Cyclooxygenase-2 (COX-2), aromatase and breast cancer: a possible role for COX-2 inhibitors in breast cancer chemoprevention

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Interest in chemoprevention in oncology using suppressants of prostaglandin (PG) synthesis has been stimulated by epidemiological observations that the use of aspirin and other non-steroidal inflammatory drugs (NSAIDs) is associated with reduced incidence of some cancers, including cancer of the breast.

The main target of NSAID activity is the cyclooxygenase (COX) enzyme. Two isoforms of COX have been identified: COX-1, the constitutive isoform; and COX-2, the inducible form of the enzyme. COX-2 can undergo rapid induction in response to many factors such as bacterial lipopolysaccharides, growth factors, cytokines and phorbol esters. COX-2 is overexpressed in some malignancies including carcinoma of the breast. It has been suggested that such enhanced expression may lead to increased angiogenesis such that the inhibition of COX-2 might have a general anticancer effect via decreased blood vessel formation. In addition, an association between COX-2, its main product PGE2, and aromatase activity in human breast cancer suggests that such inhibitors might have an additional, specific prophylactic mechanism for this tumour.

New COX-2 inhibitors are already licensed for use in the treatment of arthritis and are well tolerated. Their potential role in chemoprevention of mammary carcinogenesis in rats has already been investigated. What remains to be seen is if these findings can be extrapolated to human studies.

Key words: aromatase, breast cancer, cyclooxygenase-2, oestrogens, prevention, prostaglandins

Non-steroidal anti-inflammatory drugs, cyclooxygenase-2 and breast cancer

Non-steroidal anti-inflammatory drugs and cancer

There have been a number of studies over the past 25 years linking non-steroidal anti-inflammatory drugs (NSAIDs) and cancer incidence [1]. Much of the work relating to NSAIDs and cancer has focused on colorectal cancer, including familial adenomatous polyposis (FAP). Several studies have reported an inverse relationship between colon cancer incidence and regular NSAID use including aspirin [2–6]. Studies using animal models of intestinal tumorigenesis have shown cyclooxygenase-2 (COX-2) expression in intestinal adenomas [7–10]. Further human studies of colon carcinomas have shown marked upregulation of COX-2 in carcinomas compared with normal mucosa [11–14]. A number of retrospective and prospective studies have shown a reduced incidence of colorectal cancer with NSAID use [15, 16]. In addition a number of randomised placebo-controlled double-blind trials have produced data showing a regression in polyp size [17, 18]. This process was reversible with tumours resuming growth after removal of the NSAID.

There are fewer human data in relation to NSAID use and breast cancer incidence. A large cohort study of 89528 registered nurses in the USA found no association between regular aspirin use and the incidence of breast cancer [19]. The analyses were based on 2414 cases identified over 12 years of follow-up [relative risk (RR) 1.0]. By contrast, in their case-control study of 511 women with newly diagnosed breast cancer and 1534 women who had undergone screening mammography, Harris et al. [20] found a reduced risk of breast cancer associated with the use of any NSAID three or more times a week for at least a year (RR 0.66). The protection was similar for users of aspirin alone, ibuprofen alone and all NSAIDs combined. The most heavily exposed women had the lowest risk, although the study was limited by incomplete descriptions of the study population and the participation rates. The epidemiological data relating NSAID use and the incidence of breast cancer are summarised in Table 1.

NSAIDs: mechanisms of action

Arachidonic acid, a 20-carbon polyunsaturated fatty acid, is the precursor for prostaglandin (PG) synthesis. The first step is
the hydrolysis of phospholipids to produce free arachidonic acid catalysed by phospholipase A2 (Figure 1). The next step is catalysed by COX, which inserts molecular oxygen into arachidonic acid [21]. This reaction produces an unstable product, PGG2. PGG2 is then converted by the peroxidase activity of COX to PGH2. PGH2 is the common precursor for all other prostanoids. The production of individual prostanoids is catalysed by different, specific synthases, which may vary in their expression between different types of cells. Each of the products derived from PGH2 has a distinct biological function.

