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Review

Cyclooxygenase-2 modulates cellular growth and promotes tumorigenesis

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Abstract

Cyclooxygenase (COX) -2 and the prostaglandins resulting from its enzymatic activity have been shown to play a role in modulating cell growth and development of human neoplasia. Evidence includes a direct relationship between COX-2 expression and cancer incidence in humans and animal models, increased tumorigenesis after genetic manipulation of COX-2, and significant anti-tumor properties of non-steroidal anti-inflammatory drugs in animal models and in some human cancers. Recent data showed that COX-2 and the derived prostaglandins are involved in control of cellular growth, apoptosis, and signal through a group of nuclear receptors named peroxisome proliferator-activated receptors (PPARs). In this article we will review some of the findings suggesting that COX-2 is involved in multiple cellular mechanisms that lead to tumorigenesis.

Keywords: Cyclooxygenase -1 and -2 • prostaglandins • cell growth • tumorigenesis • nonsteroidal anti-inflammatory drugs • angiogenesis • apoptosis • peroxisome proliferator-activated receptors

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Introduction

COX (also known as prostaglandin endoperoxide H synthase), the central enzyme in prostanoid biosynthesis, catalyzes the conversion of arachidonic acid to prostaglandin (PG) H_2. The enzyme encompasses two distinct enzymatic functions: a cyclooxygenase activity, which converts arachidonic acid to PGG_2, and a peroxidase activity, which transforms PGG_2 to PGH_2. PGH_2 is converted to biologically active PGs such as PGE_2 or TXA_2 by tissue-specific isomerases such as PGE_2 synthase and TXA_2 synthase [1]. Prostaglandins are important regulators of biologic processes such as inflammation, cell proliferation, pain, angiogenesis and vascular tone [2-4].

There are two distinct isoenzymes COX-1 and COX-2, similar in size, primary sequence [critical active site amino acids are conserved in both isoenzymes), and activity. Both enzymes are blocked by nonselective nonsteriodal anti-inflammatory drugs (NSAIDs), such as indomethacin and ibuprofen [5]. In the past few years, newer isoenzyme specific inhibitors such as celecoxib, rofecoxib and SC-560 became available for both research and medical applications [6-9]. Despite the similarities, the isoenzymes exhibit differences in patterns of tissue expression and cellular function. The COX-1 mRNA could be detected in most human tissues and that led to its description as the constitutive enzyme [10]. The COX-2 gene is rapidly expressed in a variety of cell types such as human endothelial, smooth muscle, monocytic cells and fibroblasts in response to growth factors, tumor promoters, hormones, bacterial endotoxins and cytokines [11-15]. In addition, COX-2 is induced in numerous processes such as cellular growth, differentiation and inflammation [16, 17]. Even though it was called the inducible isoform, COX-2 is constitutively expressed in brain, testes, tracheal epithelia, kidney macula densa [16]. However, in some cells such as the epidermoid carcinoma cell line A431, the mRNA cannot be detected even after treatment with phorbol esters [18].

Distinct genes located on different chromosomes encode the two isoenzymes. The COX-1 gene is 22 kb in length contains 11 exons [19] and lacks a TATA box, a common characteristic among developmentally regulated housekeeping genes. Much less is known about regulation of the expression of the COX-1 gene. The gene for COX-2 is 8 kb long and contains 10 exons [20]. The COX-2 gene promoter contains several enhancer sequences and expression can be induced through multiple signaling pathways involving protein kinase A and C, tyrosine kinases, lipopolysaccharide (LPS) and v-Src [16].

Different expression patterns for the two COX isoenzymes, and apparently differential access to cellular pools of arachidonic acid [21] have led to the hypothesis that COX-1 and -2 represent two distinct pathways for prostanoid biosynthesis. COX-2 is expressed in conjunction with cellular events such as differentiation and replication [18], and it has been hypothesized to be the major source of prostanoids involved in an alleged PPAR-mediated nuclear signaling system [22, 23]. Confocal immunolocalization studies suggested that a different cellular localization of the two isoenzymes might serve to separate the activities of COX-1 and -2 [24], yet electron microscopy has shown that the two isoenzymes have the same subcellular localization [25]. In human monocytes, NIH 3T3 cells, and HUVEC, COX-1 and -2 are present in endoplasmic reticulum and both inner and outer nuclear envelope membranes in similar proportions and, at least in 3T3 cells, both enzymes generate the same products. These findings did not exclude the possibility that COX-2 could provide prostaglandins for a nuclear eicosanoid signaling system [16, 23, 26]. However, they implied that generation of products functioning in the cell nucleus should result not from different localization of the two isoenzymes but from differences in enzyme kinetics and/or differences in interaction with various phospholipases [25].

