

Cyclooxygenase-2 and Colon Cancer

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Cyclooxygenases (COX) also known as prostaglandin (PG) synthases are found in two forms, COX-1 and COX-2. While COX-1 is responsible for cytoprotective functions in a number of organs, COX-2, which is normally absent at basal levels, is induced under certain conditions. Induction of COX-2 is found in many pathophysiological states including acute inflammation, arthritis, as well as in cancer and cancer-related angiogenesis. Overexpression of COX-2 is sufficient to cause tumorigenesis in animal models and inhibition of COX-2 is sufficient to reduce tumour incidence and progression. Availability of a mouse model in which the COX-2 gene is deleted has been very useful in this regard and has provided compelling evidence that presence of COX-2 is essential for an aggressive phenotype. Indeed, COX-2 may very well be a diagnostic marker for cancer and identification of mechanisms that regulate its expression may help in designing new therapies for the treatment of cancer. COX-2 mediated prostaglandin E₂ is the primary mechanism of promoting cancer development in colon tissue. It should be noted that there are other COX-2 mechanisms, independent of prostaglandins as well as other natural prostaglandins such as 15-deoxy-prostaglandin J2 that also contribute to tumour development in other tissues. However, in the current article, we will attempt to review the recent progress in understanding the role of COX-2 dependent prostaglandin E₂ in colon cancer and mechanisms that regulate COX-2 expression.

Key Words: Prostaglandins, Angiogenesis, Apoptosis, Prostanoid Receptors, Transcription, Messenger RNA translation, RNA binding protein, 3'untranslated region, HuR, CUGBP2, Tristetraprolin

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the western world. The estimated number of new cases of colorectal cancer in the United States for 2002 was 107,000, and approximately 48,000 people would die from the cancer or its complications (Society 2003). The incidence in India had been very low in the past but more recently a rapid increase was projected due to changing food habits to a more western style diet (Mohandas 1999). Indeed, in the past, the Indian diet comprised primarily of fruit and vegetables, and included spices such as turmeric that contains the active anti-cancer ingredient, curcumin (Rao et al. 1995a, 1995b, Reddy & Rao 2002). However, western diets are high in fats and red meat and low in fibre a major risk factor for colon carcinogenesis (Mohandas 1999). Other risk factors include age (>50 years), gender (women > men) and lifestyle such as alcohol abuse, smoking and sedentary habits (DeCosse et al. 1993, Potter 1993, Potter et al. 1993,

Reddy 1988, Slattery et al. 1993, Slattery & Kerber 1993, Slattery & West 1993).

Colon cancer can be treated if identified early. Colonoscopy screening can find polyps, and removing these polyps at an early stage can prevent cancer. Even if the patient had the polyp for a long time that it may develop into a cancer, screening tests can find these lesions early, greatly increasing survival. Hence, regular colonoscopy is recommended in the United States for those over 50 years of age (Trowbridge & Burt 2002, Walsh & Terdiman 2003a, 2003b). However, the problem with colon cancer is that in about 40% of the cases, the cancer will come back within 3-5 years of treatment (Topal et al. 1999). Cancer may recur in the colon or rectum, or in another part of the body. Recurrent colorectal cancer often is found in the liver and/or lungs (Habal et al. 2000).

Colorectal cancers usually arise from the progression from normal colonic mucosa to adenomatous polyp to cancer (figure 1). Although

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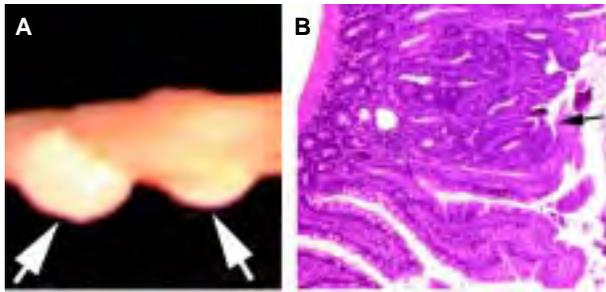


Figure 1. Colon cancer. **A**, A mouse model for colon cancer. Mice carrying a mutation in one allele of the APC gene develop multiple adenomas in the intestine as shown in the figure. Colon cancer begins with the development of polyps, which are benign growths in the epithelium as shown in the figure. Eventually the polyps get bigger and nests of malignant cells appear within the polyps. Some of these malignant cells will escape from the primary tumour and metastasize throughout the body. Arrow denotes presence of adenoma. **B**, Histological presentation of an adenoma stained with hematoxylin and eosin. The neoplastic gland is long and frond-like, and the growth is primarily exophytic (outward into the lumen). Invasion of the tumour into the subepithelial layer is not observed at this point. Arrow denotes presence of adenoma.

the mechanisms are not fully understood, at least five to seven major events occur in the progression (figure 2). Two major pathways may lead to cancer, chromosomal instability and microsatellite instability (Fenebro et al. 2002, Komarova et al. 2002). About 85% of colorectal cancers are due to events that result in chromosomal instability, and important genes involved in this pathway are adenomatous polyposis coli (APC, chromosome 5q), deleted in colon cancer (DCC, 18q) and p53 (17p) (Vogelstein & Kinzler 1993). The microsatellite instability-related cancers are mainly due to defects in DNA repair resulting from acquired or inherited mutations in DNA repair-related proteins (Lengauer et al. 1998, Kinzler & Vogelstein 1998).

