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Cyclooxygenase-2: a target for the prevention and treatment of breast cancer

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Abstract

Cyclooxygenase-2 (COX-2), an inducible prostaglandin synthase, is normally expressed in parts of the kidney and brain. Aberrant *COX-2* expression was first reported in colorectal carcinomas and adenomas, and has now been detected in various human cancers, including those of the breast. Strikingly, *COX-2* overexpression in murine mammary gland is sufficient to cause tumour formation. To date, the role of COX-2 in tumorigenesis has been most intensively studied in the colon. Thus, the relationship between COX-2 and neoplasia can best be illustrated with reference to intestinal tumorigenesis. Here we consider the potential utility of selective COX-2 inhibitors for the prevention and treatment of breast cancer. Data for cancers of the colon and breast are compared where possible. In addition, the mechanisms by which *COX-2* is upregulated in cancers and contributes to tumorigenesis are discussed. Importantly, several recent studies of mammary tumorigenesis in animal models have found selective COX-2 inhibitors to be effective in the prevention and treatment of breast cancer. Clinical trials will be needed to determine whether COX-2 inhibition represents a useful approach to preventing or treating human breast cancer.

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Introduction

Cyclooxygenase-2 (COX-2) is emerging as an increasingly promising pharmacological target for the prevention and treatment of many human cancers. COX-1 and COX-2 are prostaglandin (PG) synthases which catalyse sequential synthesis of prostaglandin G_2 (PGG_2) and PGH_2 from arachidonic acid by virtue of intrinsic cyclooxygenase and peroxidase activities (Fig. 1). PGH_2 is then converted by specific isomerases to other eicosanoids, including PGs, thromboxane (Tx) and prostacyclin. Cyclooxygenase-derived prostanoids contribute to many normal physiological processes including haemostasis, platelet aggregation, kidney and gastric function, reproduction, pain and fever. Despite the similar enzymatic activities of COX-1 and COX-2, the *COX-1* and *COX-2* genes have distinct properties, and differing expression patterns (Table 1). While *COX-1* is constitutively expressed, *COX-2* is upregulated in response to growth factors, tumour promoters and cytokines (reviewed by Herschman 1996). Additionally, *COX-2* is responsive to several oncogenes, including v-*src*, v-*Ha-ras*, *HER-2/neu* and *Wnt* genes (Xie & Herschman 1995, Subbaramaiah *et al.* 1996, Sheng *et al.* 1998*b*, Howe *et al.* 1999, Vadlamudi *et al.* 1999, Haertel-Wiesmann *et al.* 2000). Thus, increased PG synthesis is detected in inflamed and neoplastic tissues. Analysis of *COX-2*-deficient mice suggests that COX-2 is normally important for post-natal renal development and multiple female reproductive processes including ovulation, fertilisation, implantation and decidualisation (Dinchuk *et al.* 1995, Morham *et al.* 1995, Lim *et al.* 1997, 1999*a*). Aberrant *COX-2* expression has been detected in multiple human cancers, as shown in Table 2. Together, a weight of epidemiological, pharmacological, genetic and expression data combine to suggest an important role for COX-2 in tumorigenesis, particularly in colorectal cancer. There is recent evidence that COX-2 may also represent a novel target for the prevention and treatment of breast cancer.

Cyclooxygenase activity is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sulindac, which are most commonly administered for the relief of pain and inflammation. However, adverse side effects including peptic ulcer disease are associated with the use of such compounds, which are nonselective inhibitors of