Regulation of COX-2 expression

COX-2 expression is associated with many aspects of tumorigenesis such as transformation, cell growth and apoptosis, tumor angiogenesis, invasiveness and metastasis, and modulation of immune response. Thus, understanding the mechanisms involved in the regulation of expression of this enzyme will help to better understand the disease process. Although many of the tumor specific events are still unknown, revea-

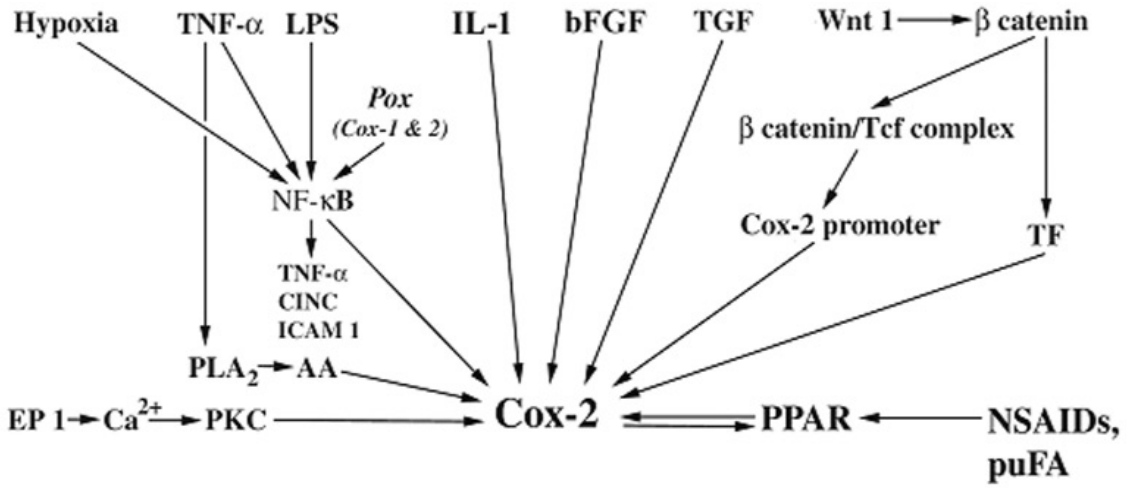

Fig. 1 Regulation of COX-2 expression. Cyclooxygenase 2 is an immediate early gene. Depending on the cell type it can be activated by a variety of stimuli. In HUVEC, COX-2 overexpression can be induced by IL-1, bFGF, TGF-α and by hypoxia through activation of transcription factor (TF) NF-κB. Additionally, NF-κB activation has been shown to mediate COX-2 mRNA expression in response to LPS, TNF-α, and peroxidase activity of both Cox-1 and -2. TNF-α also induces PLA_2 in the osteoblastic cell line MC3T3-E1 resulting eventually in COX-2 stimulation. In the RAC311 and C57MG cell lines, Wnt-1 expression induces up-regulation of the COX-2 gene possibly through activation of COX-2 promoter by the -catenin/Tcf complex. PGE_2 is a major product of COX-2 activity and it also can stimulate the COX-2 expression when bound to the prostaglandin receptor EP-1. NSAIDs are therapeutically used for their ability to block Cox activity. However, similar to polyunsaturated fatty acids (puFA), they can activate PPARα resulting in induction of the COX-2 expression.

ling these mechanisms is part of the ongoing effort (Figure 1). In the mouse mammary epithelial cell lines RAC311 and C57MG, Wnt-1 expression induced stabilization of cytosolic β-catenin, morphological transformation and transcriptional up-regulation of the COX-2 gene, resulting in increased levels of COX-2 mRNA and protein [27]. Roman et al [28] found that induction of COX-2 and the release of prostaglandins E2 and I2 from mouse colonocytes was augmented by activated Ras and transforming growth factor (TGF)-β_1. It has also been demonstrated that up-regulation of COX-2 is a downstream effect of Ras-mediated transformation in fibroblasts [29], intestinal epithelial cells [30], mammary epithelial cells [31] and non-small cell lung cancer cells [32].

In human umbilical vein endothelial cells (HUVEC) COX-2 mRNA expression can be induced by a variety of mitogens including interleukin-1α (IL-1α) [33], tumor necrosis factor (TNF) [34], LPS [15, 35], or fibroblast growth factor (FGF) [2] (Fig. 1). In normal human epidermal keratinocytes COX-2 expression is strongly upregulated by interferon-γ and TGF-α [36]. Similar to HUVEC, in endothelial cells derived from bone, COX-2 is induced by FGF and IL-1α. [37, 38]. The tumor promoter phorbol 12-myristate 13-acetate (PMA) induces an increase in the COX-2 mRNA with a minimal change in the COX-1 mRNA levels [2]. A similar effect is seen with the cytokine IL-1α [33]. Unlike IL-1α, stimulation of fibroblasts with IL-1β induced an increase in mRNA for both COX-1 and COX-2, and the accumulation of COX-2 protein is enhanced by ceramide [39]. Given that the specific inhibition of PKC or its depletion prevents COX-2 induction by PMA or IL-1α in endothelial cells [40], a possible direct regulation of COX enzymes by PKC was speculated. Potential serine and threonine targets for PKC phosphorylation exist in both COX enzymes, particularly in the18 -amino acid carboxyl-terminal extension of COX-2, which contains a PKC consensus sequence [41]. However, PKC (from rat brain) failed to phosphorylate human recombinant COX-1 and -2 *in vitro*, and in MEG-01 and NIH 3T3 cells, neither of the two isoforms where phosphorylated *in vivo* [42].