The main target of NSAID action is COX. Two isoenzymes exist in the human form. The enzyme which is now called COX-1 was first purified and characterised from bull vesicular...
glands by Miyamoto in 1976 [22]. In 1991 cDNAs for COX-2, a novel isoenzyme of COX, were isolated and sequenced by two independent groups [23, 24]. Before the discovery of COX-2 it was known that PG synthesis could be stimulated by a variety of substances including cytokines, growth factors and tumour promoters. These effects were due to activation of phospholipases, which supply arachidonic acid to COX [25]. The two isoenzymes are regulated independently: COX-1 is constitutively expressed, whereas the inducible isoenzyme COX-2 is expressed only in response to certain stimuli such as tumour promoters, endotoxin, cytokines and hormones [26, 27].

There are also data suggesting that COX-independent mechanisms may be responsible for some of the effects seen with NSAIDs in cancer models. It has been reported that NSAIDs can disrupt the binding of a nuclear transcription factor (PPAR-γ) to its *cis* recognition sequence [28]: PPAR-γ expression is increased after disruption of the tumour suppressor gene Apc and could be an important downstream effector. This NSAID effect is not likely to be related to inhibition of COX. In contrast, another report demonstrated that PPAR-γ can be activated by binding PGI2 derived from COX-2 [29]. More work therefore needs to be done to elucidate the exact role of PPAR-γ in carcinogenesis, and in particular in relation to mammary carcinogenesis.

**Transcriptional regulation of COX-2**

Increased expression of COX-2 in malignancy is likely to occur via multiple routes. COX-2 induction by lipopolysaccharide (LPS) has been shown to occur through both the mitogen-activated protein kinase (MAPK) and protein kinase C-ζ (PKC-ζ) pathways [30]. It has also been shown that ceramide-stimulated activation of MAPK can activate c-Jun N-terminal kinase (JNK), which in turn can lead to increased COX-2 gene expression in human mammary epithelial cells [31]. This occurs via a cAMP response element (CRE) in the COX-2 promoter. Further evidence for the role of MAPK and c-Jun pathways in tumour necrosis factor-α (TNF-α)-stimulated COX-2 expression in human epithelial cells was provided recently by Chen et al. [32]. These pathways are illustrated in Figure 2.

Transient transfection experiments have demonstrated that nuclear factor κB (NFκB), nuclear factor IL-6 (NF-IL6) and CRE promoter sites mediate gene transcription independently in response to LPS treatment [30]. LPS can activate different pathways to induce COX-2 gene transcription: through NFκB

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**Figure 2.** Signal transduction pathways influencing cyclooxygenase-2 (COX-2) expression. TNF-α, tumour necrosis factor-α; LPS, lipopolysaccharide; PKC-ζ, protein kinase C-ζ; MAPK, mitogen-activated protein kinase; CRE, cAMP response element.
via extracellular signal related kinase (ERK-2), p38 and JNK pathways, through NF-IL6 via a p38 pathway, and through CRE via ERK-2 and JNK pathways. Moreover, PKC-ζ signalling seems to mediate transcription after LPS treatment through all three promoter sites. Therefore, individual signalling pathways, such as ERK-2, p38, JNK or PKC-ζ, appear to be sufficient to mediate COX-2 gene transcription by virtue of their ability to recruit transcription factors to at least two promoter sites. This may indicate redundancy in the signalling pathways and promoter elements regulating COX-2 transcription, at least in endotoxin-treated cells of macrophage/macrophage lineage. Associations have also been made between mutated ras gene and COX-2 expression in human breast cancer cell lines [33], and c-myb expression is upregulated in colon tumours and breast cancers [34, 35]; c-myb overexpression causes a modest induction of COX-2 promoter activity [36].

