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Mini-review

Cyclooxygenases in cancer: progress and perspective

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Abstract

Aspirin has been used to control pain and inflammation for over a century. Epidemiological studies first associated a decreased incidence of colorectal cancer with the long-term use of aspirin in the early 1980s. Near the same time the first reports showing regression of colorectal adenomas in response to the non-steroidal anti-inflammatory drug (NSAID) sulindac were reported. In subsequent years, the use of other NSAIDs, which inhibit cyclooxygenase (COX) enzymes, was linked to reduced cancer risk in multiple tissues including those of the breast, prostate, and lung. Together these studies resulted in the identification of a new cancer preventive and/or therapeutic target-COX enzymes, especially COX-2. Meanwhile, the overexpression of COX-2, and less consistently, the upstream and downstream enzymes of the prostaglandin synthesis pathway, was demonstrated in multiple cancer types and some pre-neoplastic lesions. Direct interactions of prostaglandins with their receptors through autocrine or paracrine pathways to enhance cellular survival or stimulate angiogenesis have been proposed as the molecular mechanisms underlying the pro-carcinogenic functions of COX-2. The rapid development of safe and effective inhibitors targeting individual COX enzymes not only dramatically improved our understanding of the function of COX-2, but also resulted in discovery of COX independent functions of NSAIDs, providing important hints for future drug design. Here we review the fundamental features of COX enzymes, especially as related to carcinogenesis, their expression and function in both animal tumor models and clinical cancers and the proposed mechanisms behind their roles in cancer.

Keywords: Cyclooxygenase; Cancer; Angiogenesis; Non-steroid anti-inflammation drug; Review

1. Introduction

Aspirin was introduced as an anti-pyretic, anti-inflammatory and analgesic drug at the end of nineteenth century. Soon after, a family of drugs with similar properties were discovered and collectively termed non-steroidal anti-inflammatory drugs (NSAIDs). In the late 1960s work from

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Samuelsson and Bergstrom revealed the prostaglandin synthesis pathways [1–3] and a few years later, J.R. Vane and his colleagues identified the therapeutic target of NSAIDs as the cyclooxygenase (COX) enzyme [4]. The Noble Prize for Physiology or Medicine was awarded to Drs. Vane, Samuelsson and Bergstrom in 1982 'for their discoveries concerning prostaglandins and related biologically active substances' [5]. Both epidemiological and randomized clinical trials have indicated efficacy, albeit not uniformly, in the ability of aspirin and/or NSAIDs to decrease colorectal cancer [6–9].

A number of epidemiological studies have indicated that long term aspirin/NSAID use is associated with 30–50% reduction in risk of colorectal cancer or adenomatous polyps or death from colorectal cancer [10]. In addition, these studies suggest that the duration and the consistency of NSAID use are more important than the dosage. Other epidemiologic studies also found associations between NSAID use and a lower death rate from cancers of the esophagus, stomach, breast, lung, prostate, urinary bladder and ovary [11,12].

Meanwhile, Dr. William Waddell reported the regression of rectal polyps in a small number of familial adenomatous polyposis (FAP) patients in response to the NSAID sulindac [13,14]. This work has been extended by a number of epidemiological studies as well as clinical trials. The results from the completed randomized double-blind placebo controlled trial on FAP patients suggest that sulindac and celecoxib cause adenoma regression in some polyposis patients, and in some cases, a complete regression is seen [15–19]. Clinical trials on other high risk populations have generally shown a beneficial reduction in adenoma number and/or size, although the effects are inconsistent [9,20–24]. In young FAP patients who were entered into a randomized clinical trial prior to the development of colorectal adenomas, there was no significant effect of sulindac on preventing de novo adenoma formation [25]. In a large scale randomized clinical trial to determine the ability of aspirin to prevent myocardial infarction, there was no reduction in colorectal cancer in the patients receiving aspirin in a secondary analysis [26]. Taken together, despite the early very promising results, currently there is not sufficient evidence to recommend wide-spread use of any of these agents for primary prevention of colon cancer. More clinical trials are ongoing with aspirin, sulindac, celecoxib and refocoxib and we await the results of these trials to provide a more complete estimate of the chemo-preventative value of NSAIDs.

2. Cyclooxygenase genes and enzymes

In 1988, three different groups cloned a gene encoding cyclooxygenase, which later turned out to be the constitutive isoform—COX-1 [27–29]. Subsequently, the inducible isoform of COX was discovered and named—COX-2 [30–33]. The human gene encoding the COX-1 enzyme (*PTGS1*) is located on chromosome 9 (9q32–9q33.3), contains 11 exons and spreads across 40 kb; its mRNA is approximately 2.8 kb [34]. The gene encoding COX-2 (*PTGS2*) is located on chromosome 1 (1q25.2–25.3), contains 10 exons and encompasses 7.5 kb with a 4.5 kb transcript [35]. Despite the difference in genomic structure and transcript size, the proteins of both COX enzymes are about 600 amino acids with the calculated molecular weight as 68 kDa unmodified and about 75–80 kDa after post-translational modifications, which mainly consist of glycosylation [36].

Despite their similarities, the expression pattern and regulation of these two isomers are different [37]. While there are notable exceptions, a simplified view is that COX-1 is constitutively expressed with near constant levels and activity in many tissues, whereas COX-2 is an inducible or early-response gene. COX-2 expression is low or negative in most tissues; however, a few hours after a single stimulation, the mRNA, protein and enzymatic activity of COX-2 increase more than 10-fold and then return promptly back to the basal level. Exceptions to this include portions of the central nervous system (CNS), the kidney and the seminal vesicles, which contain constitutively high levels of COX-2. The best studied inducers of COX-2 are bacterial lipopolysaccharide (LPS), pro-inflammatory cytokines-interleukin (IL)-1β, IL-2 and tumor necrosis factor (TNF)-α [38–41]. Growth factors (e.g. epidermal growth factor (EGF), platelet derived growth factors (PDGF)) and some tumor promoters such as phorbol-12-myristate-13-acetate (PMA) also stimulate COX-2 expression [42,43]. On the other hand, anti-inflammatory

molecules such as corticosteroids, IL-13, IL-10 and IL-4 suppress the expression of COX-2 [44]. Finally, COX-1 protein can also be induced in certain cell types by either phorbol esters or dexamethazone [37]. Therefore, the simplistic notion that COX-2 is the inducible form and COX-1 is the constitutive form is probably an oversimplification.

3. Functions of cyclooxygenases

Prostaglandins were first discovered in semen or in the extract of prostate as lipid soluble compounds with potent vasodepressor and smooth muscle-stimulating activity. They were named based on the fact that they were believed to be derived from the prostate [45,46]. Now it is clear that the normal human prostate itself is not the major source of prostaglandins. The large amounts of prostaglandins in the semen are derived from the nearby seminal vesicles, which are one of the most abundant sources of prostaglandins in the body.