In HUVEC, increased COX-2 gene expression can be induced independent of chemical stimuli, by exposing cells to hypoxia, and the molecular mechanism involves the NF-κB p65 transcription factor [43]. Hypoxia also induces overexpression of vascular endothelial growth factor (VEGF) [44], suggesting a putative role for COX-2 in the biology of angiogenesis. However, the physiological significance of COX-2 induction by hypoxia in vivo remains to be established.

Role of COX-2 in tumorigenesis

NSAIDs in chemoprevention/chemotherapy

Several epidemiological studies have demonstrated a reduction in mortality from colorectal cancer (CRC) in individuals who are taking aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) [45, 46]. In the prevention side, clinical studies had demonstrated that treatment with NSAIDs in patients with Familial Adenomatous Polyposis caused regression of pre-existing adenomas [47] involving the COX enzymes in the process of polyp formation. Moreover, NSAIDs have proven to reduce tumorigenesis or to inhibit tumor growth in animal models of colon cancer [30, 45, 48].

Although the data obtained with nonselective NSAIDs implies a putative role for COX-2 in tumor biology a direct pharmacological evidence for the role of COX-2 in tumorigenesis was obtained using COX-2 inhibitors. For example, a COX-2 selective inhibitor SC-58125 decreased cell growth in both *in vitro* and *in vivo* assays only in cells expressing COX-2 [29, 30]. Furthermore, numerous groups have shown a marked reduction in tumor growth after treatment with COX-2 inhibitors [30, 49, 50]. An insight into the mechanism behind the anti-tumor efficacy of COX-2 inhibitors was provided by Zweifel *et al.* [51] who showed using a xenograft mouse tumor model that treatment with celecoxib (a COX-2 inhibitor) inhibited PGE_2 levels in the tumor. Moreover, neutralizing anti-PGE_2 antibodies also delayed tumor growth, suggesting that, at least in this system, PGE_2 is a critical effector downstream from COX-2.

Based on early experiments showing a significant reduction in xenograft neovascularization after treatment with NSAIDs [52] it has been hypothesized that COX-2 might play a role in regulation of angiogenesis associated with solid tumors. *In vitro* studies conducted by Tsujii et al [53] showed that COX-2 expression in colon cancer cells stimulates angiogenesis of co-cultured endothelial cells by up-regulating expression of VEGF, bFGF, TGF-β1, platelet derived growth factor (PDGF) and endothelin-1. Treatment with NS-398 decreased colorectal cancer cells ability to secrete these factors. Similarly the pancreatic cancer cell line BxPC-3 was capable of stimulating morphogenesis (in capillary like structures) of co-cultured endothelial cells and that effect was blocked by NS-398 and restored by adding back PGE_2 [54]. Strongly supporting the hypothesis that COX-2 plays an important role in tumor angiogenesis is the observation that selective COX-2 inhibitors reduced angiogenesis *in vivo* in several models [49, 55–59], while COX-1 inhibition had no effect on bFGF induced angiogenesis [49].

Even though NSAIDs are generally specific in their COX enzyme blocking action, there are numerous published observations suggesting COX/prostaglandin independent effects for some of these molecules, with the caveat that these results were obtained *in vitro* and using high drug concentrations. Qiao et al [60] have reported that sulindac sulfide can inhibit *in vitro* proliferation of a CRC cell line that does not express COX nor produce PGs. Along the same line, even though they lack COX enzymes, COX-1 and COX-2 null mouse embryo fibroblasts remain sensitive to the antiproliferative effects of NSAIDs [61].

Trying to explain these observations a number of researchers discovered that NSAIDs (including selective COX-2 inhibitors) could induce apoptosis [29, 62–64]. However, in some studies, NSAID-induced apoptosis was independent of COX-2 expression [62, 65–68] suggesting that NSAIDs stimulate apoptosis via both COX/PGs-dependent and -independent mechanisms [68, 69]. Possible PG independent mechanisms include inhibition of the protein kinase Akt [70] and suppression of NF-κB activation [71–74]. Another proposed explanation is that COX inhibition with NSAIDs leads to accumulation of arachidonic acid and stimulation of the conversion of sphingomyelin to ceramide, which then leads to apoptosis [75]. Some NSAIDs have been also demonstrated to act as ago-

nists/antagonists for PPARs (see below], suggesting that they may regulate gene expression [76–78].

Based on these observations we can conclude that NSAIDs, and particularly the new COX-2 selective inhibitors, are potential chemopreventive agents. The mechanism might involve control of angiogenesis or control of cell proliferation and apoptosis (see below). However, some NSAIDs could also act through COX independent mechanisms, yet at concentrations far exceeding the current therapeutic ranges [79].

Evidence that COX-2 promotes tumorigenesis

Studies aimed to explain the protective mechanism of NSAIDs in cancer revealed that COX-2 is overexpressed in CRC and other forms of epithelial cancers [80]. In CRC the COX-2 mRNA and protein levels are elevated when compared with normal adjacent mucosa [81]. In human pancreatic adenocarcinomas and cell lines derived from such tumors, COX-2 mRNA and protein expression were found to be frequently elevated [82]. Ristimaki et al [83] have found that human gastric adenocarcinoma tissue contains significantly higher levels of COX-2 mRNA when compared with paired gastric mucosa specimens devoid of cancer cells. Immunohistological detection of COX-2 protein showed cytoplasmic staining only of the gastric carcinoma cells. Similar, Uefuji et al [84] showed by immunoblotting and immunohistochemistry that COX-2 protein expression was elevated, in comparison to the normal mucosa, in 19 out of 23 (83%) human gastric adenocarcinomas examined.

