Inhibition of cyclooxygenase-2 and amphiregulin reduces cell proliferation in a colorectal cancer cell line

Abstract
Background: The role of cyclooxygenase-2 (COX-2) overexpression in colorectal cancers has engaged interest in the role of COX-2 and prostaglandin E2 (PGE₂) in cancer cell mitogenesis. Amphiregulin (AR), an epidermal growth factor receptor (EGFR) ligand, has been directly related to PGE₂, which activates the AR promoter, causing increased AR expression.

Aims: To identify if COX-2 and AR play a role in human colon cancer cell mitogenesis.
Methods: HCA-7 cells in culture were treated alone or in combination with an amphiregulin neutralising antibody (ARNA) and a selective COX-2 inhibitor (SC236). The mitogenic effects were assessed by enzyme immunoassay to quantify bromodeoxyuridine (BrdU) incorporation.

Results: ARNA (P<0.01) and SC236 (P<0.01) alone both showed a growth inhibitory effect, which was augmented by co-treatment (P<0.001) with both agents. EGFR signalling and COX-2 play roles in colon cancer cell death regulation.

Conclusions: Novel therapeutic strategies should combine elements that target both pathways.

Introduction
Colorectal cancer is one of the most common cancers, occurring in about 14% of the population with a worldwide incidence of around 945,000 cases per year. The transition from normal colonic epithelium to polyp, and then to cancer, is characterised by the acquisition of serial genetic mutations and molecular events.

One of the earliest molecular events in colorectal carcinogenesis is the overexpression of cyclooxygenase-2 (COX-2). COX-2, the inducible form of cyclooxygenase, is overexpressed in 80-90% of colorectal cancers.\(^1\)

When COX-2 is induced, there is increased incidence of colorectal adenoma and carcinoma. Evidence to support this is seen in epidemiological,\(^2\) murine,\(^3\) and in vitro\(^4\) studies. Epidemiological studies have shown a consistent 40-50% decrease in the risk of developing colorectal cancer with long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit the cyclooxygenases.\(^2\)

Celecoxib, a selective COX-2 inhibitor, inhibits the progression of adenomatous polyps and causes regression of existing polyps in patients with familial adenomatous polyposis (FAP), who are genetically predisposed to have a high risk of developing colorectal cancer.\(^2\) This observation supports the findings from the APC\(^{min/-}\) mice model for FAP, where a decreased rate of adenoma formation was seen in mice genetically engineered to lack COX-2.\(^5\)

Mechanisms for NSAID-induced inhibition of tumour progression include altered tumour cell apoptosis, cell proliferation/cell cycle arrest and inhibition of angiogenesis (Figure 2).\(^5\) Indeed, rat intestinal cells modified to increase COX-2 expression develop a resistance to apoptosis in association with increased production of prostaglandin E\(_2\) (PGE\(_2\)). Treatment with the COX-2 inhibitor sulindac sulfide decreased PGE\(_2\) levels and restored the baseline susceptibility to undergo apoptosis.\(^1\)

PGE\(_2\) is produced at high levels by COX-2 in human colorectal cancer and has been shown to increase proliferation of colorectal cancer cells.\(^5\) The mechanism may involve epidermal growth factor receptor (EGFR) signalling through a ligand-dependent autocrine pathway.\(^6\)

PGE\(_2\) can transactivate EGFR,\(^7\) and can also increase expression of the EGFR ligand AR, through the ERK-2 pathway.\(^6\)

EGFR is a type of transmembrane tyrosine kinase receptor. Seven different polypeptide ligands from distinct genes can bind EGFR. These ligands are androgen receptor (AR), cripto-1 (CR-1), epidermal growth factor (EGF), transforming growth factor-alpha (TGF–\(\alpha\)), heparin-binding EGF (HB-EGF), epiregulin, and betacellulin (BTC).\(^4\)

EGFR ligands have been detected in human colon cancer cell lines and normal mucosa, which may suggest that co-expression of EGFR ligands, and upregulation of EGFR receptors, aid colonocyte proliferation in gastrointestinal tumours.\(^8\) AR levels have been directly related to PGE\(_2\) (and indirectly to COX-2), as PGE\(_2\) can activate the AR promoter, causing increased AR expression.\(^6\) AR is expressed in 60-70% of primary and metastatic human colorectal carcinomas, but in only 2-7% of normal human colonic mucosa from the same patient.\(^6\)

The roles of EGFR and AR, and their relationship with COX-2, have not yet been fully characterised in colorectal cancer. We decided to investigate the effects of AR and COX-2 signalling pathways on a colorectal cancer cell line by antagonising their effects using AR and COX-2 inhibitors. This provided an indirect study on constitutively expressed AR and COX-2, and demonstrated their effects on controlling colorectal cancer cell proliferation. This was done using a nucleotide incorporation method, which assessed the effects of EGFR and AR inhibitors on cell proliferation in the human cancer cell line, HCA-7.