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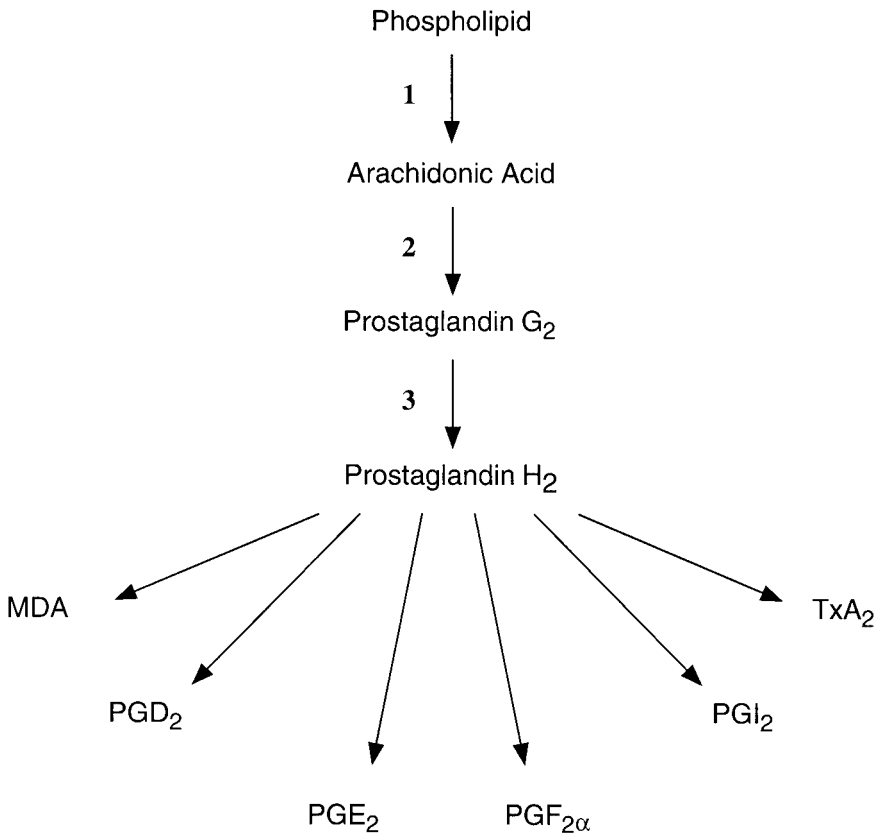

Figure 1 Biosynthesis of prostaglandins. Arachidonic acid, released from membrane phospholipids by phospholipase A_2 action (reaction 1), is metabolised by cyclooxygenases to PGH_2 in two steps. PGG_2 is generated by cyclooxygenase activity (reaction 2), then converted to PGH_2 by the peroxidase activity (reaction 3); both enzyme activities are intrinsic to COX-1 and COX-2. PGH_2 can be converted to several eicosanoids by specific isomerases. Additionally, MDA (malondialdehyde) can be produced enzymatically or by degradation of PGH_2. TxA_2, thromboxane A_2.

Table 1 Properties of COX-1 and COX-2.

	COX-1	COX-2
Expression	Constitutive	Inducible
Size of gene	22 kb	8.3 kb
mRNA transcript	2.7 kb	4.5 kb, with multiple Shaw-Kamen sequences
Size of protein	72 kDa	72/74 kDa doublet
Localisation	Endoplasmic reticulum, nuclear envelope	Endoplasmic reticulum, nuclear envelope
Expression pattern	Most tissues, including stomach, kidney, colon and platelets	Regions of brain and kidney, activated macrophages, synoviocytes during inflammation, malignant epithelial cells. Expression stimulated by cytokines, growth factors, oncogenes and tumour promoters

COX-1 and COX-2. In fact, prior to the development of selective COX-2 inhibitors, there were an estimated 100 000 hospitalisations and 16 500 deaths per year in the United States related to NSAID use (Singh 1998). Toxicity associated with the use of nonselective NSAIDs was the major stimulus to develop selective COX-2 inhibitors. Endoscopically controlled studies show that selective COX-2 inhibitors are far less ulcerogenic than classical NSAIDs (Langman *et al.* 1999, Simon *et al.* 1999). Since selective COX-2 inhibitors appear sufficiently safe to allow large scale administration on a chronic basis to healthy individuals, they represent potentially useful agents for cancer chemoprevention.

COX-2 and cancer: epidemiology and expression

Colon cancer

One of the first clues that cyclooxygenase inhibition might be an effective approach to preventing cancer came from

Table 2 *COX-2* overexpression in human tumours.