Prostaglandins and leukotrienes compose a large family of regulatory molecules termed eicosanoids, which include almost all long-chain oxygenated polyunsaturated fatty acids derived from arachidonic acid (20:4ω6) [2,47]. Prostaglandins, which are also referred to as prostanoids, are composed of the cyclic oxidized members of the eicosanoid family. Prostaglandins can be produced in almost every human cell type and act as autocrine and/or paracrine mediators through their specific receptors. De novo prostaglandin synthesis starts with the oxidative cyclization of the five carbons at the center of arachidonic acid, which is released by phospholipase A_2 (PLA_2) from the cell membrane. The free arachidonic acid is then presented to the endoplasmic reticulum (ER) and nuclear membrane, where the COX enzymes catalyze the rate-limiting step for prostaglandin synthesis—the generation of the bio-cyclic endoperoxide intermediate—prostaglandin G_2 (PGG_2) and the reduction to prostaglandin H_2 (PGH_2) [48]. In different cell types and under different physiological conditions, the down stream metabolism of PGH_2 can be dramatically different. Prostaglandin D (PGD) synthase is usually found in mast cells and in the brain; prostaglandin F (PGF) synthase is expressed in the uterus; prostaglandin I synthase (also called prostacyclin synthase, PGI) is found in endothelial cells; thromboxane synthase is commonly seen in platelets and macrophages; and prostaglandin E isomerase appears in most cell types. There are also non-enzymatic mechanisms involved in the transformation of PGH_2 into primary prostaglandins. In some cases, the COX enzymes and the subsequent prostaglandin synthase(s) are coordinately regulated. For example, during inflammatory cell activation, macrophages increase the expression of both COX-2 and prostaglandin E isomerase [49].

4. Structure of cyclooxygenases

COX-1 and COX-2 share the same substrates, generate the same products, and catalyze the same reaction using identical catalytic mechanisms. When the X-ray crystal structures of these two enzymes were solved, both human and murine COX-2 could be largely superimposed on that of COX-1, with the amino acids serving as the substrate binding pocket and catalytic site being nearly identical to each other. One exception with profound implications is that the isoleucine 590 around the substrate channel of COX-1 is replaced by valine in COX-2 [50–52], which gives COX-2 a larger substrate binding pocket and consequently a broader substrate spectrum. For arachidonic acid and dihomo-γ-linolenate, COX-1 and COX-2 are equally effective, but for other fatty acids such as linoleic acid and eicosapentaenoic acid, COX-2 is significantly more efficient than COX-1 [53]. The isoleucine/valine substitution is also the structural basis for the COX-2 selective inhibitors. Co-crystals of either COX-1 or COX-2 with its selective inhibitor showed that the smaller valine in COX-2 allows the bulk structure of COX-2 selective inhibitors to access the substrate-binding site, while the larger isoleucine in COX-1 prevents their binding [50,51]. It also explains the different degrees of inhibition that aspirin possesses towards COX-1 and COX-2. As an irreversible inhibitor, aspirin acetylates serine 530 in COX-1, completely abolishing its ability to oxidize arachidonic acid; while after similar acetylation, COX-2 can still oxidize arachidonic acid, but to 15*R*-hydroxyeicosatertraenoic acid (HETE) instead of PGG_2 [54–56]. The retention of oxygenase activity in COX-2 has been attributed to the larger overall space

available in the COX-2 active site than that in COX-1. Therefore, the acetylation of serine 530—critical for controlling the configuration of prostaglandins at the 15-carbon—can better be accommodated in COX-2. Another important structural difference between these two enzymes is that COX-2 contains an insertion of 18 additional amino acids towards its C-terminus and is missing 17 amino acids from its N-terminus in comparison to COX-1 [31]. It is known that the C-terminal insertion in COX-2 does not alter the last four amino acids, which are believed to serve as the ER-targeting signal for both proteins. COX-2 is localized to both the ER and the nuclear envelope, while COX-1 is only found in the ER. It has been suggested that the C-terminal insertion might contribute to the nuclear membrane localization of COX-2 [31,57,58]. When the X-ray crystal structures were published, the last 18 amino acids of COX-1 and the last 30 amino acids of COX-2 were unsolved, presumably due to the high flexibility of these regions even in the crystalline forms [50,52]. Further investigation is needed to elucidate the functional significance of the different termini.

5. Genetic evidence for an association between COX-2 and cancer

The studies from a murine model of FAP (mice carrying $APC^{\Delta 716}$) provided the first genetic evidence for a link between COX-2 and carcinogenesis. When $APC^{\Delta 716}$ mice were crossed with mice containing targeted mutations that inactivate the *Pgst2* gene (homozygous or heterozygous), the size and number of small intestinal and colonic polyps, especially the number of large polyps were reduced in a dose-dependent manner in comparison with the *Pgst2* wide-type littermates [59]. Deletion of the gene (*Pla2g4*), encoding the upstream enzyme PLA_2—in the same mouse model for colorectal cancer resulted in a significant decrease of the size but not the number of polyps in small intestine, and neither size or number of polyps in the colon [60]. The authors attributed the discrepancy in *Pgst2* and *Pla2g4* knockout mouse models to the fact that the arachidonic acid might be potentially provided by other PLA_2 isoforms other than that encoded by *Pla2g4* in the colon. Genetic disruption of *Pla2g4* in another mouse model for colon cancer—APC^{Min}, confirmed the protective effects of *Pla2g4* deletion in the small intestine [61]. Among the various downstream prostaglandins, PGE_2 has long been suggested as the key player for the following reasons: (i) PGE_2 concentration is increased in colon cancer tissues where COX-2 is overexpressed [62]; (ii) PGE_2 can induce angiogenesis in vitro and increases cellular resistance to apoptosis which fits into the proposed mechanisms for COX-2 to promote carcinogenesis [63]; (iii) only the prostaglandin E receptor (*Ptger*) knockout mice, but not other single prostaglandin receptor (prostaglandin D, F, I and thromboxane receptor) knockout mice, show a significant decrease in the number of aberrant crypt foci when compared with the wild type controls (Table 1). There are four subtypes of prostaglandin E receptors (EP 1–4), whose genes are designated *Ptger1–4*. The results on *Ptger1* and *Ptger2* knockouts were not consistent among different colon cancer animal models. Sonoshita et al. reported that knockout of *Ptger2*, but not *Ptger1* decreased the number and size of polyposis in $APC^{\Delta 716}$ mouse through blocking angiogenesis [64]. The decrease is parallel to the *Pgst2* knockout [59,64]. They also showed that the expression of Ptger2, but not Ptger1, 3 or 4 was elevated in polyps with reference to normal tissue from the small intestine and colon. But in the azoxymethane (AOM) induced rodent colon cancer model, *Ptger1* but not *Ptger2* knockout mice showed decreased numbers of aberrant crypt foci, which could also be recapitulated in the Min mouse model by the Ptger1 antagonist—ONO-8711 [65]. Knockout of *Ptger3* showed no effect in either the AOM-induced colon cancer model or $APC^{\Delta 716}$ models [65,66]. *Ptger4* was implicated as a key player in the AOM induced colon cancer model (not tested in $APC^{\Delta 716}$) and the Ptger4 antagonist—ONO-AE2-227 decreased the polyp number, especially the number of polyps larger than 1.5 mm in APC^{Min} mice [67]. Pai et al. reported that in addition to acting on its own receptor, PGE_2 could also activate the epidermal growth factor receptor, providing another potential mechanism for the tumor promotion effect of COX enzymes [68]. These results not only further support the role of COX-2 as a tumor promoter in the intestine, but also point to PGE_2 as the key mediator of the COX-2 related susceptibility to colon cancer. These findings suggest the PGE isomerase might be a more specific target for colorectal