One of the earliest events in cancer progression

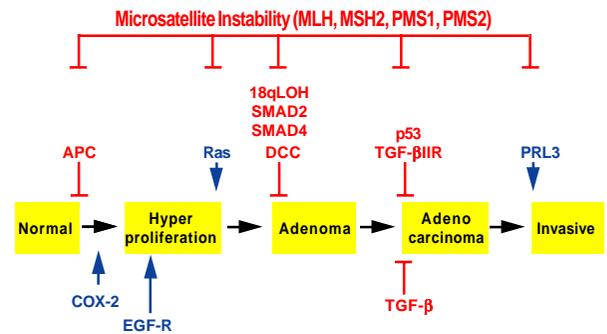


Figure 2. COX-2 overexpression in colon cancer. Genes whose mutation results in loss (red) or gain (blue) of function are shown above the schematic for progression of a normal cell to a cancer cell. Mutation in one of the four mismatch-repair genes would lead to a deficiency of mismatch-repair proteins, resulting in microsatellite instability. This in turn could result in mutations of genes such as the adenomatous polyposis coli gene (APC) and K-ras. However, mutation in the microsatellite instability related genes could occur at anytime during the progression of cancer phenotype. Genes whose overexpression (blue) or underexpression (red) is associated with the progression are shown below. COX-2 is one of the first genes that is overexpressed and this can be observed even before any gross phenotypical changes are observed.

is the loss of the APC gene (figure 2). APC, located in chromosome 5q21, is considered a gatekeeper and mutations in the gene result in loss of signal transduction and cell adhesion (Spirio et al. 1993, Brensinger et al. 1998, Pedanonte et al. 1998, Soravia et al. 1998, Laken et al. 1999). APC binds to β -catenin and targets it for ubiquitination-mediated degradation in the cytoplasm of the epithelial cell. Loss of APC results in loss of this degradation event resulting in excess accumulation of β -catenin, which in turn translocates to the nucleus and transcriptionally activates expression of genes essential for tumour progression. Other genes that are consistently observed to be mutated are p53 and K-ras (Iacopetta 2003a,b, Yamada et al. 2003, Zalewska & Tobi 2003). However, mutations in these genes alone are not the only cause of cancer. Overexpression and loss

Table 1. COX-1 and COX-2

	COX-1	COX-2
Expression	Constitutive	Inducible
Gene size	22 kb	~ 8 kb
mRNA	2.7 kb	2.8 and 4.6 kb
Expression	Most tissues, highest in stomach, kidney, colon and platelets	Regions of brain and kidney. Induced during inflammation and in tumour cells by growth factors, cytokines and protooncogenes and tumour promoters
Protein	72 kDa	74 kDa
Localization	Endoplasmic Reticulum, Nuclear Envelope	Endoplasmic Reticulum, Nuclear Envelope

of gene expression by other mechanisms can also lead to the cancer phenotype. Loss of transforming growth factor-beta occurs late in cancer progression, whereas induction of COX-2 occurs early and is sustained throughout the cancer progression (Kim & Kim 1996, Bellone et al. 2001a,b, Dimberg et al. 2001a,b, Fosslien 2001, Joo et al. 2002a,b). Furthermore COX-2 levels may be increased at an early stage in colorectal neoplasms during polyp formation and before invasion suggesting even when no other pathophysiological conditions are observed implying that COX-2 may be a marker for colon cancer progression (Williams et al. 1996).

COXs are the rate-limiting enzymes in arachidonate metabolism. They catalyze the conversion of arachidonic acid to PGH_2 , the precursor for PGs and thromboxanes (figure 3) (Williams et al. 1999a, 1999b). Two isoforms of this enzyme have been identified, COX-1 and COX-2 (table 1). Both COX enzymes are membrane bound. They are present on the luminal surfaces on the endoplasmic reticulum as well as the inner and outer membranes of the nuclear envelope (Spencer et al. 1998). COX-1 and COX-2 have structural similarities but they differ in their role in tissue biology and disease progression. COX-1 is a housekeeping gene that is constitutively expressed in many tissues and plays a major role in tissue homeostasis. On the other hand, COX-2, which was discovered in 1991, is expressed at low levels

in some tissues such as the stomach, intestine and kidney, but is highly induced by growth factors, cytokines, and inflammatory agents (Williams et al. 1999a). However, COX-2 expression levels may be varied depending on the type of cancer. For example one study determined that COX-2 levels were lower in hereditary nonpolyposis colorectal cancer (HNPCC) as compared to familial adenomatous polyposis, and sporadic colorectal cancers (Sinicropo et al. 2000). COX-2 is not only over-expressed in colon tumours but also in other neoplasias, for example mammary tumours, neuroblastoma, prostate cancer, ovarian cancer and melanoma (Denkert et al. 2001, Hoozemans et al. 2001a,b, Hayes & Rock 2002, Singh & Lucci 2002, Hussain et al. 2003). Recent studies have demonstrated a 40-50% reduction in colon carcinoma incidence due to nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin (Fischman 2003, Friis et al. 2003). Furthermore, deletion of the COX-2 gene in mice suggests that it plays a major role in the development of intestinal tumours (Chulada et al. 2000, Sasai et al. 2000, Takaku et al. 2000). In the present article, we provide an overview of how COX-2 functions both at the cellular level and in relation to colon carcinogenesis and describe our current understanding of the mechanisms that regulate COX-2 expression.

