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Celecoxib leads to G_2/M arrest by induction of *p*21 and down-regulation of cyclin B1 expression in a *p*53-independent manner

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ABSTRACT

A unique in vitro system has been developed in our lab that consists of normal enterocytes derived from the rat ileum (IEC-18 cells) and their transformed derivatives with c-K-ras (R1 cells), anti-sense bak (B3 cells) and cyclin D1 (D1 cells). R1 and B3 cells express high level of COX-2 protein and PGE_2. IEC 18 and D1 cells express negligible amount of COX-2, and produce very low level of PGE_2. A relatively low dose of celecoxib (5–10 μM) induced G_2/M arrest, followed by induction of apoptosis in the transformed but not in the normal cells. Down-regulation of cyclin B1 and up-regulation of *p*21 expressions independent of *p*53 might have cause this cell cycle block. Growth inhibition was related to COX-2 function with 90–95% reduction in PGE_2 production. These findings may be of clinical importance, since low concentration of celecoxib can be achieved in human serum following standard anti-inflammatory (100–200 mg bid) regime.

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1. Introduction

There are at least two isoforms of the cyclooxygenase (COX) enzyme. COX-1, a housekeeping protein, that is constitutively expressed in the normal gastrointestinal mucosa, while COX-2 is usually undetectable and its expression is induced by inflammatory and neoplastic stimuli. Up-regulated expression of COX-2 was observed in 40–50% of colorectal polyps and in up to 85% of colorectal cancer (CRC) [1]. The lack of COX-2 expression in the normal colonic mucosa, along with its increased expression in colonic neoplasm and the molecular differences between COX-1 and COX-2 constitute the rationale for the development of selective COX-2 inhibitors to prevent and treat colorectal tumours [2,3]. The direct cellular targets of COX-2 inhibitors responsible for their action are not completely understood and may involve several pathways resulting in the down-regulation of prostaglandin E_2 synthesis, inhibition of cell proliferation and angiogenesis, induction of apoptosis, cell cycle arrest and potentiation of the immune response (for reviews see [2,3]). Deregulation of normal cell cycle control has been implicated in the pathogenesis of most human cancers. In particular, abnormal expressions of regu-

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latory proteins that control G_1/S phase transition, a critical rate limiting step in cell cycle progression, are frequently observed. Celecoxib, a non steroid anti inflammatory drug (NSAID), is a specific inhibitor of COX-2, and was previously reported to induce G_0/G_1 arrest in many human cancer cells [4–6]. We have previously shown that celecoxib inhibits the growth of malignant but not normal cells [7].

In the current study, celecoxib, a specific COX-2 inhibitor selectively inhibited the growth of malignant cells by inducing a G_2/M and not G_0/G_1 arrest as was previously reported. The growth inhibition was COX-2, but not *p*53, dependent. These findings might be of clinically importance, since low concentrations of celecoxib can be achieved in human serum.

2. Material and methods

2.1. Cells

The parental IEC-18 cells were normal rat enterocytes derived from rat ileum [8]. These cells were immortalized but non-tumourigenic and did not induce tumours in nude mice. R1 were IEC-18 cells transfected with pMIKcys that encoded a mini-human *c-K-Ras* gene (15 kb) with a cysteine mutation at codon 12 [8]. D1 cells were IEC-18 cells transfected with pMV7-D1, encoding the 1.1 kb human *cyclin D1* cDNA [9]. B3 cells were IEC-18 cells transfected with human *bak* cDNA, cloned into the vector pMV12 in the anti-sense orientation (unpublished data). In contrast to the parental IEC-18 cells, transformed cells over-expressing K-ras, anti-sense bak and cyclin D1 proliferate faster, form colonies in soft agar, and have higher saturation density and plating efficiency. Most importantly, they form tumours when injected sub-cutaneously into nude mice [8,9]. Cells were exposed to celecoxib (5–20 μM) for 48–72 h. The control, untreated cells, received 1% of the drug vehicle DMSO.