COX-2 and carcinogenesis

A number of studies have shown overexpression of COX-2 in solid malignancies including breast [37], pancreas, prostate [38] and colon [39]. The most compelling data to support a causal relationship between overexpression of COX-2 and carcinogenesis have come from studies of the Apc716 mouse. This is an animal model for human FAP, a condition caused by a germline mutation of the Apc gene in which individuals develop numerous adenomatous colorectal polyps, which predispose to colorectal carcinomas. Several strains of mice have been developed that carry mutations in one Apc allele, including the Min mouse [40] and Apc716 [41]. Analysis of adenomatous polyps from Min mice show increased expression of COX-2 relative to normal mucosa [9]. The effect of the absence of COX-2 in the Apc716 mouse has been studied by introduction of a knockout mutation of the COX-2 gene. Removal of the COX-2 gene in the mouse reduces the number and size of intestinal polyps [42]. Genetic and pharmacological evidence has been gathered to show that specific COX-2 inhibition is more effective than traditional NSAIDs in suppressing polyposis in mouse models of FAP, where COX-2 is induced [42].

COX-2 and mammary carcinogenesis

COX-2 has been implicated in mammary carcinogenesis in several ways. COX-2 expression was detected using reverse transcription polymerase chain reaction (RT-PCR) in 13 of 13 human breast tumours, with no detectable expression in normal breast tissue [37]. A correlation was also observed between COX-2 expression and increasing tumour cell density. The COX-2 expression was localised to the epithelial compartment with no expression within the stromal component. A further study of 21 human breast tumour specimens found no detectable COX-2 expression in normal breast tissue specimens, but detectable and heterogeneous COX-2 gene expression in each of the 21 tumour specimens [43]. In addition, a significant linear association between the tumour cell density and COX-2 gene expression was found ($P < 0.0001$). In contrast Hwang et al. [44] analysed 44 samples by western blotting and only detected COX-2 protein in two of the 44 samples. A possible explanation for this is the differential sensitivity of the approaches to measuring the expression of COX-2. A study by Subbaramaiah et al. [45] looked at 29 microdissected breast cancers and found high levels of COX-2 protein in 14 of 15 HER-2/neu-positive samples. This was in contrast to the 14 HER-2/neu-negative tumours, where COX-2 was only detected in four of the cases, and at significantly lower levels than in the positive cases.

The presence and levels of tissue PGs have been studied extensively in breast cancer over the past 30 years. Early studies demonstrated a relationship between tissue PG levels in human breast tumours, poor postoperative survival and development of metastases [46–48]. The main product of COX-2, namely PGE$_2$, is found in high levels in tumour cells [49], and is synthesised by several human breast cancer cell lines. In clinical studies, high PGE$_2$ concentrations have been associated with both high metastatic potential and a lack of oestrogen and progesterone receptors [48, 50].

The inhibition of COX-1 and COX-2 has been investigated in rat models. A 35-day course of ibuprofen in female Sprague–Dawley rats with 7,12-dimethylbenzathracene (DMBA)-induced mammary carcinomas resulted in significant reduction of tumour volume ($P < 0.05$) and a significant reduction in gene expression of both COX-1 and COX-2 [51]. Other studies including studies with indomethacin have yielded similar results [52, 53]. More recently the chemopreventive effect of a specific COX-2 inhibitor, celecoxib, against DMBA-induced mammary carcinogenesis in female Sprague–Dawley rats has been investigated [54]. Dietary administration of celecoxib produced striking reductions in the incidence, multiplicity and volume of mammary tumours relative to the control group. The chemopreventive properties of another COX-2 inhibitor, nimesulide, have been assessed in a study in which COX-2 expression and mammary tumours were induced by the environmental carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, together with a 24% corn oil diet [55]. Tumour incidence was significantly reduced from 71% in the control group to 51% in the treatment group, as well as a significant reduction in the size and multiplicity of tumours. More definitive evidence has now been provided by the recent demonstration that COX-2 overexpression is sufficient to induce mammary tumorigenesis in transgenic mice [56]. Overexpression of COX-2 in the mammary glands of transgenic mice was achieved by the use of the mouse mammary tumour virus promoter. Multiparous but not virgin females showed a much higher incidence of mammary gland hyperplasia, dysplasia and transformation to metastatic tumours.