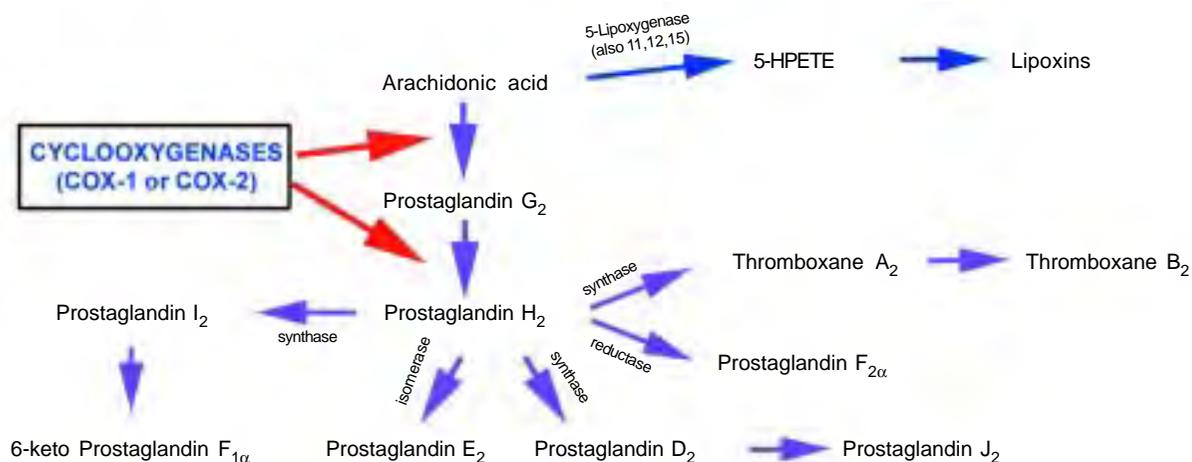


Figure 3. Cyclooxygenases are rate-limiting enzymes in prostaglandin synthesis. Arachidonic acid is the major precursor for eicosinoid hormones including prostaglandins. Cyclooxygenases (COX-1 and COX-2) catalyze the first step in the process by generating PGH_2 from arachidonic acid. Depending on the enzyme, namely synthase, reductase or isomerase different PGs are produced. The chief PG in the intestinal tract is PGE_2 , which is generated by PGH_2 isomerase. PGE_2 enhances proliferation and inhibits apoptosis of colon cancers. However, in other tissues PGI_2 also has antimicrobial and antiproliferative effects. An alternative pathway for arachidonic acid metabolism is catalyzed by 5-lipoxygenase resulting in the eventual generation of lipoxins.

Biosynthesis of Prostaglandins

Figure 3 depicts the multiple steps in the eicosanoid biosynthesis pathway. PGs are cyclopentanoic acids and the first step in the generation of PGs is the liberation of arachidonic acid, a 20 carbon polyunsaturated fatty acid from membrane phospholipids by phospholipase A2 (Sharma 2002). Alternatively, the phospholipids may be degraded to diacylglycerol by phosphoinositol-specific phospholipase C. The diacylglycerol is subsequently catabolized to arachidonic acid by diacylglycerol lipase. The next step, which is the key step in the biosynthesis pathway results in the conversion of arachidonic acid to PGH_2 by the COX enzymes or to 5-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (5-HPETE) by lipoxygenase (figure 3). The lipoxygenase related pathway results in the production of hydroxy fatty acids, leukotrienes and lipoxins. COX introduces two molecules of O_2 into arachidonic acid to form prostaglandin (PG) endoperoxides, from which the eicosanoids PGE_2 , PGD_2 , PGF_2a , PGI_2 and thromboxanes (Tx) A_2 are formed (Cook et al. 1993). These products have distinct biologic function. The types and amounts of PGs and thromboxanes are highly variable in different cell types because of differences in the distal synthases. The most important PG in the gastrointestinal tract is PGE_2 (Johansson & Bergstrom 1982, Wilson 1991, Eberhart & Dubois 1995).

PGE_2 effects are mediated by a family of G protein coupled receptors, called EP_1 to EP_4 (Chiarugi et al. 1998, Breyer & Breyer 2000a, 2000b). The mechanism by which PGE_2 interacts with specific EP receptor is not clear. However, the downstream effect of the interaction depends on the differential expression of each EP subtype in the various tissues and cells (figure 4). Functional studies on the EP receptors have been performed using subtype specific agonists/antagonists and mice lacking the different receptors. Signalling through the EP_1 receptors usually results in increased intracellular Ca^{2+} levels (figure 4) (Watabe et al. 1993). Activation of EP_3 results in reduced cAMP levels, whereas EP_2 and EP_4 signalling results in increased levels of cAMP (An et al. 1998, Yang et al. 1994, Breyer et al. 1996a,b, Hatae et al. 2002a,b). Three of the EP receptors, EP_1 , EP_2 and EP_4 have been demonstrated to play a major role in colon carcinogenesis (Barba et al. 2000, Ushikubi et al.

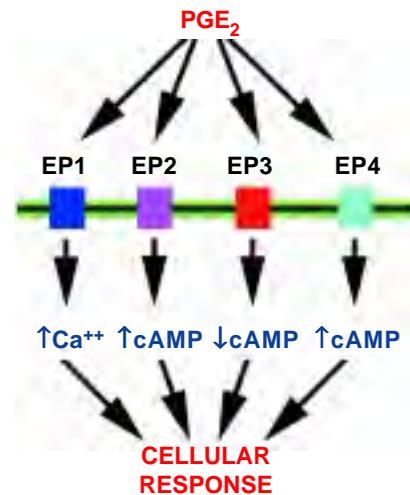


Figure 4. Prostaglandin signals through the EP receptors. Binding of PGE_2 to one of the EP receptors (EP_1 to EP_4) results in cellular changes causing either increased calcium (EP_1), or changes in cAMP (EP_2 to EP_4). Specifically, signalling through either EP_2 or EP_4 results in increased cAMP while that with EP_3 results in decreases in cellular cAMP levels. This eventually results in a cellular response that may either be pro- or anti-proliferative, depending on the status of the cell.

2000, Watanabe et al. 2000, Sonoshita et al. 2001, Takafuji et al. 2001, Kawamori & Wakabayashi 2002, Mutch et al. 2002). Furthermore, PGE_2 was observed to promote cell growth and motility through EP_2 and EP_4 receptors by activation of the protein kinase A and phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT/PKB) dependent pathways, respectively (Fujino et al. 2002).

COX-2 in Tumours

Increased COX-2 expression occurs at every stage of colon tumorigenesis, including the early stages of adenoma formation (Hao et al. 1999, Fujita et al. 2000). In chemically-induced tumours in rodents, such as treatment with azoxymethane (AOM), the tumours demonstrate high levels of COX-2 (DuBois et al. 1996). Furthermore, dietary fat induces COX-2 expression and greater numbers of aberrant crypts, while inhibition of COX-2 by chemical inhibitors results in decreased aberrant crypts in rats following AOM treatment (Reddy & Mæura, 1984, Reddy et al. 1985, 1987, Reddy & Maruyama 1986, Singh et al. 1997a, b). Similarly, intestinal tumours in $\text{APC}^{\text{min}/+}$ mice, the murine model for familial polyposis syndrome demonstrate high levels of COX-2 and treatment of these mice with COX inhibitor, sulindac also demonstrated a