In vitro, rat intestinal epithelial cells overexpressing COX-2 developed increased adhesion to extracellular matrix and resisted undergoing butyrate-induced apoptosis. This effect was reversed by treatment with NSAIDs, suggesting that overexpression of COX-2 and increased prostaglandin production is probably involved in the development and progression of colonic neoplasms [85]. Moreover, Ras-induced transformation of C57/MG cells resulted in increased levels of COX-2 mRNA and protein and increased production of PGE2 suggesting a role for COX-2 in tumorigenic transformation [31].

A direct evidence for the COX-2 involvement in tumor initiation and/or promotion was provided by genetic manipulation of COX-2. Oshima et al [86] showed that mice with a mutation in the adenomatous polyposis coli ($APC^{\Delta 716}$) gene, which developed hundreds of intestinal polyps, when bred with COX-2 null mice had a markedly reduced number of polyps when compared to the offspring of the $APC^{\Delta 716}$/COX-2 wild-type mice. In addition, we showed that selective overexpression of the human COX-2 gene in the mouse mammary glands driven by the murine mammary tumor virus promoter resulted in precocious differentiation of mammary gland and delayed mammary tissue involution [87]. Treatment with indomethacin, although causing a significant reduction in PGE_2 synthesis, failed to inhibit this phenotype. Prolonged overexpression of COX-2 in the breast led to a significantly increased incidence of mammary gland dysplasia and transformation into metastatic tumors. One notable conclusion of this study was that chronic COX-2 overexpression in mouse mammary gland was sufficient to trigger tumorigenic transformations alone or in combination with other naturally occurring factors. These two complementary experimental approaches using COX-2 deletion or overexpression clearly indicated that COX-2 is playing an important role in oncogenesis.

Possible mechanisms

A large amount of experimental data suggests that COX-2 is involved in complex processes related to cancer such as tumorigenic transformation, angiogenesis, immune response modulation, invasion and metastasis. However, the aim of this review is to focus on the cellular consequences of COX-2 overexpression and PGs action.

COX-2 and the derived prostaglandins modulate cell proliferation and apoptosis

It is well documented that overexpression of COX-2 and the subsequent increase in PG synthesis can

Table 1. Cumulative populations doublings of COX-2 expressing cells (Muristerone A stimulated) compared to unstimulated cells (Control - Ethanol).

Day	Muristerone A	Control (Ethanol)	p
4	1.527 ± 0.105	1.405 ± 0.116	0.4791
16	3.787 ± 0.162	3.503 ± 0.160	0.2803
21	5.589 ± 0.179	5.613 ± 0.152	0.9235
24	7.074 ± 0.131	7.424 ± 0.110	0.1102
27	**9.064 ± 0.123**	**9.535 ± 0.097**	**0.0397**
30	**11.158 ± 0.103**	**11.992 ± 0.069**	**0.0025**
35	**14.395 ± 0.125**	**15.616 ± 0.059**	**0.0009**
39	**16.932 ± 0.090**	**18.855 ± 0.044**	**<0.0001**
42	**19.525 ± 0.068**	**21.419 ± 0.033**	**<0.0001**
45	**21.453 ± 0.067**	**23.365 ± 0.061**	**<0.0001**
49	**24.233 ± 0.068**	**26.405 ± 0.123**	**<0.0001**
52	**26.489 ± 0.073**	**28.403 ± 0.091**	**<0.0001**

directly affect cell growth. For example, overexpression of COX-2 in COLO-320DM cells resulted in a PG dependent increase in growth rate and an induction of EGF receptor [88]. Numerous observations suggest that PGs stimulate cell proliferation alone or in combination with growth factors. For instance, it has been shown that PGE2 stimulated the proliferation of rat hepatocytes [89, 90] and human keratinocytes [91], or the proliferation of mammary epithelial cells in the presence of EGF [92]. Similar, in Balb/c 3T3 fibroblasts PGE_2 can induce mitogenic response in synergy with EGF [93]. Another prostaglandin, $PGF_{2\alpha}$, is also mitogenic for MC3T3-E1 osteoblasts [94] and Swiss 3T3 cells [95], and can induce mitogenic response in Balb/c 3T3 fibroblasts in synergy with EGF [93].

Another possible mechanism by which PGs can stimulate cell growth is by stimulating other signaling pathways that lead to cell proliferation. For example, in the breast tissue PGE_2 increased the expression and activity of aromatase [96, 97], leading to increased estrogen synthesis and indirect stimulation of cell proliferation. Also, growth response to some stimuli can involve PGs: in the rat seminal vesicles intracellular PGE_2 has been shown to be involved in the mitogenic effects of estradiol and testosterone [98, 99]. Similarly, topical application of a cancer promoting agent, 12-o tetradecanylphorbol-13-acetate, on mouse skin induced considerable PG synthesis at the site of administration and epidermal hyperproliferation. The cell proliferation was inhibited by indomethacin and this inhibition was reversed by the topical application of PGE_2 [100, 101].