Table 1
Prostaglandins and their implication in cancer

Prostaglandin	Receptor/G protein	Second messenger	Implication in cancer
PGD_2	DP/Gs	↑cAMP	DP1 null do not change the number of AOM induced aberrant crypt foci
PGE_2	EP1/unknown	↑Ca^{2+}	EP1-null-↓number of AOM induced aberrant crypt foci EP1 antagonist-↓the number of polyps in APC^{Min} EP1-null-do not change the number of polyps $APC^{\Delta 716}$ mice
	EP2/Gs	↑cAMP	EP2 null-↓number of large polyps in $APC^{\Delta 716}$ mice EP2 null-do not change AOM induced aberrant crypt foci
	EP3/Gs or Gi	↑cAMP or ↓cAMP	EP3 null do not change polyps number in $APC^{\Delta 716}$ and AOM induced aberrant crypt foci
	EP4/Gs	↑cAMP	EP4 null-↓AOM-induced colon cancer EP4 antagonist-↓number of large polyps in APC^{Min} EP4-null-do not change polyp number in $APC^{\Delta 716}$ mice Promotes proliferation through Akt pathway
PGF_2	FP/Gq	PI response	Counteracts indomethacin to restore DMBA/TPA induced skin tumors
PGI_2	IP/Gs, Gq	↑cAMP, PI response	Overexpression of PGI synthase inhibits neovacular formation in colon cancer xenograft model Transgenic over expression of PGI syn protect mice from carcinogen induced lung cancer
TXA_2	TP_α/Gi, Gq	↓cAMP, ↑Ca^{2+}	TP agonists-restore neovascular formation blocked by COX-2 inhibitors
	TP_β/Gs, Gq	↑cAMP, ↑Ca^{2+}	Overexpression of TXA synthase promotes vascular formation in colon cancer xenograft model

cancer prevention in comparison with COX-2. Various PGE receptor antagonists have been developed and are being tested in animal models.

Is constitutive expression of COX-2 sufficient to transform cells? When the *Pgst2* gene was placed downstream of the murine mammary tumor virus (MMTV) promoter, its expression was induced in the mammary gland during pregnancy and lactation. This high level of COX-2 expression causes mammary gland hyperplasia, carcinoma and eventually metastatic breast cancer in multiparous mice but not virgin mice [69]. Pregnancy and lactation associated COX-2 expression was strong in mammary gland epithelial cells and weak in surrounding stromal cells, which correlated with increased PGE_2 and PGF_2 levels. Interestingly, the increased expression of the anti-apoptotic molecule—Bcl-2 and decreased expression of its counterparts—Bax and Bcl-xL were only seen in tumor tissues, but not in the adjacent normal from the transgenic mouse. It is not clear whether this reflects an effect of transformation or actually contributes to the transformation. Given that the surrounding normal mammary gland epithelial cells with COX-2 expression did not have the altered expression of Bax and Bcl-xL, changing of apoptotic balance might not be a direct consequence of COX-2 overexpression.

Recently two transgenic mouse models have been generated to study the role of COX-2 in skin tumor initiation and promotion. *Pgst2* cDNA was inserted downstream of the keratin 5 and keratin 14 promoters to achieve constitutive COX-2 expression in the basal region of the interfollicular epidermis and the pilosebaceous unit [70,71]. Both transgenic strains developed significant alopecia, which was successfully corrected in the K14.COX2 mice by administration of the COX-2 specific inhibitor—celecoxib (not tested in K5.COX2 mice). Some K5.COX2 mice displayed spontaneous hyperplasia in the scale epidermis of the tail with focal signs of dysplasia.

No spontaneous hyperplasia was reported from K14.COX2 mice. When skin tumors were initiated in the K14.COX2 mice by topical application of 7,12-dimethylbenz [α] anthracene (DMBA) and subsequently promoted by PMA, the tumor incidence and multiplicity decreased dramatically on two different genetic backgrounds. This surprising result was bolstered by the administration of celecoxib before DMBA induction, which increased the tumor incidence in K14.COX2 mice, further suggesting a protective role of COX-2 in the DMBA/PMA tumor model. Different results were obtained, however, with the K5.COX-2 mice. While these mice also develop alopecia, they are prone to develop hyperplasia and focal dysplasia in tail skin [72]. In tumor initiation-promotion experiments, these mice readily developed tumors (squamous papillomas, squamous carcinomas, and sebaceous gland adenomas), in response to DMBA alone—they did not require subsequent PMA administration, as needed for tumor development in wild type mice [71]. However, there was a change in the proportion of the different tumor types in the DMBA alone induction experiments, with a higher proportion than usual of sebaceous adenomas. Administration of celecoxib before DMBA application or between DMBA and PMA applications both decreased the tumor multiplicity in comparison to control group, with no difference between these two treatment schedules. The authors of this study concluded that these data support a role of COX-2 in tumor promotion, but not in initiation [71]. The discrepant results found in these two different transgenic mouse models are difficult to reconcile. The different promoters (keratin 5 vs. keratin 14) used and the diverse genetic backgrounds might both contribute to the discrepancies in the results, given that strain-dependent responses by skin tumors to COX-2 inhibitors have been reported previously [73,74]. Previous studies showed that the non-specific COX inhibitor, indomethacin, was able to reduce the multiplicity of tumors induced by DMBA/PMA; furthermore, topical application of PGF_2 but not PGE_2 counteracted indomethacin [74]. Taken together, the effects of COX-2 on skin carcinogenesis in mouse models is certainly unclear at this time. Some experiments clearly show that expression or overexpression of COX-2 promotes carcinogenesis, and that inhibition of COX-2 prevents tumorigenesis. By contrast, other experiments, not only refute this concept, but actually strongly suggest the opposite—COX-2 expression may protect skin from carcinogenesis.

In an attempt to dissect the contribution of COX-2 from different cell types, Williams et al., implanted COX-1 and -2 positive Lewis lung carcinoma (LCC) grafts into genetically compatible C57/BL6 mice that were either wild type, or containing targeted disruptions of either *Pgts2* or *Pgts1*. Seven days after the engraftment, LLC tumors grown in the $Pgts2^{-/-}$ hosts started to show a statistically significant smaller size in comparison with the tumors in either wild type or $Pgts1^{-/-}$ hosts and this decrease correlated with decreased levels of VEGF and vascular density in the tumors [75]. These results implicated non-tumorigenic host cells as potential key factors of COX-2 mediated tumor growth.

Disruption of COX-2 itself or its upstream or downstream genes by means of gene knockout is not sufficient to stop the initiation of polyps in either $APC^{\Delta 716}$ mice or AOM induced colon cancer model, which suggests that COX-2 and its related pathways serve as modulators for tumor growth, but not single agent initiators. In keeping with this, in $APC^{\Delta 716}$ mice COX-2 expression only becomes obvious when the size of polyps is larger than 1 mm in diameter and positive staining cells are mostly stroma cells, not the epithelial cells in the polyps. Furthermore, COX-2 expression in $APC^{\Delta 716}$ mice correlated with the expression of angiogenesis factors (e.g. VEGF and bFGF). Together these results suggested that COX-2 and the related prostaglandin pathways affect colon polyp growth beyond 1 mm through modulating angiogenesis. In contrast to this, in all the transgenic mouse models of skin carcinogenesis mentioned above, the ectopic expression of COX-2 was in the epithelial cell components. Yet spontaneous tumor formation is only seen in the MMTV driven COX2 expressing mammary glands of multiparous mice, but in none of the K14.COX2 or K5.COX2 mice. This suggests that the expression level of COX-2 needed to transform cells is very high and other initiation factors are most likely needed to achieve the transformation. Second, the alteration in the spectrum of tumor types occurring in K5.COX2 mice treated with DMBA/PMA or DMBA alone suggests different sensitivity to COX-2 mediated tumor promotion in different cell populations. Finally, high levels of COX-2 protein are not

sufficient to induce cancer when physiologically expressed, such as in the seminal vesicles, which virtually never develop cancer.

