a significant past history of these medications. Patients on hormonal manipulation and 5-ARIs (e.g. finasteride) were also excluded, because of the uncertain effects these drugs have on prostate tumour biology, especially given the recent evidence from the Prostate Cancer Prevention Trial (6). Patients with cardiovascular risk factors were also excluded. Baseline clinical data are indicated in Table 1. Although there is no evidence that COX-2 inhibitors affect cardiovascular risk when taken for periods of less than one year, and certainly not for a period of 28 days, patients with cardiovascular risk factors were also excluded. No patient in our trial suffered an adverse cardiac event. All patients had Gleason score 6 (3+3) or 7 (3+4) disease and clinically localized prostate cancer (T1c-T2c N0 M0); the celecoxib and control groups were therefore matched in terms of histological grade and stage. There was also no significant difference in PSA values based the two arms. These baseline clinical data are indicated in Table 1.

Sample collection. Prostatic tissue was collected at the time of surgery for RP (38 laparoscopic, 7 open procedure for the entire sample series). The prostate was removed from the body prior to the vesico-urethral anastomosis being performed then sectioned vertically and needle/punch biopsies were taken of the peripheral zone of the prostate in six areas: left apex, right apex, left midzone, right midzone, right base, and left base. Biopsies were then rapidly sectioned transversely into three roughly equal parts. The middle portion was placed in RNAlater® and sent for subsequent DNA microarray analysis. The mean time from prostatectomy to RNAlater® (Qiagen Ltd., Crawley, UK) submersion for sample preservation was ≤15 minutes. Only those biopsy sections that were histologically confirmed as cancer were used for cDNA microarray analysis.

GEP using cDNA microarray analysis of prostate cancer biopsies. Freshly collected core biopsies were rapidly frozen down to −80°C for short-term storage prior to extraction. The frozen biopsies in RNAlater™ solution were thawed on ice and preservation fluid aspirated. RNA was isolated using the RNAeasy method (mini-column procedure; Qiagen Ltd.) with sample lysis using a TissueLyser (Qiagen Ltd.). 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 quantified using the Nanodrop spectrophotometer (Nanodrop Technologies Ltd). Pre-hybridization, hybridization and washing of the microarrays were carried out according to manufacturer’s instructions (Pronto Microarray hybridization kit; Corning). Cy3 and Cy5 fluorescent signals were detected with an Affymetrix 428 laser scanner and the raw Tiff images were analysed for spot signal quantification and quality evaluation with Blue Fuse software V3.1 (BlueGnome, Cambridge, UK). The generated data files were then exported into R (version 2.5.0; http://www.R-project.org), normalised (Block-median followed by Across Array Product analysis (9, 10), a technique chosen for its robustness against biological variance such as that caused by tumour and/or patient experimental designs (9). Genes with a ‘ppp’ (probability of false prediction) value (9, 10) less than 0.15 were selected as being significantly up- or down-regulated.

Real-time quantitative PCR. cDNA was made using the ImPromII reverse transcription system (Promega, Southampton, UK). Real-time quantitative PCR was performed using the Stratagene QPCRMRX3005P thermal cycler (Stratagene Europe). Reactions were performed in a 20 μl volume with 5 pmol primers and 4 mM MgCl2 using Brilliant SYBR Green QPCR Master Mix reagent (Stratagene). For survivin, the protocol was 10 min 95°C for activating the hot start Taq polymerase, then 20 s at 95°C, 20 s at 60°C.