Although in most of the occurrences PGs can stimulate cell growth, PGE_2 can inhibit blastogenesis of T-cells [102]. This, together with inhibition of the cytotoxic activity of natural killer cells [102], may contribute to the immune suppression associated with increased PG synthesis of some cancers.

COX-2 and the derived PGs can also contribute to tumorigenesis by affecting apoptosis. It is generally accepted that defective control of apoptosis is one of the central mechanisms of tumorigenesis because it allows cells that have acquired mutations to survive. COX-2 ability to confer cell resistance to apoptosis was observed in multiple studies. For example, rat intestinal epithelial cells transfected with COX-2 showed increased resistance to butyrate-induced apoptosis and demonstrated increased adhesion to the extracellular matrix proteins [85] (Table I). Similarly, in HCT-15 colon carcinoma cells stably transfected with COX-2 cDNA, COX-2 overexpression significantly attenuated apoptosis induced by 5-fluorouracil (5-FU) through mechanisms involving the cytochrome c-dependent apoptotic pathway [103].

Several studies have also established a direct role for PGs in rendering cells resistant to apoptosis. For example, in human colon cancer cells, PGE_2 induced the anti-apoptotic protein Bcl-2 and inhibited cell death caused by the treatment with the selective COX-2 inhibitor SC-58125 [29]. PGE_2 also inhibited apoptosis in human neutrophilic polymorphonuclear leukocytes [104]. Similarly, PGE_1 effectively inhibited apoptosis in

rat pheochromocytoma PC12 cells deprived of nerve growth factor [105]. Likewise, PGE_1, PGI_2 or PGD_2 decreased, in a dose-dependent manner the frequency of apoptotic nuclei and suppressed DNA fragmentation in rat hepatocytes cultured in collagen gel and treated with TGF-β1 or exposed to UV light [106].

PGs and the peroxisome proliferator-activated receptors (PPAR)

Recently, there has been a growing interest in the role on cellular proliferation of novel intracellular targets that can act as endogenous PG receptors. These receptors are PPARγ and PPARδ .

PPARγ, a critical transcription factor involved in adipocyte and monocyte differentiation [23, 107], binds to many lipophilic compounds including the endogenously generated 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) that results from dehydration of PGD_2 [108]. 15d-PGJ_2 is also the most potent activator of PPARγ discovered so far, suggesting that COX and PG synthases are responsible for the regulation of the PPARγ pathway. However, it is important to point out that high concentrations of 15d-PGJ_2 are needed to activate PPARγ thus questioning its relevance as an endogenous ligand.

PPARγ is expressed in a number of cancer cells [109, 110], macrophages [111] and endothelial cells [112, 113]. It has been reported that activation of PPARγ by 15d-PGJ_2 or synthetic agonists can markedly inhibit tumor cell growth by inducing apoptosis or terminal differentiation [114, 115]. In a recent study Girnun and co-workers [116] showed that heterozygous loss of PPARγ causes an increase in β-catenin levels and a greater incidence of chemically (azoxymethane) induced colon cancer. However, mice with a mutated APC (that is incapable of regulating β-catenin), develop tumors independent of PPARγ status, suggesting that PPARγ can suppress β-catenin levels and prevent colon carcinogenesis but only with a normal functioning APC/β-catenin pathway.

15d-PGJ_2 was also found to stimulate the expression of VEGF (a potent angiogenic factor) in vascular smooth muscle cells [117], the human androgen-independent PC 3 prostate and the 5637 urinary bladder carcinoma cell line [118], suggesting that it can indirectly stimulate (tumor) angiogenesis. However, at the endothelial cell level 15d-PGJ_2 and other ligands of PPARγ are anti-angiogenic both *in vivo* and *in vitro* by mechanisms that involve decreased expression of VEGF receptors [113] and apoptosis of endothelial cells [112].

PPARδ is almost ubiquitously expressed and may be an intracellular receptor for PGI2. PGI_2 is one of the most abundant PGs in vasculature [119] with potent anti-aggregatory and vasodilating activities [120, 121]. PGI_2 is also the most abundant PG in early pregnancy and, signaling through PPARδ, plays a critical role in embryonic implantation [122].

PPARδ expression is increased in primary human colorectal adenocarcinomas, rodent colorectal tumors, as well as preneoplastic colonic mucosa [76, 123, 124]. Interestingly, in colorectal cancers PPAR was co-localized with COX-2 within the tumor [76, 123]. Co-expression of COX-2 and PPARδ has also been reported in endometrial adenocarcinoma [125]. This co-localization might have significant functional importance as indicated by a study in which co-expression of COX-2 and prostaglandin I synthase led to endogenous PGI2 production and transcriptional activation of PPARδ [123], suggesting that proper coupling of COX-2 and prostaglandin I synthase leads to increased endogenous production of PGI_2 that uses PPARδ as its receptor.