While most of the focus has been on COX-2, APC^{Min} mice carrying inactivated *Pgts1* genes also had a 80% reduction of tumor multiplicity in comparison to the $Pgts1^{+/+}$ littler mates [76]. In addition, inhibitors that preferentially block COX-1 (e.g. piroxican) have protective effects against colon cancer in both animal experiments and epidemiological studies. On one hand, these data reinforce the role of prostaglandins as common mediators for COX enzymes related to tumor promotion, yet also raise the question regarding how much each isoform contributes to tumor promotion.

When examining the data regarding the function of cyclooxygenase genes in mouse models, some of the surprising phenotypes of these mice are of interest. First, despite the long standing belief of COX-2's primary function in the inflammatory response, *Ptgs2* knockout mice show a normal response to acute inflammation induced by arachidonic acid or PMA in an ear-edema test [77,78]. Second, although inhibition of COX-1, not COX-2, has been proposed to be responsible for the renal deficiency associated with using non-specific COX inhibitors, especially aspirin, *Ptgs1* knockout mice showed no defect in kidney function, unless the kidneys were compromised by other disease (e.g. diabetes, hypertension etc.) [78,79]. However, *Ptgs2* knockout mice showed unexpected developmental abnormalities in the kidney and eventually led to reduced life span [80]. Third, long term use of aspirin causes gastric ulcer formation and bleeding in patients due to the inhibition of COX-1, which is a protective factor for the gastric mucosal layer. But *Ptgs1* knockout mice showed no spontaneous gastric erosion or injury [77]. One possible explanation is that the early loss of COX-1 might cause an adaptive increase of other protective mechanisms (e.g. calcitonin gene related peptide, NO), which is different from losing COX-1 function due to NSAID intake in adulthood. This is not to say that the knockout models have no features consistent with presumed functions of COX-1 or COX-2. The female reproductive deficiency and pain sensation loss are consistent with the predictions based on the known functions of COX-2 [77,81].

6. Proposed mechanism for the role of COX-2 in carcinogenesis

6.1. Role of COX-2 in angiogenesis

The ability to induce angiogenesis is essential for most solid tumors to grow beyond 2–3 mm in diameter. Angiogenesis may also provide an important path for metastasis. Tumor angiogenesis, as with other neovascular formations, includes destabilization of pre-existent blood vessels, proliferation of vascular endothelial cells, invasion by endothelial cells into the extracellular matrix (ECM) and finally the migration and positioning of endothelial cells. One of the earliest observations regarding COX-2 and angiogenesis was made while studying the anti-tumor effect of existing COX inhibitors. In a study published on 1997, Seed et al. noticed that a non-selective COX inhibitor, diclofenac suppressed the growth of COX-2 positive colon-26 cells in nude mice through blocking angiogenesis [82]. Subsequently, studies on corneal models indicated that COX-2 specific inhibitors block new vessel formation and this effect is reversed by adding a TXA_2 receptor agonist [83]. Numerous studies showed co-localization of angiogenesis factors, such as VEGF, PDGF, basic fibroblast growth factor (bFGF) and tumor growth factor-β (TGF-β) with COX-2 by immunohistochemical staining in different cancer types [84]. In breast and cervical cancers, enhanced COX-2 expression has been further associated with increased micro-vascular density (MCD) and with poor prognosis [85,86].

To further explore COX-2 related angiogenesis, using colon cancer cell lines co-cultured with vascular endothelial cells Tsujii et al. demonstrated that COX-2 supported angiogenesis at multiple steps both directly and indirectly [87]. First, COX-2 up-regulation leads to prostaglandin production. Since each prostaglandin has distinct roles for angiogenesis, the profile is important to determine the end effects on different cell types and under different circumstances [88]. For example, TXA_2 is particularly efficient at promoting endothelial cell migration [83]. Second, overexpression of COX-2 in tumor cells directly stimulates the production of angiogenic factors from these cells. Overexpression of COX-2 in a colon cancer cell line induced the production of VEGF, PDGF, bFGF and TGF-β. Through these angiogenesis

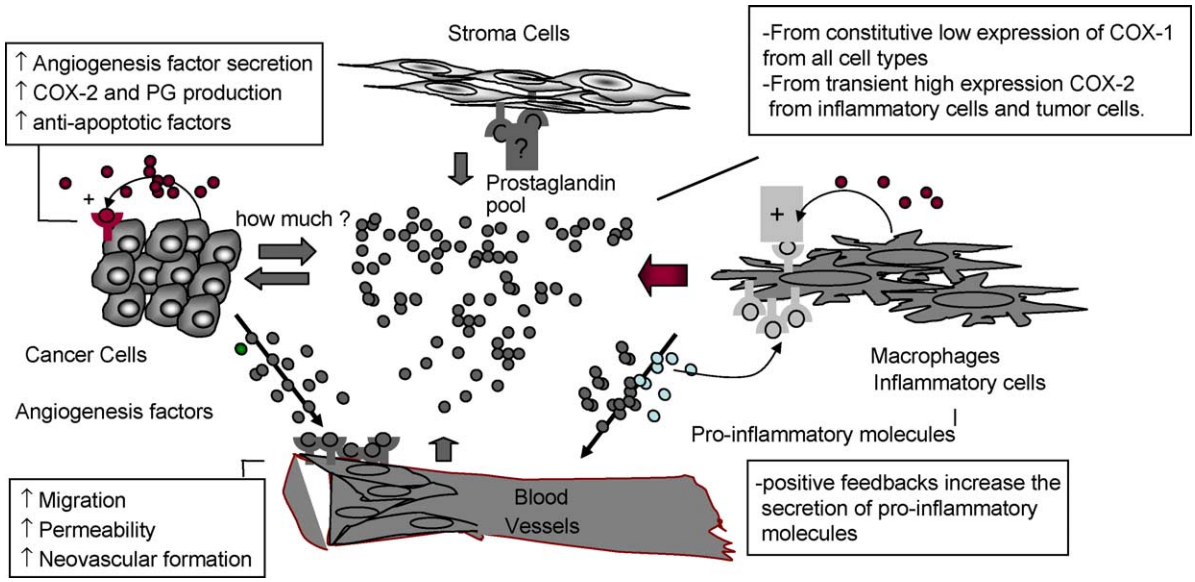

Fig. 1. COX-2 in angiogenesis. This figure models the interactive relationship among cancer cells, endothelial cells and infiltrating inflammatory cells at the site of tumorigenesis. The prostaglandin pool is contributed to by all three different cell types and occasionally stromal cells. The positive feedback through prostaglandin receptors increases COX-2 expression and ensures the continued generation of prostaglandins. In the cancer cell, prostaglandin signaling also results in the production of multiple angiogenesis factors, through which they stimulate neovascular formation at the site of tumorigenesis. In inflammatory cells, prostaglandin signaling stimulates the generation of pro-inflammatory molecules such as IL-2, which further recruits additional circulating monocytes and amplifies the inflammatory response. As a response to increased levels of prostaglandins, angiogenesis factors and pro-inflammatory molecules, endothelial cells proliferate, migrate and undergo tubal formation, providing additional nutrients for oncogenesis as well as a potential route for metastasis.

mediators and their receptors on the endothelial cells, COX-2 increased vascular permeability and induced endothelial cell proliferation and migration (Fig. 1). In vitro overexpression of COX-2 in colon cancer cell lines stimulated tube formation and extension of co-cultured endothelial cells [87]. This effect could be blocked by both the COX-2 specific inhibitor—NS398 and the non-selective inhibitor—aspirin. In other studies, COX-2 overexpression led to the production of matrix metalloproteinase (MMPs), which have been implicated in ECM invasion [89]. Furthermore, COX enzymes are essential for maintenance of the migration and attachment of endothelial cells through integrin pathways [90]. Anti-sense oligonucleotides against COX-1 were able to reduce the tube formation of endothelial cells co-cultured with colon cancer cells that were producing angiogenesis factors [87]. This might explain why COX inhibitors may slow down tumor angiogenesis even though the cancer cells themselves do not express COX-2 and why in some cases, COX-2 nonselective inhibitors, but not COX-2 selective inhibitors can better reduce growth by inhibiting tumor angiogenesis [84].