Table I. Baseline clinical comparison of control versus celecoxib-treated patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group N=13</th>
<th>Celecoxib-treated group N=27</th>
<th>Median difference (95% CI and interquartile range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>8.06 (3.47)</td>
<td>8.25 (4.62)</td>
<td>0.197 (−2.30 to 2.69)</td>
</tr>
<tr>
<td>5 μg total RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
<td>2.07 (0.26)</td>
<td>2.10 (0.31)</td>
<td>0.03 (−0.14 to 0.21)</td>
</tr>
<tr>
<td>Gleason sum</td>
<td>6.53 (0.74)</td>
<td>6.33 (0.71)</td>
<td>−0.20 (−0.66 to 0.26)</td>
</tr>
<tr>
<td>Values shown are the means with the standard deviation shown in parentheses. *All patients had clinical stage T1c-T2c N0 M0 disease; All patients had Gleason sum 6/7.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Instructions. A reference labelling reaction was also prepared with 5 μg total Universal Human Reference RNA (Stratagene Europe, Amsterdam, The Netherlands) and used as an internal control for the normalization of the microarray data. Cy3 (test sample) and Cy5 (reference) were obtained from GE Healthcare (Amersham, Buckinghamshire, UK) and used in conjunction with the ChipShot kit. The amount of Cy3 and Cy5 incorporation (frequency of incorporation; FOI) in the cDNA was quantified using the Nanodrop Spectrophotometer. Printing of the 3000 Human Cancer oligo subset V2.0 (Operon Biotechnologies GmbH, Germany) was carried out using UltraGAPS-coated glass microarray slides (Corning B.V. Life Sciences, The Netherlands). The 70mer oligonucleotide probes were dissolved in Pronto cDNA/Long Oligo Spotting Solution (Corning) and spotted onto a glass slide with a Qarray2 microarray spotter (Genetix, UK) and ScoreCard controls (GE Healthcare). Pre-hybridization, hybridization and washing of the microarrays were carried out according to manufacturer’s instructions (Pronto Microarray hybridization kit; Corning). Cy3 and Cy5 fluorescent signals were detected with an Affymetrix 428 laser scanner and the raw Tiff images were analysed for spot signal quantification and quality evaluation with Blue Fuse software V3.1 (BlueGnome, Cambridge, UK). The generated data files were then exported into R (version 2.5.0; http://www.R-project.org), normalised (Block-median followed by Across Array Product analysis (9, 10), a technique chosen for its robustness against biological variance such as that caused by tumour and/or patient experimental designs (9). Genes with a ‘ppp’ (probability of false prediction) value (9, 10) less than 0.15 were selected as being significantly up- or down-regulated.
60˚C and 20s at 72˚C for 45 cycles; for p73, 95˚C for 5 min then 40 cycles of 94˚C 30 s, 60˚C for 30 s, 68˚C for 2 min; for cyclin B1, 95˚C for 5 min then 40 cycles of 95˚C for 20 s, 60˚C for 60 s. For SRP72kDa, 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 2 separate occasions. Relative expression of genes was normalized to that of actin and gene expression in each sample calculated as 2^{-ΔΔCt}.

Primer sequences were designed using Primer 3 software and supplied by Invitrogen (Paisley, UK). For survivin: forward: 5’ACCAGGTGAGAAGTGAGGGA-3’, reverse: 5’-AACAGTAGAGGAGCCAGGGA-3’; for cyclin B1: 5’-CTCCTGTCTGTGTTGGAGAGGA-3’, reverse 5’-CTGTACCAGAATAACACCTGA-3’; for p73: forward: 5’-TCTTTTCAGGAGGTGCATCTG-3’, reverse: 5’-TCCCGTATAGTGTTCTCACAG-3’; for SRP72kDa: forward: 5’-GTCTAACAGAGGAGGCTTGTAA-3’, reverse: 5’-CAGGGACTTTGGGACTGTAAA-3’; for β actin forward: 5’-GCATCCATG AAACTACCTTC-3’, reverse: 5’-CAGGAGGCAAATGATCTTG-3’.

Statistical analysis. For comparison of all the q-PCR data obtained, 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

Baseline clinical parameters obtained for control and celecoxib-treated patients. Data are shown in Table I. Microarray analysis and Q-PCR validation: For the microarray part of the study we used a cut-off of ± 50% change in expression relative to the control arm. Table II lists the up-regulated genes. The data obtained revealed a list comprising 24 genes that showed statistically significant (see methods) >1.5-fold increments between celecoxib-treated and control untreated patients. A variety of genes with different functional significance were up-regulated in the celecoxib-treated patient group. Figure 1 shows the subsequent q-PCR evaluation of 3 up-regulated genes that were on the list (cyclin B1, p73 and SRP72kDa). The data help to verify the microarray analysis with levels of significance for the variation between control and celecoxib-treated group as being significant or very close to significant for SRP72kDa, where a high standard deviation for the levels of mRNA obtained in the celecoxib-treated patient biopsies was seen. Table III lists the down-regulated genes. A further list comprising 3 genes showed significant <0.5-fold decrease in celecoxib treated versus control patients. Due to our previous observation that survivin mRNA and protein were markedly down-regulated in COX-2-expressing human prostate cancer LNCaP cells (11), we also considered this gene in our subsequent q-PCR evaluation in the present study. The mRNA levels proved to be significantly reduced in patients from the celecoxib-treated group versus controls (see Figure 1).

Discussion

In order to investigate the effects of celecoxib on GEPs, we analysed biopsies from patients treated with celecoxib as well as control patients. After statistical processing, we compiled a list of 24 up-regulated and 3 down-regulated genes in celecoxib-treated versus control arm patients. In addition, to the gene list, we looked at the anti-apoptotic factor survivin as this was shown to be a very sensitive indicator of the effects of celecoxib in COX-2-expressing prostate cancer cells in our previous studies (11). Due to the stringent filtering criteria used (spot quality, statistical
significance as opposed to fold change alone) for the microarray data analysis, certain genes may not have been identified. We aimed to look at the survivin gene in our patient dataset and so performed q-PCR analysis including other genes that had been identified as being up-regulated in the celecoxib-treated prostate cancer patients.