2.2. Flow cytometry and cell cycle analysis

The measurement of DNA content using propidium iodide (PI) staining and flow cytometry was used to determine cell cycle distribution and apoptosis as previously described [7]. In brief, cells were plated at a density of 7×10^5/10 cm dish. Following drug exposure (5, 10 μM), $1–2 \times 10^6$ cells were collected, washed, fixed and stained with 45 μg/ml PI for 1 h before FACS analysis (necrotic cells were counted and eliminated using trypan blue staining before fixation). Data acquisition was performed on a FACS caliber and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). The apoptotic cells were considered to constitute the sub-G_1 population. All the experiments were performed independently at least three times, and the results were expressed as mean ± SD.

2.3. Reverse-transcriptase PCR (RT-PCR)

Total RNA was purified, following exposure to celecoxib (10 μM), using Nucleospin® RNA II kit (Clontech, Palo Alto, California, USA) according to the manufacturer protocol. RT-PCR reaction was preformed, with 50 *η*g, on PTC-100 programmable thermal controller (MJ Research Inc, USA). Primers were designed using the LightCycler Primer Designer program (Roche Diagnostics, Mannheim, Germany) and were as follows:

GAPDH (housekeeping gene): Forward, 5′-GGA GAT TGT TGC CAT CAA CG-3′; Reverse, 5′- TTG GTG GTG CAG GAT GCA TT-3′. *p*53: Forward, 5′-TTG CAG AGT TGT TAG AAG GC-3′; Reverse, 5′-GAA GAT TCC CAC TGG AGT CT-3′. PCR conditions were as follows: 2 min denaturation at 94 °C; each cycle consisting of 1 min at 94 °C, 1 min 56 °C; 1 min and 15 s at 72 °C; and a final extension of 5 min at 75 °C. The PCR products were separated in 2% agarose/GelStar gel and visualized under UV light.

2.4. PGE_2 synthesis measurement

Cells were exposed to celecoxib (10–20 μM) for 48–72 h. Released PGE_2 in the medium was determined by a commercially available PGE_2-specific enzyme-linked immunoassay (R&D biosystems, Abingdon, UK) according to the manufacturer's protocol.

2.5. Cellular protein analysis

Exponentially growing cells were exposed to celecoxib (10 μM) for 48 h then collected and protein was extracted as previously described [7]. For western blotting, 50 μg of total cell lysate were loaded onto 12% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were transferred to Hybond-C membranes (Amersham, Arlington Heights, IL, USA). Membranes were treated with blocking solution (3% BSA in PBS 0.2% Tween-20) and incubated with various antibodies, including goat anti-actin (Santa Cruz Inc.), rabbit anti-*p*21 (Santa Cruz Inc.), mouse monoclonal anti-cyclin B1 (DAKO, Trappes, France) and mouse anti-*p*53 (NeoMrkers, Fermont, USA). Horseradish peroxidase-conjugated secondary antibody was used for immune detection using the ECL detection system (SuperSignal, Pierce, Rockford, USA). Densitometric quantification was performed using TINA 2.10 g program. Bands were normalized regarding actin levels.

3. Results

3.1. Effect on cell growth

The effect of increasing concentrations of celecoxib (up to 25 μM) for 72 h was assessed in IEC-18, R1, B3 and D1 cell lines (Fig. 1). Growth inhibition, in a dose dependent manner, was seen in R1 and B3 cells. No growth inhibition was seen in IEC-18 and D1 cell lines.

3.2. Celecoxib induces a G_2/M arrest

Most of the IEC-18 cells were at G_0/G_1 stage, and their cell cycle was not altered by exposure to celecoxib. In contrast, growth inhibition in a dose-dependent manner in R1 and B3 cells was associated with G_2/M arrest after 48 h exposure to the drug. After 72 h, the transformed cells entered apoptotic phase (Fig. 2). There was no effect on the growth of the transformed D1 cells that did not express COX-2.