6.2. COX mediated resistance to apoptosis

Increasing resistance to apoptosis has been proposed as another major mechanism for the effect of COX-2 in tumorigenesis. The first hint came from the observation that NSAIDs could induce apoptosis in cultured cells [91]. Later in 1995, Tsujii and DuBois engineered a rat intestinal epithelial cell line to express COX-2 constitutively. These cells demonstrated an increased resistance to butyrate-induced apoptosis that was mediated by increased expression of the anti-apoptotic factor BCL-2 and TGF-β. Treatment of cells with a non-selective COX inhibitor, sulindac, reversed this phenotype [92]. Since then numerous studies using cultured cells and animal models have supported a role for COX-2 in promoting cell survival under unfavorable growth conditions. Interestingly, overexpression of COX-1

or just simply adding PGE_2 into the culture medium could also increase the resistance to apoptosis. These results suggested that increased prostaglandin production itself might account for the resistance to apoptosis [93,94]. COX-2 but not COX-1 is usually upregulated in tumors. Multiple NF-κB binding sites, Sp-1 sites and a cAMP-response element are located in the *PGTS2* promoter and enhancer region, which provide target DNA binding sites for transcription factors to rapidly induce mRNA expression under stress conditions. These features are not present in the *PGTS1* gene. The COX-2 transcript also contains multiple repeats of a sequence within its 3′ untranslated region (AUUUA) that mediates rapid mRNA degradation [95].

The notion that the anti-apoptotic effects of selective or non-selective COX inhibitors are always mediated through the COX enzymes themselves has been challenged recently. Given that there are now known COX-2 independent functions (see below) of those inhibitors, it is not clear if inhibition of COX-2 enzymatic function alone is responsible for the increased apoptosis in each case. It will be interesting to test the effect of NSAIDs on $Ptgs2^{-/-}$ and $Ptgs1^{-/-}$ animal tumor models or even double knockouts, such as $APC^{Min/-}$ $Ptgs2^{-/-}$ compound mice, to tease out the COX independent function of NSAIDs. Recently Song et al. showed that cells lacking *PGTS1* or *PGTS2* were viable and sensitive to celecoxib-induced apoptosis. In addition, a derivative of celecoxib, which is incapable of inhibiting COX-2, also induced apoptosis in these cells at a similar concentration [96].

7. Expression of COX enzymes in human normal tissues and in cancer

7.1. Expression in normal tissues

Although COX-2 protein is undetectable by immunohistochemistry in many human tissues under normal physiological conditions, there are several known exceptions. The seminal vesicles are known to have the high levels of constitutive expression of COX-2. PGE_2 and its 19-hydroxy metabolites are the major components of primate semen [97]. COX-2 is also constitutively expressed in the kidney with positive staining in glomeruli and small blood vessels. The limited evidence on human subjects suggests that COX-2 is involved in sodium regulation and kidney perfusion under stress, but not in maintaining basal renal blood flow [98,99]. The CNS contains both constitutive and inducible COX-2 expression in both neuronal and non-neuronal cells in the cortex, hippocampus, hypothalamus and spinal cord, where COX-2 is involved in the establishment of pain sensation and body temperature control [100]. COX-2 is also expressed in ovarian follicles upon gonadotrophin stimulation, in uterine epithelial cells and surrounding stromal cells at the site of blastocyst attachment during implantation and decidualization [81].

7.2. Colorectal cancer

In 1994, Eberhart et al. first reported COX-2 overexpression in human colon cancer, followed by two other groups in the next year [101–103]. In their papers, they described that COX-1 expression was weak, universal and unchanged in both normal and cancerous colon, while COX-2 expression was only seen in tumors. COX-2 overexpression was also reported in the tumors generated from APC^{Min}, $APC^{\Delta 716}$ and the AOM-induced colon cancer models [59,104]. These results in combination with the encouraging information from $APC^{Min\ or\ \Delta 716}$ $Ptgs2^{-/-}$ mice have bolstered motivation for clinical trials on COX-2 selective inhibitors for colon cancer prevention. There are still, however, several areas that remain somewhat unclear. First, the percentage of COX-2 positive cells among clinical colon cancer samples tested varied from 40 to 100% between different studies. Even though most studies reported that colon cancers occurring in FAP patients often express COX-2, there was a great deal of variation among sporadic cases. Second, it is not clear at what time during carcinogenesis COX-2 expression is induced and how it changes during tumor progression. In general COX-2 overexpression has been considered to be an early event in colon cancer development, which correlates well with the prophylactic effect of NSAIDs. But how early it is and its temporal relationship with other early events, in particular the loss of the wild type APC allele, is undetermined. While studying $APC^{\Delta 716/+}$ mice,

Oshima et al. reported that COX-2 expression was only seen in the large established adenomas, not in the uninvolved colon nor in the adenomas smaller than 2–3 mm diameter [59], while all the adenomas genotyped had already lost the wild type allele of APC. However, COX-2 upregulation was described in uninvolved colon epithelium from Min mice [104]. In the clinical setting, the distal non-involved polyps from FAP patients showed minimal COX-2 staining, but the cancer from corresponding cases showed strong staining for COX-2. Increased COX-2 staining correlated with larger polyp size and progression to invasive carcinomas as well [105,106]. Third, the actual cell-type expressing COX-2 within colon cancer is largely debatable. Many published studies suggested that the carcinoma cells themselves express COX-2, especially in the early studies. Others suggested that most of the expression was found in infiltrating macrophages within the tumors [107,108]. Other cancer types expression of COX-2 by vascular endothelial cells [109], fibroblasts [110] and smooth muscle cells around the cancer, and even neuroendocrine cells [111] has all been reported. Oshima et al. replaced one allele of the *Ptgs2* gene with the bacterial β-galactosidase (*lac*Z) to generate gene knockout mice in which the *lac*Z expression was under the control of endogenous *Ptgs2* promoter. When the mice were crossed with $APC^{\Delta 716}$ mice, only the interstitial cells with large ovoid and light stained nuclei were lacZ positive, but the epithelium itself was negative [59]. Although it was not directly shown, the identity of many of these cells was consistent with that of lamina propia macrophages. This is also consistent with the result from studying clinical samples in which the vast majority of strong COX-2 immunoreactivity was present in the lamina propia macrophages directly subjacent to the surface adenomatous epithelial cells [107]. Genetic differences between the study groups, artifacts introduced during sample handling and storage, and variations between the antibodies and staining protocols all could potentially lead to the discrepancies. The generation of tissue and cell-type specific *PGTS2* knockout mice might provide some insights regarding these questions, and shed some light on either paracrine or autocrine mechanisms contributing to tumorigenic function of COX-2.