A hallmark of cancer cells versus normal cells is uncontrolled cell division as a consequence of dysregulated...
cycelins and cyclin-dependent kinases (CDKs). CDKs are necessary for proper progression of the cell cycle through mitosis. A study by Gomez et al. (12) established a positive correlation between cyclin B1 protein and apoptotic cell death. Stable overexpression of cyclin B1 in LNCaP and PC-3 cells induced a chemosensitizing effect and conversely an siRNA approach was shown to decrease apoptosis. In the present study, we were able to demonstrate a clear increase in cyclin B1 in prostate cancer tissue taken from celecoxib-treated patients, an effect confirmed using q-PCR analysis. Our data are suggestive of a proapoptotic effect orchestrated by p73 and its interaction with p53 and apoptosis remains unclear, we show a clear induction of wild-type p73 as a consequence of celecoxib treatment in the present study. A recent report by Beitzinger et al. (18) lends further support to the hypothesis that p73 has a tumour suppressor role, independent of the p53 tumour suppressor, and is associated with inhibition of malignant transformation by limiting anchorage-independent growth.

A gene that was shown to be reduced in celecoxib-treated prostate cancer tissue was myosin light chain kinase. Inhibition of this enzyme has been shown to reduce the growth of mammary and prostate cancer cells in vitro and in vivo models with chemosensitization of cells to the effects of etoposide (19). Thus, the effect of celecoxib has been shown to reduce tumorigenic potential in prostate cancer cells and this has been borne out in a clinical setting in the present study. We have previously shown a very significant reduction of survivin ubiquitination and stabilisation. A report by Yoo et al. (20) showed that elevated IAPs were a common feature of prostate cancer including PIN, but they showed no relationship with Gleason grade or PSA levels. Krysyan et al. (21) showed that survivin levels correlated positively with COX-2 expression levels in non-small lung cancer (NSCLC) cells and COX-2 was shown to modulate survivin ubiquitination and stabilisation. A report by Yoo et al. (20) showed that elevated IAPs were a common feature of prostate cancer including PIN, but they showed no relationship with Gleason grade or PSA levels. Krysyan et al. (21) showed that survivin levels correlated positively with COX-2 expression levels in non-small lung cancer (NSCLC) cells and COX-2 was shown to modulate survivin ubiquitination and stabilisation. A report by Yoo et al. (20) showed that elevated IAPs were a common feature of prostate cancer including PIN, but they showed no relationship with Gleason grade or PSA levels. Krysyan et al. (21) showed that survivin levels correlated positively with COX-2 expression levels in non-small lung cancer (NSCLC) cells and COX-2 was shown to modulate survivin ubiquitination and stabilisation. A report by Yoo et al. (20) showed that elevated IAPs were a common feature of prostate cancer including PIN, but they showed no relationship with Gleason grade or PSA levels. Krysyan et al. (21) showed that survivin levels correlated positively with COX-2 expression levels in non-small lung cancer (NSCLC) cells and COX-2 was shown to modulate survivin ubiquitination and stabilisation. A report by Yoo et al. (20) showed that elevated IAPs were a common feature of prostate cancer including PIN, but they showed no relationship with Gleason grade or PSA levels. Krysyan et al. (21) showed that survivin levels correlated positively with COX-2 expression levels in non-small lung cancer (NSCLC) cells and COX-2 was shown to modulate survivin ubiquitination and stabili
al. (22) described the effects of aspirin (a non-specific-COX-2 inhibitor) in promoting TRAIL-induced apoptosis via down-regulation of survivin due to inhibition of E2F-1 binding activity to the survivin promoter region. A study by Febbo et al. (23) described a trial involving weekly docetaxel for 6 months in patients with high-risk localized prostate cancer in the neoadjuvant setting, prior to RP. A gene expression profiling approach was also used by these authors to assess the biological effects of the chemotherapy on prostate cancer tissues taken from those patients taken at RP and compared with untreated controls. The magnitude of the gene expression changes seen between the two groups of patients in that particular report was of a similar order to that in the present study, which essentially compared two groups (control and celecoxib-treated) of cancerous tissue, rather than normal adjacent versus cancerous tissue, where higher differentials of gene expression have been reported. Nevertheless, the study by Febbo et al. (23), taken together with the data described herein, suggest that microarray analysis following neoadjuvant therapy in prostate cancer is feasible and can also form a valuable line of enquiry when exploring the effects of novel targeted therapy. With regard to celecoxib, other clinical studies have shown the beneficial effects in prostate cancer using different endpoints. For example, Pruthi et al. (24) demonstrated stabilisation of PSA levels in patients who had been treated with celecoxib for various time periods of up to 18 months, following radiation therapy or radical prostatectomy.

We have successfully completed a randomized clinical trial investigating celecoxib in patients with primary, localized prostate cancer prior to RP. Overall, this study suggests that celecoxib has anticancer effects affecting cell proliferation, promotion of apoptosis, cell communication and integrity, and supports our earlier findings (5, 11). Our results suggest that celecoxib is worthy of further investigation as a novel therapy in prostate cancer.

References

Sooriakumaran et al: Celecoxib Effects on Gene Expression Profiling of Prostate Cancer


Received January 9, 2009
Accepted January 14, 2009