Methods
Cell culture
HCA-7 colorectal cancer cells were seeded in T\(_{75}\) flasks at a density of 2.0 x 10\(^6\) and split when confluent (every other day) with trypsin EDTA. The cells were maintained on Dulbecco’s Modified Eagle’s Medium, containing 10% foetal bovine serum (FBS), kanamycin, and sodium pyruvate. Prior to treatment, HCA-7 cells were counted using a haemocytometer and 5,000 cells were then seeded into each well on a 96-well sterile culture plate and cultured overnight in serum free media.

Reagents
Cells were treated (in triplicate wells) with various concentrations of an AR neutralising antibody (RnD system) [0.1mg/ml, 1.0mg/ml, and 10.0mg/ml], COX-2 inhibitor SC236 [\(5\mu\)m], a combination of AR neutralising antibody [10.0mg/ml] and SC236 [\(5\mu\)m], or with vehicle control, which enabled comparison of the inhibitory effects in the treated cells with untreated control cells.

Chemiluminescent assay
After 24 hours, the treated cells were pulse labelled with BrdU and allowed to incorporate for one hour. BrdU incorporation allowed detection of proliferating cells in living tissues and was assessed by chemiluminescent enzyme immunoassay (Roche) according to the manufacturer’s instructions. Briefly, following pulse labelling, cells...
were then denatured and fixed to the plate. They were incubated with an anti-BrdU antibody for 90 minutes. They were washed with a wash buffer three times and 100 μl/well of substrate was added with a multi-channel pipette. After there was evident colour change (blue), H₂SO₄ [1mm] was added to each well to stop the reaction. Absorbance was measured with a Wallac plate reader at 450 nm.

Controls

A number of controls were used, including two positive controls (consisting of cells cultured in media with 10% FBS) and various negative controls. The first and second positive controls were treated in a different location on the 96-well plate to ensure that the absorbance results for controls did not vary across the plate. The first negative control used had anti-BrdU antibody without cells in the wells (thus detecting non-specific binding of antibody to the plate), and the second had unlabelled cells (no BrdU incorporation). The empty wells had no antibody or cells to determine the absorbance of the plate.

Statistical analysis

The average across three wells for a given condition was then subtracted from the average of the blank wells. The mean absorbance relative to the control for each condition was calculated, and results for replicate experiments (n=5) were averaged for the purpose of statistical analysis. The differences across treatments were analysed using one-way analysis of variance with post-test Bonferroni correction. A P value <0.05 was considered significant.
Results

HCA-7 cells are a colorectal cancer cell line that has been shown to have high levels of constitutive COX-2 expression and basal PGE_2 production, and to secrete the EGFR ligand AR. They were therefore an excellent in vivo model for examining the interplay of prostaglandin and EGFR signalling. The effect of COX-2 inhibitors on cell proliferation has been previously shown, and we once again showed a significant reduction in cell proliferation (P<0.01) following treatment with the COX-2 inhibitor SC236. At low concentration (0.1–1.0mg/ml) the AR neutralising antibody had no significant effect; however, at higher concentrations (10mg/ml) a small but significant effect (20% reduction, P<0.01) on cell proliferation was observed. The effects of a COX-2 inhibitor and AR neutralising antibody in combination were examined to investigate if this resulted in an additive reduction in cell proliferation, and a reduction in cell proliferation greater than 50% was observed by combining the two agents (P<0.001).

The first negative control used measured the non-specific binding of anti-BrdU to the plate and showed no significant difference in absorbance to blank wells. The second negative control had unlabelled cells to measure the level of non-specific binding of antibody to the cells and showed only fractional increases in absorbance relative to the blank wells. Positive controls showed an increase in absorbance values in each assay.

Discussion

COX-2 inhibitors, such as celecoxib and rofecoxib, have displayed chemopreventive and proapoptotic effects in cancer treatment. However, since both celecoxib and rofecoxib inhibit the COX-2 enzyme, the production of prostaglandins in endothelial cells can decrease, leading to ineffective anticoagulation and vasodilatation. This may explain the observation that AR and COX-2 are responsible for stimulating HCA-7 cell growth.

More recently, EGFR receptor tyrosine kinase inhibitors have shown anti-tumour effects in gastrointestinal cancer and have started clinical phase I-III studies. Patients with gastrointestinal carcinomas may benefit by blocking signal transduction through EGFR.

An increasing body of evidence suggests an interplay between COX-2 and EGFR signalling in colorectal cancer and these results appear to support such a notion, suggesting that both AR signalling and prostaglandins can regulate the growth of HCA-7 cells. When AR and COX-2 were inhibited together, an additive reduction in cell proliferation of more than 50% was observed, demonstrating the substantial combined effect that these two signalling pathways have in HCA-7 colorectal cancer cell proliferation. However, our observation that AR and COX-2 are responsible for stimulating HCA-7 cell growth proliferation is new and needs to be confirmed. Future studies aimed at directly identifying AR and COX-2 expression in the cancer cell line are necessary.

These results would suggest that future therapeutic strategies should target these pathways in combination in order to achieve additive or even synergistic effects in terms of anti-tumour activity. In the meantime, we are extending our study to look for a possible relationship between COX-2 and AR expression in human cancer tissue by examining the levels of overall expression and the degree of co-expression of these two important effectors of cancer cell growth.

Acknowledgements

We are grateful for financial support from the Health Research Board of Ireland.


References