7.3. Breast cancer

Increased prostaglandin concentration in breast cancer, especially PGE_2 and TxA_2 was reported in the early 1980s [112]. Long-term use of NSAIDs has also been associated with reduced risk of breast cancer [113]. In the initial study, Kargman et al. did not find expression of COX-2 in any of the three breast tumor/normal pairs by immunohistochemistry, but they did detect significant expression of COX-2 in colon cancer samples [102]. In 1998, the first study that focused on COX-2 expression in breast cancer was published using both immunohistochemistry and Western blotting. Only two out of the forty-four cases studied had strong, definitive COX-2 expression, mainly in the tumor epithelial cells. Meanwhile among these cases, thirty of them had elevated COX-1 expression, but mainly in the stromal cells [114]. In another study, Costa et al. reported that COX-2 was expressed in eight out of forty-six carcinomas studied, and the expression of COX-2 staining correlated with microvessel density, lymph node metastasis, apoptotic index, and shorter disease-free survival time [115]. Furthermore, Half et al. reported COX-2 expression in the epithelial cells of 43% of invasive breast cancers, 63% of ductal carcinoma in situ and 80% normal appearing breast tissues that were adjacent to cancer [116]. RT-PCR revealed an average ninefold increase of COX-2 mRNA in cancer vs. proximal normal tissues. From this, the authors proposed that COX-2 upregulation might be an early event in mammary gland tumorigenesis, but the continued expression might become less important after an invasive tumor was formed.

7.4. Prostate cancer

The expression and function of COX-2 in prostate tissues and prostate cancer has been the subject of multiple reports [117–122]. In general the results of these studies suggest that COX-2 expression in normal prostate tissue is either weak or negative and prostate cancer tissue has an elevated level of COX-2 protein. Based upon these data, it was hypothesized that that the effects of NSAIDs on prostate cancer are mediated by inhibition of the enzymatic activity of COX-2 in the prostate cancer cells. COX-2 expression was not

seen in the normal prostatic cells in mice, but appeared in prostate tumors from TRAMP mice—a probasin-SV40 large T antigen transgenic prostate cancer model [123], where the established tumors are largely neuroendocrine in phenotype. Nevertheless, a consensus has not been reached regarding expression of COX-2 in prostate cancer. A recent study from our group confirmed that COX-2 expression is very low or undetectable in the normal prostate [124]. However, in contrast to the previous reports, we found that the expression of COX-2 was not elevated in prostatic intraepithelial neoplasia—the proposed precursor lesions, or in established prostate cancers studied ($n=144$ cases) [124] (Fig. 2). In limited cases, when staining for COX-2 was observed in prostate cancer, the extent of positive staining did not correlate with established clinical and/or pathological risk factors—Gleason score or pathological stage. By contrast to the neoplastic tissue, we did find consistent expression of COX-2 protein in proliferative inflammatory atrophy lesions, which have been proposed as an important etiological factor for prostate cancer [125]. The expression was seen in the atrophic lumenal epithelial cells themselves and occasionally in infiltrating macrophages (Fig. 2). These results suggested that if NSAIDs are indeed chemopreventive and/or chemotherapeutic for prostate cancer, their effects are likely to be mediated by modulating COX-2 activity in non-PCa cells (either inflammatory cells or atrophic epithelial cells) or by affecting a COX-2-independent pathway.

Since these results were different from most previously published studies, a number of control experiments were performed to determine the sensitivity and specificity of the immunohistochemical staining. Tissue culture cell lines with inducible expression of COX-2 were used as positive and negative controls for staining. Northern blots, Western blots and quantitative RT-PCR were performed in clinical samples to access the expression of COX-2 at both mRNA and protein levels. Three different antibody sources were tested for staining. Significant background staining was discovered with some antibodies. These results suggested that inadequate quality control of the staining protocols

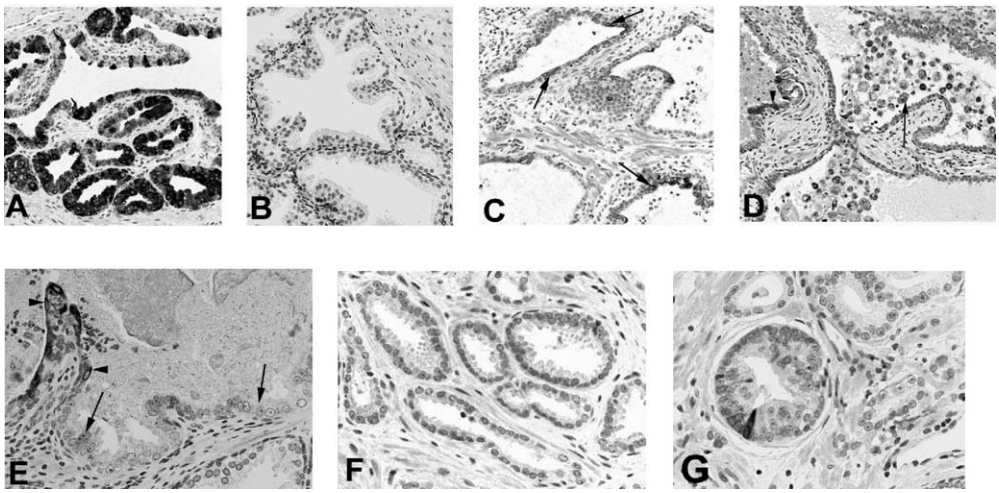

Fig. 2. Immunohistochemical analysis of COX-2 in prostate (Reprinted with permission, from Zha et al., Cancer Research). (A) Ejaculatory duct showing intense staining (200×). (B) Normal prostate epithelium from the same patient specimen as in A demonstrating lack of significant staining (200×). (C) Focus of proliferative inflammatory atrophy (PIA) with several cells staining positive. Arrows indicate positive luminal epithelial cells (200×). (D) Macrophages in the lumen of another more inflamed PIA lesion staining positive (arrow). Arrowhead indicates positive epithelial cell in PIA (200×). (E) Focus of high grade intraepithelial neoplasia (HGPIN) demonstrating infrequent staining. This lesion shows PIA (upper left) merging with HGPIN. Arrowheads indicate PIA cells with strong COX-2 staining. Arrows indicate area of HGPIN cells with no staining. Note negative staining in normal appearing acinus in lower right part of photograph (400×). (F) Focus of adenocarcinoma (PCa) from same specimen as in E demonstrating negative staining (400×). (G) Heterogeneous area of PCa, primarily staining negative but with some cells staining positively (arrow) (400×).

might contribute to the variability in reported results of COX-2 staining [124]. Another recent study has arrived at the same general conclusion—most prostate cancers do not express COX-2, although there was some overexpression in higher grade tumors in this study [126].

The mechanisms underlying the marked decrease in COX-2 protein and mRNA levels in prostate cancer and PIN lesions as compared to PIA lesions has yet to be determined. However, one recent study, using a quantitative methylation specific PCR assay, found that the CpG island upstream of the COX-2 gene is methylated in greater than 85% of primary and metastatic prostate cancers and in 7 of 7 tested prostate cancer cell lines, but not in benign prostate cells and tissues [127] (Fig. 3). Additionally, tumor-adjacent benign tissues containing regions of high grade PIN showed a low frequency of methylation (Fig. 3). This study also demonstrated that an accumulation of methylated copies of the COX-2 CpG island predicted an increased risk of prostate cancer recurrence (Fig. 3) and that this correlation was independent of Gleason score and pathological stage [127]. These data suggest that DNA methylation may play an important role in the silencing of COX-2 in prostate cancers. In addition, along with the results from the transgenic K14.COX2 mice suggesting that COX-2 expression may actually inhibit carcinogenesis (see above description), these results suggest that maintenance of COX-2 may be protective against transformation in the prostate and that inactivation of the *PGTS2* promoter by hypermethylation may be selected for during prostate carcinogenesis.