reduction in intestinal polyps (Beazer-Barclay et al. 1996, Boolbol et al. 1996, DuBois et al. 1996, Chiu et al. 1997). Definite proof that COX-2 overexpression is essential for tumorigenesis came from studies with mice genetically modified to lack COX-2 that were crossed with APC^{min/+} mice (Oshima et al. 1996, Vane 1997, Chulada et al. 2000). These COX-2^{-/-} APC^{min/+} mice demonstrated a significant reduction in tumours (Oshima et al. 1996, Vane 1997, Chulada et al. 2000). The mechanism responsible for the increased level of COX-2 in the adenoma is still not clear. Further studies have determined the presence of both transcriptional and post-transcriptional mechanisms in inducing COX-2 gene expression (discussed below). COX-1, which is constitutively expressed in normal tissues, may also affect tumorigenesis. Initial studies have demonstrated that COX-1 is not upregulated in colorectal cancers (Williams et al. 1999a). However, deletion of both alleles of the COX-1 gene in APC^{min/+} mice (COX-1^{-/-} APC^{min/+}) demonstrated reduced number of intestinal tumours, similar to that observed with COX-2, suggesting that COX-1 may also play a role in adenoma formation in the murine intestine (Chulada et al. 2000). While confirmation of the role of COX-1 is awaited, its expression was not induced in human colorectal tumors (Khan et al. 2001).

Chronic inflammation is a risk factor for epithelial carcinogenesis. Examples of chronic inflammation leading to cancer include gastric adenocarcinomas following *Helicobacter pylori* infection, hepatocellular carcinoma and hepatitis B virus, colon carcinoma following chronic colonic inflammation and finally bronchial adenocarcinoma following asbestos-induced irritation (Ohshima & Bartsch 1994, Park et al. 1998, Rosin et al. 1994, Blaser 2000, Schwartsburd 2003). During inflammation, COX-2 is upregulated resulting in increased PGE₂ synthesis. Colony stimulating factors released by tumour cells also activate monocytes and macrophages to synthesize PGE₂, which in turn inhibits the production of immune regulatory lymphokines, T cell and B cell proliferation, and the cytotoxic activity of natural killer cells (Heidenreich et al. 1989a,b, Khan et al. 1990, Fu et al. 1991, Joshi et al. 2001, Fulton & Chong 1992). Furthermore, PGE₂ inhibits the production of tumour necrosis factor alpha (TNF- α) and induces the production of IL-10, an immunosuppressive

cytokine (Kambayashi et al. 1995, Demeure et al. 1997, Takano et al. 1998). Recently, when APC^{min/+} mice were treated with dextran sodium sulphate they demonstrated that the germline mutation in APC contributed significantly to the development of colitis-associated neoplasia (Cooper et al. 2001). Similarly, mice defective in mismatch repair also developed colitis-induced neoplasia when treated with dextran sodium sulphate (Kohonen-Corish et al. 2002). Furthermore, colitis markedly accelerated the development of colonic dysplasia and cancer in these mice.

Prostaglandins Role in Tumorigenesis

Treatment of colon carcinoma cells with exogenous PGE₂ leads to a significant reduction in the percentage of cells undergoing apoptosis (Sheng et al. 1998, Wilson & Potten 2000). It has been proposed that this is due to induction of Bcl-2 expression, a protein that acts to stabilize mitochondrial membrane integrity by preventing cytochrome-c release (Kroemer et al. 1998, Susin et al. 1998, Gottlieb 2000). Increased Bcl-2 results in reduced caspase-3 and caspase-9 activation, thereby contributing to inhibition of apoptosis (Susin et al. 1999, Tirhofer et al. 2001). In addition, PGE₂ treatment also leads to marked activation of MAPK (Chen et al. 1999, Guan et al. 1999, Faour et al. 2001). Furthermore PGE₂-mediated activation of MAPK is required for induction of Bcl-2 expression (Sheng et al. 1998).

A big quagmire is in understanding the role of EP₂ and EP₄ in the PGE₂ signalling process in colon cancer. Since signalling through either one results in increased intracellular cAMP (Breyer et al. 1996a, Hatae et al. 2002), it might be assumed that they have similar activities. However, a recent report points to potential differences in the signalling program between EP₂ and EP₄ receptors. While both receptors activate T cell factor (TCF)/lymphoid enhancer factor (LEF)-mediated transcriptional activation, this occurs via different pathways. Activation of TCF/LEF signalling by the EP₂ receptor occurs through the activation of a protein kinase-A dependent pathway whereas EP₄ receptors activate the signalling mainly through the PI3K/AKT pathway (Fujino et al. 2002). These data, while intriguing, suggest that the cells have evolved to encode independent mechanisms of turning on gene expression in order to ensure that signalling

functions occur. It remains to be seen, however, whether this is restricted to only a subset of pathways or whether it is a global phenomenon.

COX-2 and Angiogenesis

Prostaglandins exert their effects locally in both an autocrine and a paracrine manner (figure 5). An increase in tumour mass beyond 1-2mm in size must be preceded by an increase in vascular supply to deliver nutrients and oxygen to the tumour. Angiogenesis is one of the important mechanisms that support tumour development (Folkman 2002). Studies of several tumour models revealed that induction of angiogenesis is a discrete component of the tumour phenotype, which is often activated during the preneoplastic stages of the tumour. Cancer-induced angiogenesis is the result of increased expression of angiogenic factors, or decreased expression of anti-angiogenic factors, or a combination of both events (Strohmeier 1999, St Croix et al. 2000, de Fraipont et al. 2001, Fosslien 2001, Reimuth et al. 2003). Early studies with chicken embryos demonstrated that PGE_2 might be a key factor in various neovascular reactions (Form & Auerbach 1983). Furthermore, none of the other PGs, PGA_2 , PGF_2 , or TXB_2 were found to be angiogenic suggesting the specificity of PGE_2 and its signalling pathway is specifically involved in the angiogenesis process (Form & Auerbach 1983). COX-2 expression also correlates with both VEGF expression and tumour vascularization of small intestine and colon (Tsujii et al. 1998, Jones et al. 1999, Williams et al. 1999, Fosslien 2001, Oshima & Taketo 2002). In fact, in colon cancer all three types of cells, namely neoplastic cells, stromal fibroblasts, and endothelial cells overexpress COX-2 resulting in high levels of PGE_2 (figure 5) (Tsujii et al. 1998, Williams et al. 1999, Fosslien 2001, Walsh & Terdiman 2003). It also believed that overexpression of COX-2, and thereby PGE_2 , in epithelial cells leads to increased production of vascular growth factors and the formation of capillary-like networks. Furthermore, over-expression of COX-2 in colon carcinoma cells stimulates endothelial motility and tube formation through increased production of a pro-angiogenic factor, which can be reversed by inhibiting COX-2 activity (Gullino 1986, Gately 2000, Li et al. 2002). COX-1 also plays a role in the neovascularization process. Treatment of endothelial cells with a non-specific COX inhibitor or antisense