Studies of breast cancer cell lines have also provided data supporting the overexpression of COX-2 in breast cancer. A study of the differential expression of COX-1 and COX-2 in
human breast cancer cell lines found that in the MCF7 oestro-
gen receptor-positive cell line, COX-2 was barely detectable, whereas in the highly invasive, metastatic cell line MDA-MB-231 there was a low level of COX-1 but a high level of COX-2 expression [57]. The concentration of PGE2, the major COX-2 product, correlated well with the level of COX-2 protein.

**COX-2 overexpression and mammary carcinogenesis: potential mechanisms**

The expression of COX-2 in human solid cancers is not confined to the epithelial component of the tumour. The neovascularure associated with colonic adenomas, carcinomas and metastatic liver lesions also demonstrates COX-2 staining by immunohistochemical methods [58]. This expression seems to be a general characteristic of epithelial tumours including head and neck [59] and pancreas [60]. Significant correlations between COX-2 and tumour vascularisation (P <0.0001), microvessel density (P <0.007) and vascular endothelial growth factor (VEGF) in 35 head and neck cancers have been reported [61]. Uefuji et al. [62] found that microvessel density positively correlated with COX-2 expression in 42 cases of primary human gastric adenocarcinoma. The COX-2-overexpressing cases showed significantly elevated levels of PGE2 compared with normal gastric mucosa.

The effects of specific COX-2 inhibitors have been tested in animal models of angiogenesis and celecoxib, a specific COX-2 inhibitor, has been shown to cause inhibition of the angiogenic response in fibroblast growth factor (FGF)-induced corneal angiogenesis in rats [58]. Celecoxib reduced both the number and length of sprouting capillaries in a dose-dependent fashion. These data suggest COX-2 overexpression is associated with increased PGE2 biosynthesis and angiogenesis in gastric cancer, and provide supportive data for COX-2 overexpression being functionally significant in tumour angiogenesis.

The mechanism by which COX-2 expression produces these effects is unclear, however. Activated human microvascular endothelial cells produce a number of eicosanoid products including thromboxane A2 (TXA2) [63]. Selective COX-2 antagonists have been shown to inhibit TXA2 production and endothelial migration as well as corneal angiogenesis, an effect that is reversed with the use of a TXA2 agonist U46619 under COX-2-inhibited conditions [63]. TXA2 may represent an important intermediary of the angiogenic process. It has also been suggested that combined expression of inducible nitric oxide synthase and COX -2 may contribute to tumour angiogenesis. The exact mechanism by which this may occur is far from understood. Inducible nitric oxide synthase and COX-2 have been positively correlated with VEGF in non-small cell lung cancers [64]. In addition a study of 100 patients with resectable hepatocellular carcinoma showed that tumours that were negative for inducible nitric oxide synthase and COX-2 by immunohistochemical assess-

ment had a significantly better overall (P = 0.041) and recurrence-free survival (P = 0.018) [65].

A recent study by Dormond et al. [66] investigated the potential links between αVβ3 integrin, an adhesion receptor critically involved in mediating tumour angiogenesis and COX-2. They demonstrated that inhibition of endothelial-cell COX-2 by NSAIDs suppresses αVβ3-dependent activation of the small GTPases Cdc42 and Rac, resulting in inhibition of endothelial-cell spreading and migration in vitro and suppression of FGF-2-induced angiogenesis in vivo. These results provide a functional link between COX-2, integrin αVβ3 and Cdc42/Rac-dependent endothelial-cell migration.

Thus, COX-2 appears to be related to induction of angiogenesis in a variety of tumours, which provides a general target for the chemopreventive use of COX-2 inhibitors for a variety of malignancies.