7.5. Other cancer types

COX-2 up-regulation has also been frequently reported in other cancer types, particularly in the skin, lung, bladder and pancreas [128–132].

8. Other unresolved issues and opportunities in NSAID mechanisms of action

The fact that chronic or acute inflammation is commonly associated with cancer also complicates the interpretation of COX-2 expression in cancer. On one hand, the tissue disruption and cell death in cancer

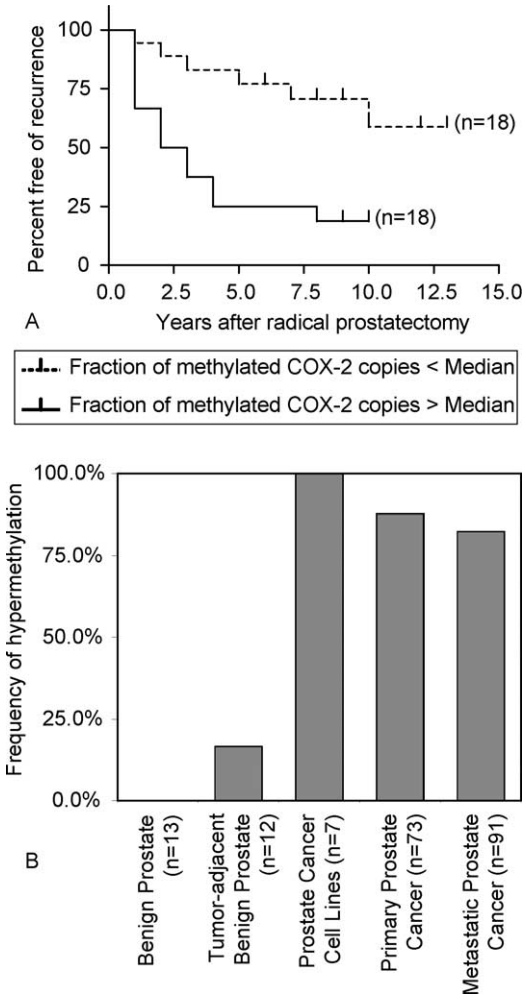

Fig. 3. The frequency of methylation at the CpG island upstream of the COX-2 gene in various tissues. (A) Kaplan–Meier curve assessment of risk of biochemical recurrence as a function of the fraction of hypermethylated COX-2 CpG island copies. This analysis was performed for $n=36$ patients treated with radical prostatectomy for whom clinical recurrence data was available. The difference in the rates of recurrence for each group was statistically significant by Logrank test ($P=0.0017$). (B) Benign prostates were obtained from 13 transplant organ donors at the time of organ harvest. Prostate cancer cell lines included LNCaP, PC-3, DU-145, LAPC-4, CWR22Rv1, C42B, and VCaP. Primary prostate cancer tissues were taken from 73 men undergoing radical prostatectomy for the treatment of clinically localized prostate cancer. Matched tumor-adjacent benign prostate specimens were isolated from 12 of these 73 subjects with primary prostate cancer. These tumor-adjacent benign regions included regions of inflammation and high grade PIN, but did not contain any regions of adenocarcinoma. Ninety-one anatomically distinct metastatic prostate cancer lesions were obtained from 36 subjects with advanced prostate cancer.

recruit pro-inflammatory cells and lead to inflammation. On the other hand, some types of infections or chronic inflammation are causative for the initiation of certain cancers, such as chronic hepatitis, chronic gastritis and chronic ulcerative colitis. Prostaglandins generated as a result of COX-2 overexpression can also act as paracrine as well as autocrine growth regulators (Fig. 4). Prostaglandin receptors are expressed in most endothelial cells, macrophages, stroma and epithelial cell types. It is known that at least some prostaglandin-receptor interactions (e.g. PGE_2–PTGER2) can send positive feedback signals to increase COX-2 mRNA levels. If this is the case, regardless of the initial trigger, once COX-2 expression begins, prostaglandins could mediate a wave of COX-2 expression not only in cancer cells but also in the surrounding stroma, macrophages and endothelial cells. At any given time, one particular cell or cell type may or may not express COX-2, but specific prostaglandins may be present. This may explain why prostaglandin level elevation is relatively consistently observed between studies. Thus it may be very difficult to separate COX-2 expression caused by inflammation and that caused by transformation. If tracking the expression of COX-2 longitudinally in a particular cell type becomes possible, it would clarify some of the confusion. The development of tissue-specific COX-2 knockouts would be an excellent tool to study the effects of COX-2 expression on the initiation and progression of cancer. Macrophage-specific loss of COX-2 expression would be an especially powerful way to address the relationship between inflammation and cancer.

In tissue culture settings, NSAIDs induce apoptosis in multiple tumor cell lines and suppress the expression of angiogenesis factors [133]. However, the ability of NSAIDs to induce apoptosis does not always correlate with their ability to inhibit the COX enzymes. Therefore several COX independent mechanisms have been proposed in the past years. The first hint came from the study of sulindac metabolites. Sulindac is usually given as the parental drug and it is metabolized to sulindac sulfide (an active COX inhibitor) and sulindac sulfone (not an inhibitor of COX). But both metabolites induced apoptosis with similar efficiency in cell culture models [134,135]. This result indicated the existence of a COX independent mechanism of apoptosis induction. Recently Song et al. generated PC3 prostate cancer cells with varying levels of COX-2 protein expression. The sensitivity to apoptosis induced by both celecoxib and its non-COX-2 inhibiting derivatives was similar regardless of the levels of COX-2 protein, which support the COX independent function of NSAIDs, even the COX-2 selective groups [96].

Peroxisome proliferation activating receptors (PPARs) could serve as the intracellular receptors for some prostaglandins as well as some NSAIDs [136]. Reduced PPARγ and over activation of PPARδ/β have been associated with colorectal cancer. He et al. suggested that sulindac could interfere with the DNA-binding of PPARδ/β, and other groups proposed the possibility for NSAIDs to cause accumulation of an endogenous as yet undiscovered PPARγ ligand [136]. In terms of another potential mechanism, sulindac has also been reported to reduce the levels of the anti-apoptotic factor BCL-xL, tilting the balance between the pro-apoptotic factor BAX and BCL-xL and subsequent programmed cell death. Therefore cells containing inactive BAX gene are resistant to sulindac induced apoptosis [137]. Aspirin and salicylates might also suppress NF-κB related survival signaling by inhibiting IκKα activation, leading to apoptosis. Sulindac sulfide can inhibit both IκKα and β [138]. Yet, other NSAIDs, such as indomethacin or ibuprofen, did not interfere with NF-κB signaling in the colon cancer cell line tested (HCT-115) [139]. These results suggested additional COX-2 independent mechanisms that contribute to the apoptosis resulting from NSAID treatment. Another COX independent mechanism may involve inhibiting cGMP-specific phophodiesterases PDE_2 and PDE_5 [140]. In most cases, the COX independent effects of NSAIDs are relatively specific for each individual inhibitor and have been tested only in limited samples. Further investigations are called for to elucidate the particular structural feature of each group of NSAIDs.