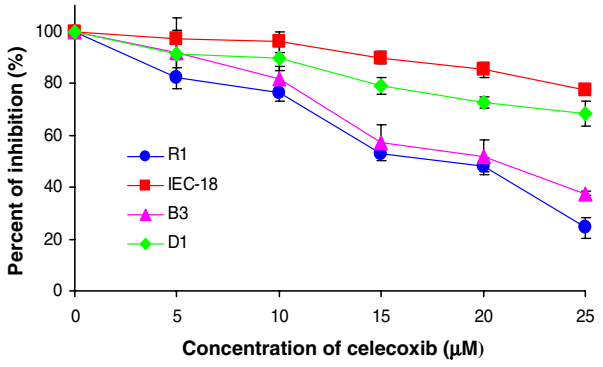

Fig. 1 – Celecoxib inhibits the growth of COX-2 expressor cells.

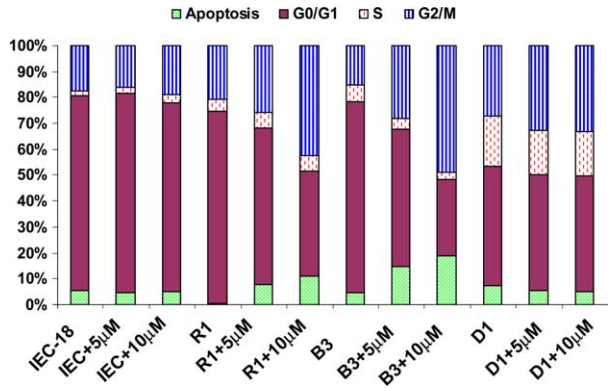

Fig. 2 – Celecoxib induces a G2/M arrest in COX-2 expressor cells.

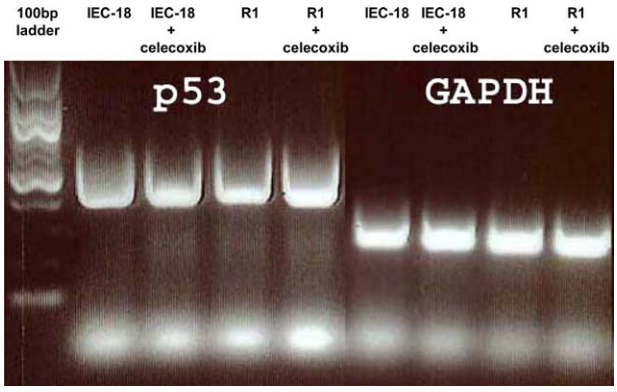

Fig. 3 – *p53* mRNA expression after treatment with celecoxib.

3.3. *The growth inhibition is independent of p53*

IEC-18 and R1 cells harbouring *p53* wild-type gene [10] had abundant *p53* mRNA (Fig. 3). At the same time, *p53* protein was not detected in the transformed cells even after exposure to celecoxib (10 μM) (data not shown). Hence, the growth inhibition in the transformed cells, seen in this model, was *p53* independent. It is suggested that mutated K-*ras* has an important role on the cell cycle, and eliminated *p53* activity by translation inhibition or induction of its degradation.

3.4. *Celecoxib induces p21 and down regulate cyclin B1 expression*

It has been previously described that *p21* induces cell cycles arrest by both *p53*-dependent and independent pathways [11]. Western blot analysis demonstrated a two-fold decrease in the expression of cyclin B1 and two-fold increase of *p21* in R1 and B3 cells as compared to IEC-18 and D1 cells, following celecoxib treatment (10 μM) (Fig. 4). These results were confirmed by immunoflourescence methods (data not shown).