Transcription of PPARδ is normally suppressed by wild-type APC. In colorectal cancer cells that have an inactivating APC mutation, the transcription of PPARδ is up-regulated through enhanced β-catenin/Tcf-4 binding to TCF-4-responsive elements in the PPARδ promoter [76]. It has been suggested that PPARδ might contribute directly to tumorigenesis: in a nude mouse xenograft model PPARδ (-/-) colon cancer cells exhibited a decreased ability to form tumors compared with PPARδ (+/-) and wild type controls [126]. However, a recent study [127] reported that PPARδ is dispensable for polyp formation in the intestine and colon of APC^{min} mice, but disruption of PPARδ gene appears to reduce the size of polyps, suggesting that further studies are required in order to fully unveil the role of PPARδ in colorectal neoplasm.

Further insight into the involvement and possible roles of PPARs in tumorigenic transformation was provided by the recent study of Shao and co-workers

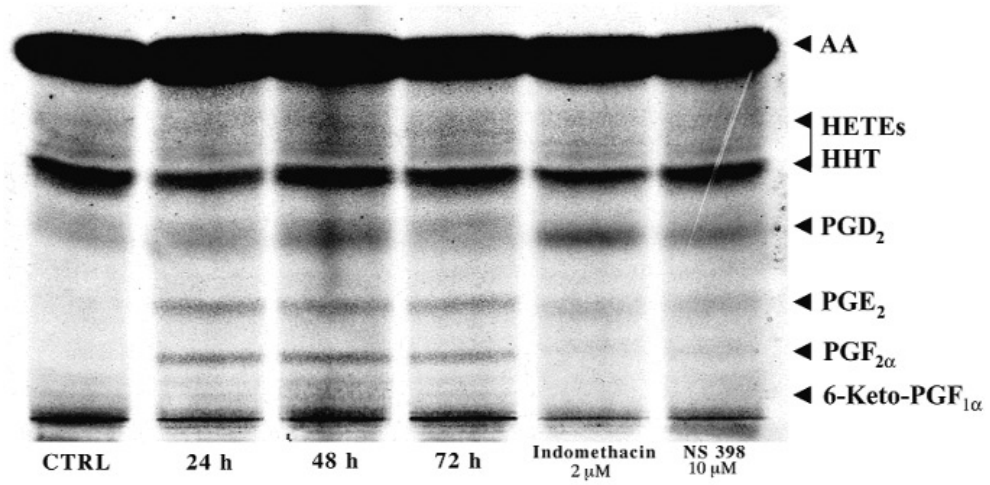

Fig. 2 COX-2 overexpression results in enhanced prostaglandin production**.** An inducible COX-2 expressing ECV-304 cell line designated C5 was used to evaluate the effect of COX-2 expression on prostaglandin production. Untreated cells, cells stimulated with Muristerone A for 24, 48 or 72 hours, or stimulated with Muristerone A for 24h and treated with 2μM Indomethacin or 10μM NS-398 were incubated with 12.5 μM [1-14C] Arachidonic Acid (AA) (Amersham Pharmacia Biotech) in serum-free medium. After 15 min at 37 °C, medium was collected and acidified with 1M HCl, then subjected to lipid extraction with 6 volumes of chloroform/methanol (2:1 v/v) and separation on silica G TLC plates in the solvent system Iw (ethyl acetate:isooctane:acetic acid:water, 11:5:2:10) as described [133]. TLC plates were then autoradiographed for 4 weeks. Radioactive eicosanoid bands were compared with cold standards (Cayman Chemical Co) visualized by staining with phosphomolybdic acid.

[128]. They found that in conditionally K-Ras-transformed rat intestinal epithelial cells, the level and activity of PPARδ were markedly increased requiring mitogen-activated protein (MAP) kinase activity and endogenous production of PGI_2 via the COX-2 pathway. Another interesting finding of that study was that activation of PPARγ in Ras-transformed cells resulted in a delayed transit through G1 phase of the cell cycle, suggesting that during Ras-transformation, PPARγ seems to control growth inhibition and differentiation, while PPARδ is directly up-regulated by Ras and activated by COX-2-derived PGI_2. However, a complete understanding of the mechanism by which COX-2 derived prostaglandins and PPARs promote neoplastic transformations in certain tissues warrants further study.

COX-2 overexpression causes delayed cell growth

Our work has also revealed an interesting effect of COX-2 overexpression. The bulk of our studies were conducted on ECV-304 cells, believed to be spontaneous immortalized HUVECs [129]. Later on, genetic analysis confirmed that ECV-304 and the bladder carcinoma cell line T-24 are identical, though ECV-304 should be considered a variant of T-24 [130].

When unstimulated, ECV-304 expressed very low levels of COX-2 or COX-1 mRNA. After transfection with COX-2 cDNA in the expression vector pCDNA/Neo and antibiotic selection, very few colonies were formed, and these exhibited very poor survival. After several weeks of culture, the COX-2 expression was lost [131]. This study suggested that COX-2 overexpression in ECV cells results in a growth disadvantage. Using the ecdysone inducible system [132], we obtained an ECV-304 cell line overexpressing COX-2 only upon Muristerone A (a synthetic analog of Ecdysone) administration (Figure 2). Several weeks of culture with continuous COX-2 overexpression led to a significant growth disadvantage (Table 1).