Organ site	References
Breast cancer	Parrett *et al.* (1997), Hwang *et al.* (1998), Masferrer *et al.* (2000), Subbaramaiah *et al.* (1999*b*), Soslow *et al.* (2000)
Cervical dysplasia and cancer	Kulkarni *et al.* (2001)
Prostate carcinoma	Gupta *et al.* (2000), Yoshimura *et al.* (2000)
Bladder transitional cell carcinoma	Mohammed *et al.* (1999)
Hepatocellular carcinoma	Koga *et al.* (1999)
Pancreatic cancer	Molina *et al.* (1999), Okami *et al.* (1999), Tucker *et al.* (1999)
Skin cancer	Buckman *et al.* (1998)
Lung cancer	Hida *et al.* (1998), Wolff *et al.* (1998) Ochiai *et al.* (1999)
Head and neck cancer	Chan *et al.* (1999)
Colorectal adenomas and carcinomas	Eberhart *et al.* (1994), Kargman *et al.* (1995), Sano *et al.* (1995), Kutchera *et al.* (1996)
Gastric cancer	Ristimaki *et al.* (1997)
Barrett's oesophagus and oesophageal cancer	Wilson *et al.* (1998)

epidemiological studies. Several studies reported an inverse correlation between colon cancer incidence and regular use of NSAIDs including aspirin (Thun *et al.* 1991, Greenberg *et al.* 1993, Logan *et al.* 1993, Suh *et al.* 1993, Reeves *et al.* 1996). Since NSAIDs are known to function, at least in part, by inhibiting cyclooxygenase enzyme activity, these observations suggested that aberrant PG biosynthesis might contribute to colorectal neoplasia. This led to an analysis of *COX* expression in colorectal neoplasms. Levels of *COX-1* were not increased in colorectal carcinomas relative to adjacent normal mucosa (Eberhart *et al.* 1994, Kargman *et al.* 1995, Sano *et al.* 1995). In contrast, striking *COX-2* upregulation was observed in colon carcinomas compared with the virtually undetectable expression in normal mucosa (Eberhart *et al.* 1994, Kargman *et al.* 1995, Sano *et al.* 1995, Kutchera *et al.* 1996). Eberhart *et al.* (1994) also detected *COX-2* expression in 9 of 20 adenomas examined. In carcinomas, COX-2 protein localised predominantly to the epithelial component, but could also be detected in tumour-associated fibroblasts, vascular endothelial cells, and inflammatory mononuclear cells (Sano *et al.* 1995, Kutchera *et al.* 1996). *COX-2* expression has also been detected in intestinal adenomas from rodent models of intestinal tumorigenesis (Boolbol *et al.* 1996, DuBois *et al.* 1996*a*, Williams *et al.* 1996, Singh *et al.* 1997).

Together, these epidemiological and expression studies suggested a role for COX-2 in colorectal tumorigenesis. This idea is supported by the results of clinical trials. Treatment with the NSAID sulindac or with celecoxib, a selective COX-2 inhibitor, causes a decrease in the size and number of polyps in familial adenomatous polyposis patients (Giardiello *et al.* 1993, Steinbach *et al.* 2000). Thus, overexpression of *COX-2* appears to contribute to colorectal cancer and cyclooxygenase inhibitors are likely to be useful chemopreventive agents.

Breast cancer

In contrast to colon cancer, the role of COX-2 in breast cancer is less clear. Epidemiological studies conducted to investigate the relationship between NSAID use and breast cancer incidence have reported conflicting findings. Several studies have failed to find a significant relationship between aspirin use and breast cancer risk (Paganini-Hill *et al.* 1989, Thun *et al.* 1991, Egan *et al.* 1996). However, other analyses have revealed an association between NSAID consumption and decreased breast cancer incidence. Friedman & Ury (1980) found significantly reduced breast cancer incidence in 4867 women who used indomethacin, compared with age-matched controls. Harris and colleagues (1996) compared NSAID use in 511 women with newly diagnosed breast cancer with 1534 population control subjects, and found that the relative risk of breast cancer was reduced to 66% in women using NSAIDs at least 3 times per week for at least one year. Two additional studies also found that NSAIDs protected against breast cancer (Schreinemachers & Everson 1994, Sharpe *et al.* 2000). The basis for the lack of consistency among different studies is unclear. One potential explanation is that some NSAIDs may have restricted bioavailability in breast tissue. Thus, conflicting data obtained in separate studies may reflect the usage of different NSAIDs in the populations examined. Another potential complication is that significant *COX-2* overexpression may be limited to a subset of human breast cancers, which could certainly confound epidemiological analyses. Approximately 85% of human colorectal adenocarcinomas overexpress *COX-2* (Eberhart *et al.* 1994, Kargman *et al.* 1995, Sano *et al.* 1995, Kutchera *et al.* 1996). This could account for the strong correlation between regular NSAID use and reduced cancer incidence (Thun *et al.* 1991, Greenberg *et al.* 1993, Logan *et al.* 1993, Suh *et al.* 1993, Reeves *et al.* 1996). In contrast, as discussed below, *COX-2* is not abundantly overexpressed in the majority of human breast cancers (Hwang *et al.* 1998, Subbaramaiah *et al.* 1999*b*). With this in mind, it is predictable that the results of epidemiological studies would be less clear-cut for breast than colon cancer even if NSAIDs were active against COX-2-positive breast cancers.