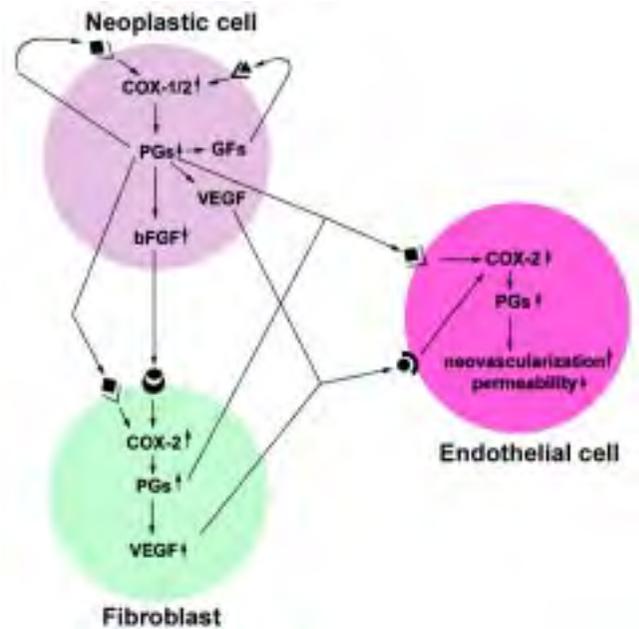


Figure 5. COX-2 and angiogenesis. Overexpression of COX-2 in a neoplastic cell results in increased PGs. The PG can then induce COX-2 and other growth factors in the neoplastic or fibroblast and endothelial cells in function in an autocrine or paracrine fashion, respectively. The PG can also induce VEGF in the neoplastic and fibroblast cells which then bind to specific receptors in the endothelial cells to induce neovascularization and increase permeability of the neoplastic cells in the surrounding stroma.

oligonucleotide to COX-1 messenger RNA (mRNA) inhibits COX-1 expression and activity, which in turn results in suppression of vascular tube formation. Thus, COX-1 and COX-2 may regulate colon carcinoma induced angiogenesis by two mechanisms; while COX-2 modulates production of angiogenic factors by colon cancer cells, COX-1 regulates angiogenesis in endothelial cells (Tsujii et al. 1998). When COX-2 expression is induced in stromal cells surrounding a polyp, increased PGE_2 is generated and signals through the EP_2 receptor located on the membrane surface (Sonoshita et al. 2001). This results in elevated cAMP levels, which in turn stimulates VEGF production, and thereby promotes neovascularization and tumour angiogenesis (Sonoshita et al. 2001). Therefore, one can speculate that COX-2 inhibitors and EP_2 antagonists might prove to be effective chemotherapeutic agents in preventing tumour angiogenesis.

The administration of sulindac, a non-selective COX-2 inhibitor not only brings the levels of COX-2 and PG formation to baseline but also

restores normal levels of apoptosis (Boalbol et al. 1996). This was observed to be comparable to that induced by cyclohexamide, a potent inducer of apoptosis. While the mechanism is not completely clear, it is believed that both non-specific (aspirin or sulindac) and specific (celecoxib or rofecoxib) nonspecific anti-inflammatory drugs (NSAIDs) also induce neutral sphingomyelinase (figure 6). This results in the conversion of sphingomyelin into ceramide, a known mediator of apoptosis (Chan et al. 1998). Ceramide can act on at least two target molecules, a membrane-bound serine/threonine kinase, referred to as CAP kinase (ceramide-activated protein kinase) and a CAP phosphatase, which is a serine/threonine protein phosphatase (Story & Kodym 1998). Ceramide, via CAP phosphatase activation or okadaic acid activation downregulates c-myc by blocking transcription elongation. Ceramide is also indirectly responsible for the cleavage and activation of a Ced-3/ICE-like cysteine protease called CPP32 or caspase-3. Caspase-3 then cleaves poly (ADP-ribose) polymerase (PARP) during apoptosis. In addition, ceramide induces the stress-activated protein kinase (SAPK/JNK) pathway signalling but the role of SAPK/JNK in apoptosis signalling is currently unknown (Story & Kodym 1998).

Regulation of COX-2 Expression

Transcription

The human COX-2 gene is localized in the long arm of chromosome 1 at map position 25.2-25.3 (Kosaka et al. 1994). The gene spans approximately 8 kb and consists of 10 exons (figure 7) (Appley et al. 1994, Kosaka et al. 1994). Transcription initiation occurs 134 nt upstream of the translation initiation (ATG) codon (Kosaka et al. 1994). The region within the 800 nt upstream of the transcription start site contains a canonical TATA box located 31 nt upstream of the transcription start site, as well as response elements for transcription factors SP1, AP2, NF κ B, GATA-1 and cAMP-response element binding protein, suggesting that expression of this gene may involve the complex interaction of various transcription enhancing factors (Tazawa et al. 1994). The first 200 nt of the human COX-2 promoter demonstrated ~65% homology with both mouse and rat COX-2 promoter but no homology to COX-1 promoter, suggesting that this