In addition to angiogenic effects, COX-2 expression may have effects on apoptosis. The specific COX-2 inhibitor celecoxib induces apoptosis in human prostate cancer cells, whereas the COX-1 inhibitor piroxicam has no appreciable effect [67]. The forced expression of COX-2 inhibits programmed cell death, and the growth of COX-2-positive human colon cancer xenografts has been shown to be inhibited by a COX-2 inhibitor (SC-58125) [68]. PGE2 inhibited the SC-58125-stimulated apoptosis and also induced expression of the anti-apoptotic protein Bcl-2 but did not affect expression of BAX, the death-promoting member of the Bcl-2 family, in human colon cancer (HCA-7) cells. In the human colorectal cancer cell line HCT116, which contains normal p53 and BAX genes, deletion of the BAX gene provides profound protection against the apoptosis induced by the NSAIDs, sulindac and indomethacin [69]. This mechanism may also explain resistance to NSAID-induced apoptosis in conditions with a hereditary predisposition such as hereditary non-polyposis colorectal cancer, where inherited defects of BAX may be present. The enhanced tumorigenesis seen in transgenic mice carrying COX-2 under the control of the mouse mammary tumour virus promoter is preceded by reduced levels of BAX and Bcl-xL and increased levels of Bcl-2 in the mammary epithelial cells [56]. Thus the interactions between COX-2, Bcl-2 and other members of the Bcl-2 family may represent a further mechanism by which COX-2 expression influences tumour growth, including that of the mammary gland.

**Intratumoural aromatase, COX-2 and breast cancer**

**Intratumoural aromatase and breast cancer**

Several areas of research have implicated sex hormones in the aetiology of breast cancer. Laboratory studies have shown that hormones, and in particular oestrogens control the growth of breast epithelial cells and affect the course of established disease. In premenopausal women, studies of serum oestradiol
and its correlation with breast cancer risk have been inconsistent [70]. Variations in concentrations throughout the menstrual cycle may account for this inconsistency. A number of studies [71–75], however, have reported an association between high serum concentrations of oestradiol and increased risk of breast cancer in postmenopausal women. Some studies, e.g. Thomas et al. [71], report a steep gradient of association. If the association is causative, this gradient would result in a small decrease in serum oestradiol concentrations leading to substantial reduction in breast cancer risk.

Oestrogens are produced from androgens by the action of the enzyme aromatase. In postmenopausal women, plasma oestrogens result from peripheral aromatisation, particularly in adipose tissue. Many breast cancers, however, also contain aromatase, and studies using radioactive isotopes indicate that both of these sources contribute to the oestrogen detectable in breast cancers [76]. The proportional significance varies markedly from patient to patient but the mean contribution appears to be approximately equal from the local and distal sources. This capacity of certain breast cancers to synthesise oestrogens by intratumoural aromatase activity has been known for many years [77], but the exact cell types or breast tumours which contain functionally active aromatase remain an area of debate. In different studies, aromatase has been detected in both epithelial and stromal cells [78], in just the stromal elements [79] and only in breast cancer epithelial cells [80]. Aromatase is also detectable in normal breast tissue. The association between high levels of aromatase in the quadrant of normal tissue containing a breast carcinoma has been suggested as causative [81]. A small number of studies have suggested that breast carcinomas with aromatase activity show an increased response to aromatase inhibitors [82, 83] but larger studies are needed to confirm this. Aromatase-transfected MCF7 breast cancer cells can be mitogenically stimulated with androgen and their proliferation can be suppressed with aromatase inhibitors [84]. All of these findings provide support for the role of intratumoural aromatase being of importance in breast cancer incidence and progression.

**Regulation of breast aromatase gene expression and transcription**

Transcriptional regulation of the aromatase gene is complex and differs between tissues, providing tissue-specific control.
of the enzyme. At least eight exons I have been reported. In breast carcinomas exons 1.3 and II are the most frequent exons I [85], suggesting that promoters 1.3 and II are the major promoters directing aromatase expression in the malignant and surrounding tissue. This contrasts with the adipose stromal cells and fibroblasts of normal breast tissue, in which exon 1.4 is the dominant exon I [86, 87]. Exon 1.4 is preceded by a TATA-less promoter and upstream CRE and Sp1 sequences, which are required for upregulation of transcription by serum glucocorticoids [88]. A series of in vitro experiments point to exons 1.3 and II being under the control of cAMP [89, 90]. Thus the concept has been developed of promoter switching on the aromatase gene between normal and malignant tissues (Figure 3). PGE2, which is synthesised by many breast carcinomas, as noted above, is a known stimulant of CAMP in breast cancer cells [91]; its production by breast cancer may be instrumental in the switching of aromatase promoters. If these transcriptional relationships are important in breast cancer, a relationship between COX-2 and aromatase activity might be expected.