Enhanced *COX* expression in breast cancer was first suggested by reports of elevated PG levels in breast tumours (Tan *et al.* 1974, Bennett *et al.* 1977, Rolland *et al.* 1980). PG production was increased in human breast cancers, particularly in those from patients with metastatic disease (Bennett *et al.*

1977, Rolland *et al.* 1980). PG production and *COX-2* expression have also been detected in breast cancer-derived cell lines (Schrey & Patel 1995, Liu & Rose 1996, Gilhooly & Rose 1999). Interestingly, there appears to be a correlation between invasiveness/metastatic potential and PG production/*COX-2* expression in both cell lines and tumour specimens (Bennett *et al.* 1977, Rolland *et al.* 1980, Liu & Rose 1996). However, there are conflicting data regarding the frequency of *COX-2* expression in breast cancers. Parrett *et al.* (1997) detected *COX-2* expression in 13/13 human breast tumours by reverse transcriptase-coupled polymerase chain reaction (RT-PCR), compared with no detectable expression in normal human breast tissue, and observed a correlation between *COX-2* expression and increasing tumour cell density. Immunohistochemistry revealed COX-2 protein in the epithelial cells of the tumours, with no expression in the stromal compartment. In contrast, Hwang and colleagues (1998) analysed 44 tumour samples by Western blotting but only detected COX-2 protein in 2 of the 44 samples. These apparently discrepant observations can be reconciled by consideration of the following. First, the failure to detect COX-2 by Western blotting or RNAse protection (Hwang *et al.* 1998) may reflect the relative insensitivity of these techniques compared with RT-PCR. The second important caveat is that *COX-2* expression may be predominantly associated with certain subsets of human breast cancers (Gilhooly & Rose 1999, Subbaramaiah *et al.* 1999*b*). We have examined *COX-2* expression in 29 microdissected human breast cancers using a coupled immunoprecipitation/Western blotting assay, which confers increased sensitivity relative to direct Western blotting of lysates. High levels of COX-2 protein were detected in 14 of 15 *HER-2/neu*-overexpressing breast cancers. In contrast, COX-2 was detected in only 4 of 14 HER-2/neu-negative breast cancers, and was expressed at significantly lower levels than in the HER-2/neu-positive samples (Subbaramaiah *et al.* 1999*b*). Immunohistochemistry localised COX-2 protein to epithelial cells and the vasculature. Thus, it seems likely that significant overexpression of *COX-2* may be largely confined to those breast cancers in which *HER-2/neu* is overexpressed, or in which the signalling pathways normally activated by HER-2/neu are activated by an alternative event such as *ras* mutation (Gilhooly & Rose 1999). Since *HER-2/neu* overexpression is limited to 20–30% of human breast cancers, conflicting epidemiological data may reflect differing proportions of HER-2/neu-positive cancers in the various studies. Based on these recent findings, it would be of considerable interest to compare the efficacy of NSAIDs in preventing HER-2/neu-positive and -negative breast cancers.