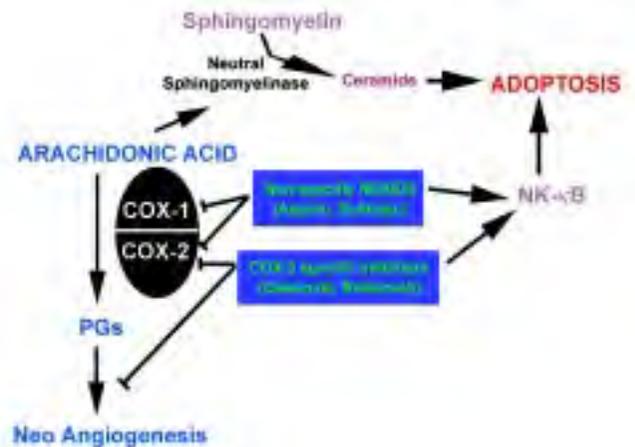


Figure 6. Mechanism of apoptosis by COX-2 inhibitors. Inhibition of arachidonic acid conversion to PG by either non-specific (sulindac or aspirin) or specific (celecoxib or rofecoxib) NSAIDs results in the induction of sphingomyelinase. This results in the conversion of sphingomyelin to ceramide—a potent mediator of apoptosis. The NSAIDs may also exert their effect on apoptosis independent of COX-2 inhibition by inducing NF- κ B activation.

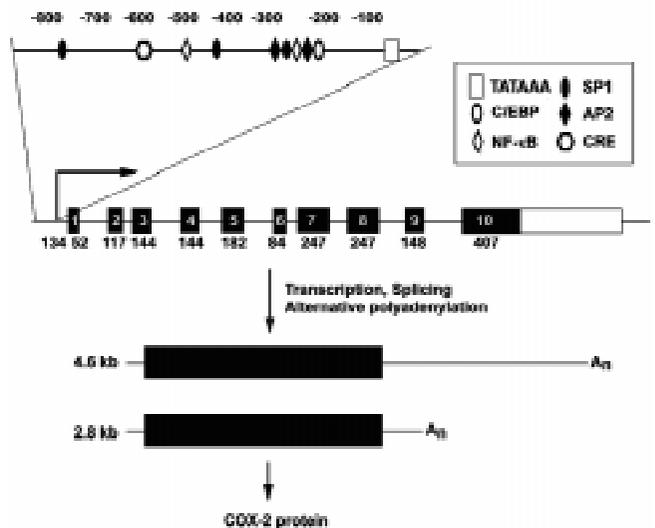


Figure 7. COX-2 gene. Schematic representation of the human COX-2 gene. The exons are shown by a black box and their sizes are indicated below. Location of the transcription start site is depicted by an arrow. The 800 nt region upstream of the transcription start site has been expanded and the various transcription factor binding sites that bind to this region and induce transcription from the TATA A box is shown. Use of different polyadenylation sites in exon 10 results in the generation of two distinct transcripts differing in the length of the 3'UTR. This results in varying amounts of mRNA stability for the transcripts. Translation of either COX-2 mRNA results in COX-2 protein production.

region is critical in regulating COX-2 expression (Kosaka et al. 1994). Promoter analysis studies have demonstrated that a 460 nt sequence upstream of the transcription start site are sufficient to drive

the expression of a luciferase gene in a human vascular endothelial cell line (Tazawa et al. 1994). COX-2 is also a target of NF κ B and NF-IL6 in cells in response to TNF- α , while dexamethasone completely inhibited IL-1-induced COX-2 mRNA expression (Geng et al. 1995, Crofford 1997, Berg et al. 2000).

The 5' flanking region of COX-2 gene has two NF κ B binding sites and a strong correlation between COX-2 and NF κ B expression was observed in colorectal tumours (Charlanbous et al. 2003). In normal cells NF κ B activity is mainly controlled at the posttranscriptional level. It is silenced by sequestration and degradation in the cytoplasm by the inhibitory protein I κ B α but migrates to the nucleus following activation. Transient transfection of the two NF κ B subunits, p65 and p50 enhances COX-2 promoter activity, an activity also seen when cells were stimulated with phorbol ester TPA (Kaltschmidt et al. 2002). However, in a murine neuroblastoma cell line that has constitutively active NF κ B, COX-2 expression was not further induced by TPA suggesting that TPA mediated activation was through NF κ B (Kaltschmidt et al. 2002). Bacterial lipopolysaccharide (LPS) and interferon- γ (IFN γ) also induce COX-2 expression by inducing I κ B degradation resulting in activation of NF κ B as well as by turning on interferon-regulatory-factor-1 (IRF-1) (Blanco et al. 2000, Berg et al. 2001, Zhang et al. 2002). Furthermore, LPS was unable to induce COX-2 in IRF-1 deficient mice suggesting that LPS mediated induction of COX-2 is mediated by IRF-1 (Zhang et al. 2002). Collectively these data provide a role for NF κ B and IRF-1 in regulating COX-2 gene transcription.

Tumour suppressor protein p53 is a transcription factor that is important in the suppression of cellular growth and transformation (Eliyahu et al. 1989, Finlay et al. 1989, Baker 1990, Diller et al. 1990, Agarwal et al. 1995, Ko & Prives 1996). p53 can either increase or suppress the expression of a number of target genes and it was observed to inhibit COX-2 gene transcription (Ginsberg et al. 1991, Seto et al. 1992, Mack et al. 1993, Raginov et al. 1993, Deb et al. 1994, Ko et al. 1996, Werner et al. 1996, Subbaramiah et al. 1999). p53 suppresses transcription from TATA containing promoters probably by interacting with components of the basal transcriptional machinery (Ginsberg et al. 1991, Seto et al. 1992, Mack et al.

1993). This may explain why COX-2 mRNA is not detected in normal intestinal cells whereas it is overexpressed in colon cancer cells, suggesting an important link between p53 and cancer.

More recently, COX-2 was shown to be regulated by the Wnt and ras pathways. Loss of functional APC in APC^{min/+} mice activates Wnt signalling pathway resulting in accumulation of β -catenin, which then binds to TCF-4/LEF and turns on COX-2 (Araki et al. 2003). Given that EGF₂ induces TCF-4/LEF by signalling through EP₂ or EP₄ receptors, this implies that a closed positive feedback occurs between EGF₂ and COX-2 (Araki et al. 2003). Furthermore, PEA-3, a transcription factor of the Ets family is upregulated by Wnt and PEA-3 induces COX-2 expression (Howe et al. 2001). Taken together, these data suggest that multiple mechanisms may be in place for inducing COX-2 expression, and this is dependent on the inducer that is present near the target cell.