Using a COX-2 green fluorescent protein (COX-2/GFP) chimeric protein functionally

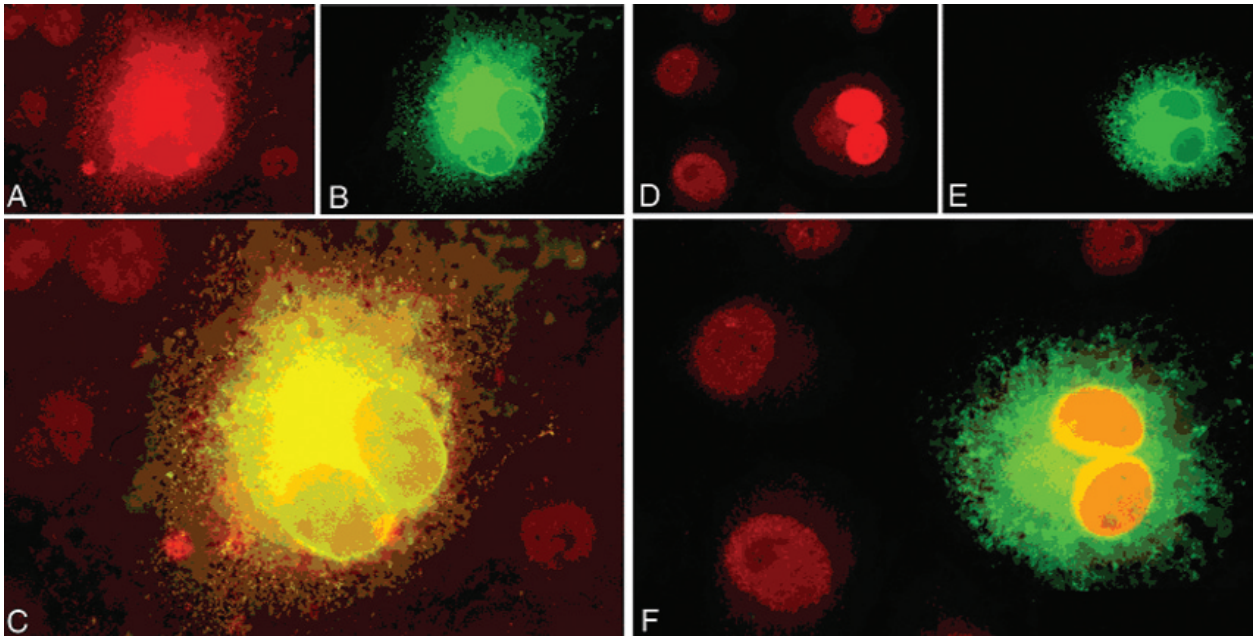

Fig. 3 COX-2 overexpression results in increased expression of p21 (A-p21 staining; B-COX-2/GFP; C composite image) and p27 (D-p27 staining; E-COX-2/GFP; F composite image). ECV-304 ells were grown on glass cover slips and were subjected to transient transfection with COX-2/GFP using lipofection reagents. Forty-eight hours later, cells were washed with PBS and fixed for 15 min with a 4% paraformaldehyde solution in PBS. Cells were then permeabilized for 5 min with 0.2% Triton X-100, washed with PBS and incubated for 90 min with anti-p21 or anti p27 (Santa Cruz Biotechnology) primary antisera. After several washes, the secondary antibody TRITC- conjugated antibody was incubated with the sample for 30 min. All specimens were mounted in 80% glycerol and photographed with a Zeiss-Axiovert 100 fluorescence microscope.

indistinguishable from wild-type COX-2, we had shown that COX-2 overexpression induced a delayed transit through G1 phase in a variety of cell types [133]. One interesting aspect of our observation was that the cell cycle arrest was independent of prostaglandin production. Pretreatment with 2 μM Indomethacin and 10 μM NS-398 for 48 hours had no effect on the cell cycle arrest caused by the COX-2-GFP transfection. However, such treatment resulted in virtually complete (> 95%) inhibition of prostanoid secretion from the cells transfected with COX-2/GFP.

To further demonstrate the distinction between cell growth arrest and the prostanoid synthesis we utilized two cyclooxygenase active site mutants of COX-2, S516Q and S516M [134]. Unlike S516Q, the S516M mutant possessed a partial oxidative activity, resulting in the production of cell-associated 15-(R)-HETE. Overexpression of S516M and S516Q mutants also induced cell-cycle arrest. All together, these findings excluded the involvement of the cyclooxygenase activity in the cell growth delay caused by COX-2 overexpression. A possible mechanism of action could involve the CDK inhibitors $p21^{cip-1}$ and $p27^{kip-1}$ as suggested by an increased immunoreactivity in the in COX-2 expressing ECV-304 cells (figure 3).

Recently, Zahner and co-workers [135] showed that overexpression of COX-2 in mesangial cells resulted in a significantly reduced proliferative capacity at 4h and 24 h comparative with mock-transfected cells. In these experiments cell cycle arrest was also prostaglandin independent since neither indomethacin nor NS-398 treatment had an effect on cell proliferation of COX-2 trasfected cells. Furthermore, COX-2 overexpression in mesangial cells inhibited PDGF induced proliferation. Interestingly, the delay in normal or PDGF-induced cell growth of COX-2 expressing

Table 2. Effects of COX-2 overepression on apoptosis, growth rate, invasiveness and cell adhesion in various cell lines.