COX-2 is expressed in intestinal and mammary tumours in rodents

Rodent models of intestinal tumorigenesis can be divided into carcinogen-induced tumour models, and those in which tumour formation is induced by introduction of germline mutations into tumour suppressor genes such as *Apc*. In humans, germline mutation of the *APC* gene is responsible for familial adenomatous polyposis (FAP), in which individuals develop numerous adenomatous colorectal polyps, which predispose to colorectal carcinomas. In addition, *APC* is mutated in approximately 85% of sporadic colorectal carcinomas. Several mice strains have been developed which harbour mutations in one *Apc* allele, including the Min mouse (Moser *et al.* 1990), $Apc^{\Delta 716}$ (Oshima *et al.* 1995), Apc1638N (Fodde *et al.* 1994), and $Apc^{\Delta 474}$ (Sasai *et al.* 2000). These mice consistently develop intestinal adenomas, although these are more prevalent in the small intestine than in the colon. Analysis of adenomatous polyps from Min mice revealed increased *COX-2* expression relative to normal mucosa (Williams *et al.* 1996). Elevated expression of *COX-2* was also detected in colonic mucosa and tumours from rats treated with azoxymethane (AOM) (DuBois *et al.* 1996*a*, Singh *et al.* 1997). Thus *COX-2* is commonly overexpressed in both human colorectal cancers and animal models of colorectal cancer. The cellular localisation of COX-2 in both human and rodent tumours continues to be investigated.

Rodent models have also been used to examine *COX-2* expression in mammary tumours. In the rat, *COX-1* is ubiquitously expressed in virgin, pregnant, lactating, and post-lactational mammary glands, but *COX-2* is only detectable in the mammary glands of lactating animals (Badawi *et al.* 1999). Treatment of ovariectomised animals with oestradiol and progesterone causes induction of *COX-2* and PG synthesis (Badawi & Archer 1998, Badawi *et al.* 1999), suggesting that *COX-2* transcription is susceptible to hormonal regulation. COX-2 protein has been detected in rat mammary tumours induced by various carcinogens, including *N*-nitrosomethyl urea (NMU), dimethyl-benz[a]anthracene (DMBA) and 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP) (Robertson *et al.* 1998, Hamid *et al.* 1999, Nakatsugi *et al.* 2000). Based on immunohistochemical analyses, COX-2 protein was observed in the epithelial cells within the mammary tumours (Robertson *et al.* 1998, Nakatsugi *et al.* 2000). Interestingly, dietary administration of n-6 polyunsaturated fatty acids (PUFAs) in the form of safflower oil stimulated *COX-2* expression in rat mammary glands, suggesting a potential mechanism by which n-6-PUFAs may contribute to mammary tumorigenesis (Badawi *et al.* 1998).

In addition to these rat studies, COX-2 protein levels have also been examined in mammary tissues from transgenic mice strains that develop mammary tumours due to mammary-targeted oncogene expression. Significant amounts of COX-2 protein were detected in mammary tumours from mice overexpressing *neu* (K Subbaramaiah and A J Dannenberg, unpublished observations), consistent with our findings in *HER-2/neu*-overexpressing human

breast cancers (Subbaramaiah *et al.* 1999*b*). We have also found increased COX-2 protein in mammary tumours from *Wnt-1* transgenic mice, relative to the levels in normal mammary gland (Fig. 2; Howe *et al.* 2001). Consistent with this, *COX-2* is transcriptionally upregulated in mouse mammary epithelial cell lines engineered to express *Wnt-1* (Howe *et al.* 1999), and expression is also increased in response to transformation by other oncogenes (Subbaramaiah *et al.* 1996).