Other results confirm the importance of COX in NSAID action, but introduce different explanations for effects on apoptosis. Cao et al. suggested that an increase in the concentration of unesterified arachidonic may be responsible for NSAID induced apoptosis [141]. In support of this, introduction of fatty acid-CoA ligase—another enzyme that uses free arachidonic acid as its substrate—can produce NSAID resistance. Also multiple studies suggest

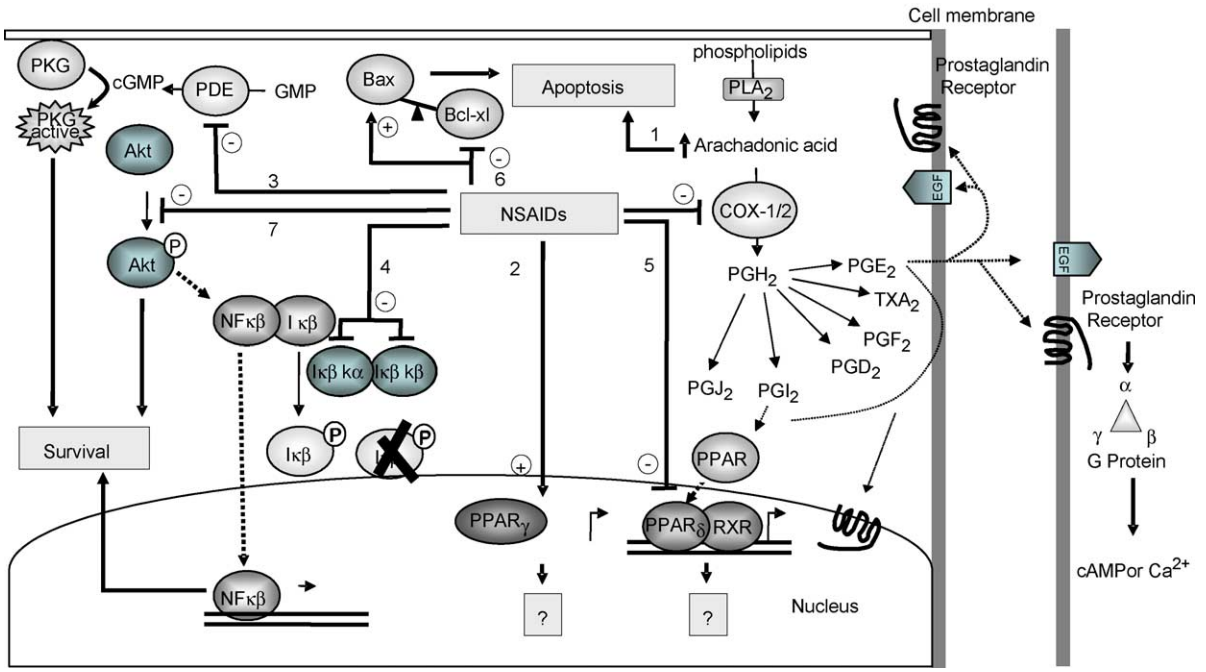

Fig. 4. Molecular mechanisms for COX-2 and NSAIDs. The right part of the model illustrates the prostaglandin synthesis pathway as well as the subsequent receptor signaling—the specific prostaglandin receptors as well as the non-canonical EGF receptor pathway. As the result of inhibiting COX enzymes, accumulation of arachadonic acid would directly promote apoptosis and attenuation of positive feedback to proliferation and survival through receptors. The rest of the figure demonstrates several COX-2 independent mechanisms proposed for NSAIDs. Since, not all NSAIDs are able to act through these mechanisms in every cell type, a brief table is attached to summarize the particular NSAIDs used in each experiment as well as the cell lines involved.

No	Mechanism	NSAID (concentration)	Cell line system	Reference
1	Accumulation of AA causes apoptosis	Sulindac (200 μM), indomethacin (300 μM)	HT29, HEK293	[141]
2	Serve as ligands for PPARγ	Indomethacin (40 μM), flufenamic acid (100 μM), fenoprofen (100 μM), ibuprofen (100 μM)	Fibroblast (C3H10T1/2)	[145]
3	Inhibits PED	Sulindac sulfone (165 μM)	SW480	[140]
4	Inhibits I-κ B kinase β	Aspirin, sulindac sulfide, not indomethacin	HCT16, Cos, etc.	[146]
5	Blocks DNA binding of PPAR δ/β	Sulindac sulfide (100–250 μM)	HCT116, SW480	[136]
6	Suppresses Bcl-xl	Sulindac (120 μM)	HCT116	[137]
7	Blocks Akt activation	Celecoxib (25–50 μM)	PC-3, LNCaP	[147]

NSAIDs can not only act on the enzymatic activity level of COX, but can also repress the induction of COX-2 at the transcriptional level [142].

9. Summary

More than a century after the introduction into the market, aspirin is still a somewhat ‘magical’ drug that can not only prevent inflammation, and reduce pain, but can also prevent cancer. In the past 10 years, our understandings of the molecular biology of COX enzymes, from structure to catalytic mechanisms, have begun to provide evidence from multiple angles to support the pro-carcinogenic role of COX enzymes. One of the most important major issues that remain relates to the expression pattern of the COX-2 enzyme in preneoplastic and neoplastic lesions. It is still not

clear, for example, exactly which cells the inhibitors are acting on since there is often controversy regarding which cells express the enzyme. Further studies of the expression and function of COX-2 in clinical samples and animal models, with emphasis on proper control experiments, are needed to further clarify this important issue. In addition, we submit that the most interpretations of why NSAIDs prevent cancer have perhaps underemphasized the importance of chronic inflammation in cancer development—most reports have ignored the strong possibility that the mechanism of action of NSAIDs in cancer prevention may often proceed via inhibition of the inflammatory response. Another potential issue regarding COX-2 is that in at least one animal model, the K.14-COX-2 transgenic mouse, ectopic overexpression of COX-2 dramatically prevented cancer. In addition, the finding that *PGST2* is apparently silenced during prostate carcinogenesis by hypermethylation of the CpG island in its promoter region raises the question that this gene is targeted for inactivation during prostate carcinogenesis [127]. Thus the simple view that COX-2 expression is always acting to increase cancer risk may have to be revised. This is also bolstered by the very high levels of constitutive COX-2 expression in the seminal vesicles, which have an extremely low rate of cancer development.

Future work using cell-specific gene knockout and transgenic animals may help elucidate specific temporal and spatial relationships between COX-2 expression, the particular prostaglandin profile and tumor initiation and progression in various organ systems. These types of studies may also help to address the specific functions of each of the COX enzymes: COX-1, COX-2 and COX-3—the newly identified isoform of COX-1 [143,144], and their contribution to NSAID mediated tumor regression. Chemical approaches with the effects of various structural derivatives on these animal models, and models with alterations in the prostaglandin receptors, should further refine the specific and non-specific effects of NSAIDs. Finally, results of ongoing and future prospective placebo-controlled double blind studies of various inhibitors in human studies are needed to provide definitive information regarding what types of patients can benefit from the various types of inhibitors.

Note added in proof

A recent study (Y.G. Crawford, M.L. Gauthier, A. Joubel, K. Mantei, K. Kozakiewicz, C.A. Afshari, T.D. Tlsty, Histologically normal human mammary epithelia with silenced $P16^{INK4a}$ over express COX-2, promoting a premalignant program, Cancer Cell 5 (2004) 263–273.) provides new evidence for a role of COX-2 in breast carcinogenesis.

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