3.5. *Growth inhibition is COX-2 dependent and is associated with inhibition of PGE_2 synthesis*

In order to ascertain if the drug effects were dependent on COX-2, we used tumourigenic cells (R1 and B3) that had high COX-2 activity and expressed high levels of PGE_2, in contrast to the parental IEC-18 cells that have negligible COX-2 activity and very low level of PGE_2. Treatment with celecoxib (5–20 μM) for 48–72 h inhibited more than 95% of PGE_2 production (Fig. 5), suggesting that the action of celecoxib, in this model, is PGE_2 dependent. Furthermore, the growth of transformed D1 cells, that did not express COX-2, was not inhibited and there was no change in their cell cycle. Also in D1 cells, celecoxib did not induce apoptosis even at high doses of the drug (20 μM) (Fig. 2).

4. Discussion

In this study, celecoxib inhibited the growth of transformed cells expressing COX-2 protein (R1 and B3 cells), but not that of normal cells or transformed cells that did not express COX-2 (D1 cells). It confirmed our previous observations that celecoxib inhibits the growth of malignant cells only [7].

Most of the IEC-18 cells were in G_0/G_1 phase, and their cell cycle was not altered by exposure to celecoxib. Celecoxib inhibited cell growth by induction of apoptosis, even following exposure to low concentrations of the drug (5 μM). At the same time, no effect was seen in the parental IEC-18 cells, nor in transformed cells (D1) expressing low level of COX-2.

This is the first report describing that celecoxib can also induce G_2/M arrest in transformed intestinal epithelial cells over-expressing COX-2 (R1 and B3), before directing the cells into the apoptotic phase.

The findings are intriguing as previous reports suggested that celecoxib causes a G_0/G_1 block [4–6,12]. The different results presented here might be due to different dosing; relatively low doses (5–20 μM) in the current study as compared to higher concentrations (25–100 μM) in the other studies. Similar findings of G_2/M block was reported by Ding [13]. However, their findings were obtained in oral cancer cells and followed exposure to significantly higher dose (50 μM) of celecoxib, that are not clinically achievable. There is only one study that has used similar low dose (5 μM) of celecoxib [14], and they demonstrated that very low (5 μM) clinically achievable concentrations of celecoxib inhibited the growth of prostate cancer cells, that lacked COX-2, by induction of G_0/G_1 block. It might be that celecoxib inhibits cell growth differently in different organs (intestine versus prostate). The G_2/M block induced in this study, was achieved in numerous re-

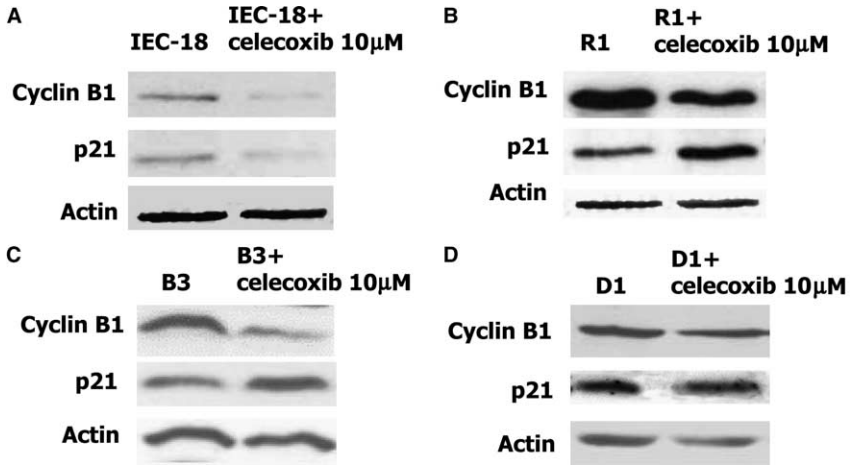

Fig. 4 – Upregulation of *p*21 and downregulation of cyclin B1 expression following exposure to celecoxib.