**COX-2 and aromatase in breast cancer**

COX-2 expression has been found to correlate with aromatase expression within human breast cancer tissue. A study of 23 human breast tumours found that COX-2 and aromatase expression, as measured by semi-quantitative RT-PCR showed a significant positive correlation [43]. The highest levels of COX-2 expression were in tumours with high cellularity and those that showed evidence of invasion. A strong linear association between aromatase expression and the sum of COX-1 and COX-2 expression was also found. The COX-2 product PGE2 and cytokines such as interleukin-6 (IL-6) or TNF-α can regulate aromatase activity [90]. Studies of aromatase activity in fibroblasts from breast tissue proximal to a carcinoma have shown a higher basal aromatase activity than those derived from the tumour itself, and the ability of PGE2, TNF-α and IL-6 to stimulate aromatase activity was greater in the tissue adjacent to the tumour [92, 93].

**Prospects for COX-2 inhibitors in breast cancer prophylaxis**

The body of evidence cited above supports a link between the overexpression of COX-2 and mammary carcinogenesis which may at least in part be dependent on the induction of aromatase by PGE2. The selective inhibition of COX-2 may modulate a critical step in the initiation and promotion, as well as progression of breast cancer. COX-2 inhibitors are well tolerated and have minimal side-effects, and as such may be well suited to prophylactic use.

The long-term use of COX-2 inhibitors has been evaluated in several large randomised trials including the Vioxx Gastrointestinal Outcomes Research Study (VIGOR) [94] and the Celecoxib Long-term Safety Study (CLASS) [95]. These studies confirm the improved gastrointestinal safety profile compared with conventional NSAIDs. Recent concerns have been raised, however, about possible cardiovascular risks associated with COX-2 inhibitor usage [96]. An increased RR of thrombotic cardiovascular events (2.38; P = 0.002) was seen with the use of rofecoxib compared with naproxen in the VIGOR study. This difference was not seen between celecoxib and other NSAIDs in the CLASS study. Whether these findings reflect a prothrombotic effect of rofecoxib or a greater antithrombotic effect of naproxen is uncertain. Further prospective evaluation is needed to fully characterise any increased risk and these data may affect the prospects for COX-2 inhibition in breast cancer prophylaxis.

Aromatase inhibitors are in pilot trials to evaluate their possible utility in chemoprevention, but their profound suppression of oestrogen throughout the body may lead to unacceptable side-effects. In contrast, the possible link between the COX-2 products and breast aromatase in and around malignant breast tissue suggests a possible means for selective suppression of oestrogenic stimulation of the breast. It is likely that such targeted oestrogen suppression will lead to only partial withdrawal of oestrogen, but the epidemiological data indicate that there may be a steep dose relationship between oestrogen exposure and breast cancer incidence, suggesting that partial oestrogen suppression may have profound effects on breast cancer incidence. Thus the current understanding of the role of COX-2 in breast cancer suggests that COX-2 inhibitors may have a role in chemoprevention which is based in part on the generic issues of anti-angiogenesis and pro-apoptotic processes, and in part on a tissue-specific inhibition of oestrogen synthesis (Figure 4). It might be considered...
that the effects on angiogenesis and apoptosis may be more important since these would provide a means of non-oestrogen-dependent prevention, while current strategies for prevention in breast cancer are targeted at oestrogen-dependent effects.

Pilot studies of the chemopreventive use of COX-2 inhibitors are merited to test these concepts. The presurgical setting offers an opportunity to test the effects of a short course of these compounds on biological markers of angiogenesis, apoptosis, proliferation as well as conventional markers such as oestrogen, progesterone and HER-2 receptor status in human breast cancer tissue. This would provide direct data to indicate whether these compounds have biological effects of potential clinical significance within human breast cancer tissue, and would provide the platform for larger chemopreventive studies.

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