Post Transcriptional Control

In addition to the extensive transcriptional regulation, COX-2 is tightly regulated at the posttranscriptional levels of mRNA stability and translation. The first important point in this regard is the presence of two distinct transcripts that differ in the length of the 3'UTR (figure 7) (Ristimaki et al. 1996, Newton et al. 1997). This is the result of alternative polyadenylation resulting in transcripts of 2.8 and 4.6 kb, respectively. The major difference in the two transcripts is that the 4.6 kb COX-2 mRNA is degraded at a faster rate compared to the 2.8 kb mRNA when HCA-7 cells were treated with dexamethasone (Newton et al. 1997). This implies the presence of dexamethasone degradation element in the 3'UTR of 4.6 kb mRNA that is missing in the 2.8 kb mRNA. In addition IL-1 β has been shown to increase the half-life of COX-2 mRNA in mesangial cells through activation of the JNK and MAPK pathways (Guan et al. 1997, Guan et al. 1998, Diaz-Cazorla et al. 1999). This was associated with binding of cellular factors within the first 150 nt of the COX-2 3'UTR, suggesting a major role for this region in modulating COX-2 mRNA stability (Srivastava et al. 1994). More recently, Neeraja and colleagues have identified that in spermatogonial cells of mature rat testis, only the 2.8 kb isoform of COX-2 mRNA is expressed and that testosterone and follicle

stimulating hormones increased expression of COX-2 protein (Neeraja et al. 2003). It remains to be seen whether the hormones affected COX-2 mRNA stability and/or translation since COX-2 expression is tightly regulated in this manner (discussed below).

An important mechanism for posttranscriptional gene regulation in mammalian cells is rapid degradation of mRNAs mediated by AU rich elements (AREs) in their 3'UTR (Chen & Shyu 1995). The enhanced mRNA stability in tumour cells suggests that altered recognition of AU rich sequences in neoplasia may lead to improper function of AREs. Inspection of the COX-2 3'UTR revealed the presence of multiple AUUUA sequence motifs (figure 8) (Appleby et al. 1994, Cok & Morrison 2001). There are 22 copies of AUUUA throughout human COX-2 3'UTR (Appleby et al. 1994, Cok & Morrison 2001). Of these, many are located as tandem repeats in the first 60 nucleotides of the COX-2 3'UTR and form the minimal ARE sequence (Appleby et al. 1994, Cok & Morrison 2001, Mukhopadhyay et al. 2003). Deletion analysis has identified the first sixty nucleotides as a major mRNA stability and translational control element (figure 8) (Cok & Morrison 2001). In addition, other downstream regions of the COX-2 3'UTR are involved in mRNA stability and translational control (Figure 8) (Cok & Morrison 2001). These regions also contain scattered AUUUA sequences but do not conform to the canonical ARE that may be essential for ARE-mediated degradation activity (Cok & Morrison 2001). This suggests that a complex system of regulation exists for modulating COX-2 expression at the posttranscriptional level, which would include the *cis*-acting elements in the 3'UTR as well as cellular *trans*-acting factors that may bind to these elements to modulate activity (discussed below).

The stability of COX-2 RNA also depends upon the binding of different cytoplasmic factors to the 3' UTR of COX-2. Currently, three cellular proteins have been identified to play an important role in COX-2 mRNA stability and translation, namely HuR, Tristetraprolin (TTP) and CUGBP2. HuR is a ubiquitously expressed mammalian protein that is an ortholog of the *Drosophila melanogaster* protein, embryonic lethal abnormal vision (Antic & Keene 1997). HuR was originally identified to bind and

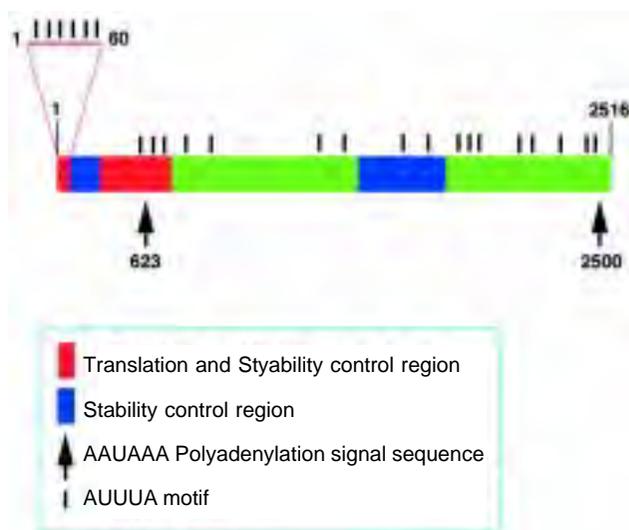


Figure 8. Post-transcriptional control of COX-2 gene expression. Multiple control elements are present in the COX-2 3'UTR that regulate COX-2 gene expression at the post-transcriptional level. Two polyadenylation signals located at nt positions 623 and 2500 of the 3'UTR (arrows) are responsible for the generation of two distinct COX-2 mRNAs as shown in figure 7. Multiple AUUUA sequence motifs (vertical lines) are present in the 3'UTR, which regulate either mRNA stability and/or translation. The first sixty nt of the COX-2 3'UTR is loaded with AUUUA sequence and is a primary modulator of mRNA stability and translation, based on reporter analyses (Cok et al. 2001, Mukhopadhyay et al. 2003). Regions of COX-2 3'UTR that regulate both mRNA stability and translation are depicted in red, while those that regulate mRNA stability only are shown in green. Regions that affect neither stability nor translation are shown in blue.

stabilize AU-rich containing mRNAs such as c-myc, c-fos and TNF- α (Ma et al. 1997, Fan & Steitz 1998, Levy et al. 1998, Peng et al. 1998, Xu et al. 1998). Recently, overexpression of HuR in HT-29 cells, a colon cancer cell line was shown to increase COX-2 expression (Dixon et al. 2001). Furthermore, it was observed to bind to COX-2 ARE sequences in lysates of these cells, and upon binding was able to increase the stability of a chimeric mRNA containing the coding region of firefly luciferase and the 3'UTR of COX-2 mRNA (Dixon et al. 2001). HuR binding to the ARE containing messages and stabilizing them suggests that a decrease in HuR level would result in a decline in COX-2. Indeed, reduction of HuR levels using antisense oligonucleotide or silencer RNA resulted in a significant decrease in COX-2 protein levels (Sengupta et al. 2003). These data suggest that a critical amount of HuR is essential for optimal expression of COX-2. Therefore, regulation of COX-2 by HuR is an important event in colorectal carcinogenesis.