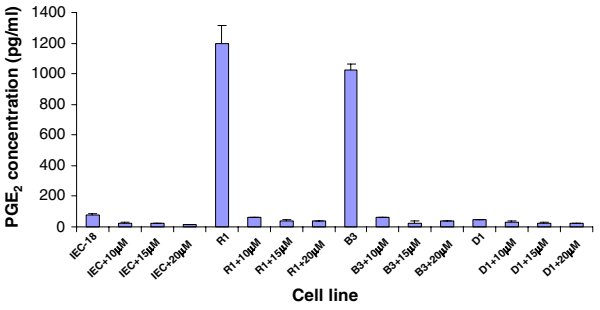

Fig. 5 – Celecoxib inhibits PGE_2 production in COX-2 expressor cells.

peated experiments. Indeed the changes in cell cycle players in this study (down-regulation of cyclin B1 and up-regulation of *p*21), are typical to the expected changes that are achieved when cells block G_2/M phase.

The transition from G_2 to M phase is thought to be triggered mainly by cyclin-dependent-kinase 1 (CDK1) following its binding to its regulatory subunit, cyclin B1. The levels of cyclin B1 rise during G_2 phase and peak in mitosis [15]. In this study, cyclin B1 was up-regulated in R1 and B3 cells, and may partially explain their tumourigenic phenotype. Since the complex CDK1/cyclin B1, promote the G_2/M transition, its down-regulation following celecoxib treatment may be responsible for the cell cycle arrest at the G_2/M phase. Kardosh [16] and Grosch [4] demonstrated similar down-regulation of cyclin B1 and increase expression of *p*21 in glioblastoma and colorectal cancer cells following exposure to celecoxib.

*p*53 plays an important role in regulating the G_1/S and the G_2/M transitions. G_1 arrest depends on the ability of *p*53 to activate transcription of specific genes, like $p21^{Waf1/Cip1}$ [17], while G_2/M arrest involves repression of the transcription of CDK1 and cyclin B1 [18] and probably alterations of the intracellular transport of cyclin B1 as well [19]. IEC-18, R1 and B3 cells harbour *p*53 wild-type gene [10] with abundant *p*53 mRNA. At the same time, *p*53 protein was not detected in transformed cells even after exposure to celecoxib. Hence, growth inhibition in the transformed cells seen in this model was *p*53 independent. The finding that *p*53 does not appear to play a role in the growth-inhibitory effects of celecoxib is not surprising, as numerous papers have demonstrated that celecoxib has growth-inhibitory effects in almost all cell lines investigated, whether or not these cells harbor wild-type or mutant *p*53.

It was previously described that *p*21 induces cell cycle arrest by both *p*53-dependent and independent pathways [11]. As a proliferation inhibitor, *p*21 is poised to play an important role in preventing tumour development. Up-regulation of *p*21 along with down-regulation of cyclin B1 in R1 and B3 cells, following celecoxib treatment, can promote G_2/M arrest and induction of apoptosis in a *p*53-independent pathway.

It is well known that celecoxib inhibits tumor growth in both COX-2 dependent and independent manners [2,20]. Treatment with celecoxib inhibited more than 95% of PGE_2 production in tumourigenic cells that had high COX-2 activity, suggesting that the action of celecoxib in this model is PGE_2 dependent. Furthermore, growth of the transformed D1 cells, that did not express COX-2, was not inhibited; apoptosis was not induced; and there was no change in their cell cycle even at fairly high dose of celecoxib. These observations further support the notion, that although numerous non COX-2 targets do exist in the intestine, COX-2 is an important target of celecoxib.

In summary, celecoxib inhibits cell growth by inducing a G_2/M arrest followed by induction of apoptosis. Increased *p*21 levels and down-regulation of cyclin B1 expression may be the underlying mechanism for this G_2/M arrest. This effect is COX-2 dependent and *p*53 independent. These results are clinically important since the drug concentration used can be achieved in human serum following standard anti- inflammatory (100–200 mg) and anti-neoplastic (400–800 mg) regimens range between 3.9–8 μM [21,22]. These novel effects of celecoxib provide new insights into the molecular mechanisms responsible for the anti-tumour effects of this compound, and may encourage further application of this and related compounds in the prevention and treatment of COX-2 expressors cells.

Conflict of interest statement

None declared.

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