Cellular effects		Cell type	Cell line description	Experimental model	Mechanism/ Observations
Apoptosis	Increase	HUVEC	Human umbilical vein endothelial cells		PPARγ
		ECV-304	Urinary bladder carcinoma (variant of T24)	15d-PGJ_2	PPARγ
	Resistance	HCT-15	Human colon cancer carcinoma	COX-2 transfection, 5-FU induced apoptosis	cytochrome c, Bcl-2
		RIE 1	Rat intestinal epithelial cells	COX-2 transfection	Reversed by NSAIDs
Growth rate	Increase	COLO 320 DM	Human colon cancer carcinoma	COX-2 transfection	Reversed by NSAIDs
		MCF-7	Human breast cancer	15d-PGJ_2	
		HCA-7	Human colon cancer carcinoma	COX-2 constitutively expressed	15d-PGJ_2, reversed by NSAIDs
		HCT-15	Human colon cancer carcinoma	15d-PGJ_2	Cox-null cell line
		PC-3	Prostate carcinoma	PGE_2	Reversed by NSAIDs
		MC3T3-E1	Osteoblastic cell line	PGE_2	Reversed by NSAIDs
	Decrease	HEK 293	Human embryonic kidney cells	COX-2 transfection	PG independent
		COS-7	Transformed monkey kidney cells	COX-2 transfection	PG independent
		NIH 3T3	Fibroblast cell line	COX-2 transfection	PG independent
		BMEC	Bovine microvasculature endothelial cells	COX-2 transfection	PG independent
		ECV-304	Urinary bladder carcinoma (variant of T24)	COX-2 transfection	PG independent
Increased invasive capacity		Caco-2	Human colon cancer carcinoma	COX-2 transfection	Metalloproteinase, reversed by NSAIDs
		RIE 1	Rat intestinal epithelial cells	COX-2 transfection	Increased urokinase, involves TGF- β1, reversed by NSAIDs
Increased cell adhesion		RIE 1	Rat intestinal epithelial cells	COX-2 transfection	Reversed by NSAIDs

mesangial cells seems to be mediated through an induction of the tumor suppressor gene p53 and the CDK inhibitors $p21^{cip-1}$ and $p27^{kip-1}$ partially independent of prostaglandin production.

These data suggest that cyclooxygenase activity and prostaglandins are not necessarily involved in the growth arrest induced by COX-2 overexpression in various cell lines. The general understanding is that COX-2 overexpression and activation of the relevant phospholipases result in the extracellular secretion of prostanoids such as PGE_2 [2, 136]. However, the peroxidase activity of the COX-2 isoenzyme may function in signal transduction; for example, it was shown that the redox-sensitive transcription factor NF-κB is regulated by the peroxidase activity of the COX enzyme [137]. Thus, such a pathway may be involved in the growth arrest mechanism. Alternatively, a direct interaction of COX-2 with cell-cycle regulatory proteins in the ER or the nuclear envelope can result in the modulation of cell growth. A novel protein-protein interaction should not be such an unexpected occurrence since both COX-1 and -2 were shown to bind to nucleobindin, an apoptosis regulatory protein [138]. Certainly, further studies will be necessary to explain the relevance of these results in the context of tumorigenic transformation and tumor growth.

Perspective

Current data implies COX-2 involvement in many steps of cancer progression. Although is unclear how COX-2 elevation exerts its oncogenic effect at the molecular level, COX-2 derived PGs could signal in an autocrine or paracrine manner triggering a wide variety of complex cellular processes that lead to tumor growth (table 2). Over the past few years a great deal of effort was concentrated on establishing the relationship between COX-2 and angiogenesis. A growing body of evidence suggests that COX-2 and the derived prostaglandins are involved in the control of angiogenesis, a process required for the growth of tumors beyond a certain size. Is not very clear whether COX-2 expression in the cells associated with the blood vessels (i.e. stromal cells, endothelial cells), or in the tumor cells, is important for angiogenesis. The results published so far in the literature suggest that COX-2 overexpression in all these cells may contribute to the angiogenic process. An important therapeutic implication of these findings is that COX-2 inhibitors could prove beneficial for the treatment of all types of cancers that depend on angiogenesis rather than only malignancies characterized by high levels of COX-2 expression.

The anti-cancer properties of COX-2 specific inhibitors and NSAIDs in general make them attractive tools in cancer prevention and treatment. The epidemiological data, clinical trials and data from animal models strongly suggest that NSAIDs, and in particular COX-2 inhibitors, are emerging as a novel class of anti-cancer agents that could be used in combination with other anti-tumor drugs with non-overlapping mechanisms of action [50]. Although the anti-tumor properties of these drugs are most likely derived from their effect on COX-2, there is a possibility that NSAIDs, in addition to decreasing PG production, could also modulate cell growth by regulating PG-independent pathways. However, at least for celecoxib, under regular treatment conditions the plasma concentrations achieved in animal models are consistent with inhibition of PG synthesis and inhibition of angiogenesis [139, 140]. Future clinical studies will be required to prove the efectiveness of such drug combinations in human cancer patients.

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