More recently, HuR has been shown to bind to three high affinity binding sites in COX-2 3'UTR located between nt 48-54, 1155-1187 and 1249-1256 (Sengupta et al. 2003). We and others investigators have previously demonstrated that the first 60 nucleotides (nt), which contains the first site where HuR binds (nt 48-54) is very AU-rich and contains Class II ARE sequences (Gou et al. 1998, Cok & Morrison 2001, Mukhopadhyay et al. 2003). Furthermore, the first sixty nt of the COX-2 3'UTR is highly conserved during evolution suggesting a very important role for this region in regulating COX-2 gene expression (Sengupta et al. 2003). Moreover this region was identified as the minimal element required for COX-2 mRNA stability in response to p38 MAPK (Gou et al. 1998). On the other hand, the nt site between 1249-1256, which also interacts with HuR is essentially made up of U-rich sequences (Sengupta et al. 2003). The significance of this finding is currently unknown. However, as alluded to above, this region has been shown to regulate translation of COX-2 mRNA (Cok & Morrison 2001).

The second protein that was recently found to play a role in COX-2 mRNA stability is TTP. Also known as TIS11 and Nup475, TTP is an immediate early gene and a prototype for a family of zinc-binding Cys(3)His motif proteins that is required for proper regulation of TNF α -alpha mRNA stability in macrophages (Carballo et al. 1997). In addition, TTP was observed to precede the adaptive hyperplastic response after small bowel resection (Ehrenfried et al. 1995, Sacks et al. 1995). In contrast to HuR, TTP was found to bind to AREs of RNAs from immediate early genes such as c-fos, IL-8 and TNF- α and destabilize these messages resulting in rapid degradation (Carballo et al. 1998, Mahtani et al. 2001, Raghavan et al. 2001, Worthington et al. 2002). Similarly, TTP was found to be upregulated in HCA-7, a colon cancer cell line in a confluence-dependent fashion, and upon induction was observed to bind to COX-2 3'UTR (Sawacka et al. 2003). Furthermore, TTP was found to bind to an nt region between 3125 and 3432, which is present only in the 4.6 kb COX-2 mRNA but is lacking in the 2.8 kb mRNA (Sawacka et al. 2003). Consistent with the reported destabilizing effect of TTP on other genes, TTP

was shown to induce degradation of COX-2 mRNA and decrease COX-2 protein expression (Sawacka et al. 2003).

A third protein that we have described to affect COX-2 expression is RNA binding protein CUGBP2. CUGBP2, also known as ETR-3, BRUNOL3 and NAPOR2 is a prototype of the CELF family of RNA binding proteins was originally identified to bind to expanded CUG triplet repeats in the 3'UTR of an mRNA encoding a protein kinase involved in myotonic dystrophy (Choi et al. 1998, God et al. 2000, Ladd et al. 2001). CUGBP2 is ubiquitously expressed and is induced in intestinal epithelial cells when the cells were subjected to radiation treatment (Mukhopadhyay et al. 2003). COX-2 mRNA is also induced in these cells following radiation but mRNA translation is inhibited (Mukhopadhyay et al. 2003). This was found to be due to CUGBP2 binding to ARE in the first 60 nt of COX-2 3'UTR and stabilizing the mRNA but inhibiting COX-2 mRNA translation. This comes as a surprise and reflects a paradigm shift in the traditional concept that more mRNA does not necessarily mean more protein is expressed. It remains to be seen, however, whether this is a global phenomenon or is it unique to this situation. In any case, this inhibition of mRNA translation without enhancing degradation, as was seen with TTP makes the mechanism of CUGBP2 action rather unique.

Another RNA binding protein, T-cell internal antigen-1 (TIA-1) and TIA-1-related protein (TIAR) have been shown to bind to transcripts and promote polysome disassembly, thereby allowing the complex to be transported into stress granules (Kedersha et al. 1999, Kedersha & Anderson 2001, Anderson & Kedersha 2002a, 2002b). Since binding of CUGBP2 to the COX-2 mRNA results in polysome disassembly, it would not be too far fetched to speculate that CUGBP2 bound COX-2 mRNA is transported to stress granules when the cells are subjected to high levels of radiation stress.

Future Perspectives

COX-2 is a major controller of carcinogenesis and is highly induced in many epithelial tumours. Inhibition of COX-2 results in decreased PG synthesis thereby resulting in a reduction in

neovascularization as well as tumour growth. Hence, this pathway has become a great therapeutic target for the treatment of not only colon cancer but also in other cancers such as hepatocellular carcinoma, breast cancer, and head and neck cancer. Most non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COX-2 also affect COX1, which has remained a problem. There is a widespread recognition that people who take NSAIDs have serious gastrointestinal events such as perforation and bleeding thus limiting its use. However, with the discovery of COX-2 specific inhibitors such as celecoxib and rofecoxib, there has been hope that these may have therapeutic potential for cancer treatment. One does not really know the long-term effects of such drugs. Accordingly, it would be better if we could identify other ways of

inhibiting COX-2 as a way of treating cancer cells. This could be by way of inhibiting COX-2 completely, for which we need a greater understanding of the mechanisms that regulate its expression. More and more data is accumulating that COX-2 gene is not only regulated by transcription control mechanisms but also by the posttranscriptional mechanisms of mRNA stability/decay and translation. The 3'UTR fragment plays a key role in this process and may be a future therapeutic target for cancer treatment.

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