Mechanisms of *COX-2* upregulation

There is evidence that *COX-2* is upregulated in both neoplastic and stromal cells within tumours. Hence, multiple mechanisms are likely to account for overexpression of *COX-2* in these different cell types. It is relevant, therefore, to evaluate the effects of different stimuli in various cell types. *COX-2* expression is normally regulated at both transcriptional and post-transcriptional levels, and can also be regulated by the rate of protein synthesis and/or degradation. The human *COX-2* promoter contains multiple transcription factor binding sites, including a cAMP response element (CRE), and potential binding sites for Myb, nuclear factor interleukin-6 (NF-IL6), nuclear factor κB (NF-κB), and Ets factors (Genbank Accession Number 505116). Of these, the sites proximal to the transcription start site (Fig. 3) have been shown to be differentially responsive to various stimuli. Induction of *COX-2* by v-*src*, serum, platelet-derived growth factor (PDGF) and ceramide requires activation of both Ras/Raf-1/ERK and Ras/MEKK1/JNK signal transduction pathways and is predominantly mediated via the CRE (Xie & Herschman 1995, 1996, Subbaramaiah *et al.* 1998*a*). In contrast, the NF-IL6 and NF-κB sites are required for induction of *COX-2* in response to tumour necrosis factor (TNF) in osteoblasts (Yamamoto *et al.* 1995). The NF-IL6 and CRE sites have been identified as being critical for the induction of *COX-2* in response to other stimuli, including lipopolysaccharide (LPS) and immunoglobulin E receptor aggregation (Inoue *et al.* 1995, Reddy *et al.* 2000*b*, Wadleigh *et al.* 2000). Other studies have implicated the NF-κB site as being important for LPS- and benzo[a]pyrene-mediated induction of *COX-2* (Hwang *et al.* 1997, Yan *et al.* 2000). The expression of *COX-2* can also be increased by stabilisation of the *COX-2* transcript (Ristimaki *et al.* 1994, Sheng *et al.* 1998*b*). The 3′ untranslated region of *COX-2* mRNA contains a 116 nucleotide AU-rich sequence element (ARE) which can negatively regulate transcript stability and modulate translation (Dixon *et al.* 2000).

During tumorigenesis, increased expression of *COX-2* is likely to be a consequence of multiple effects. For example, transcriptional activation is likely to occur in response to growth factors and oncogenes. Moreover, since wildtype p53 decreases *COX-2* transcription, loss-of-function p53 mutations may contribute to *COX-2* upregulation (Subbaramaiah *et al.* 1999*a*). Dixon *et al.* (2000) speculated that ARE-binding proteins which normally negatively regulate transcript stability may be defective in tumour cells. This, too, could result in increased levels of COX-2. The relative importance of these different factors is likely to vary in different tissues. In mouse skin carcinogenesis, promoter activation by upstream stimulatory factor (USF) and CCAAT/enhancer binding proteins (C/EBPs) appears to be important (Kim & Fischer 1998). By contrast, bile acids, which have been implicated in the promotion of

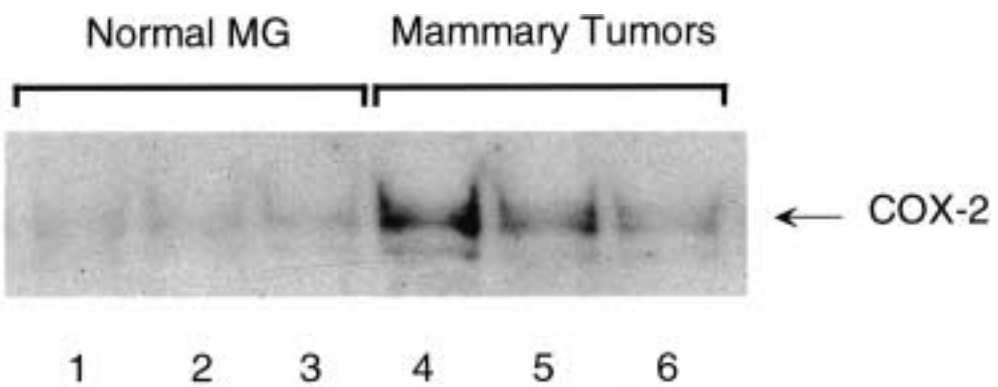

Figure 2 COX-2 protein is increased in *Wnt-1*-expressing mammary tumours. COX-2 protein was analysed in lysates prepared from mammary tumours from three *Wnt-1* transgenic female mice (lanes 4–6) and from mammary glands (MG) isolated from three strain-matched wildtype female mice (lanes 1–3). Lysates were prepared from 10 mg of each tissue sample. COX-2 protein was immunoprecipitated, and immunoprecipitates were analysed for COX-2 by Western blotting. The arrow indicates the position of a COX-2 standard. Little COX-2 protein was detectable in the wildtype mammary glands (lanes 1–3). In contrast, appreciable COX-2 protein was observed in all three tumour samples (lanes 4–6). Adapted and reproduced with permission from Howe *et al.* (2001).

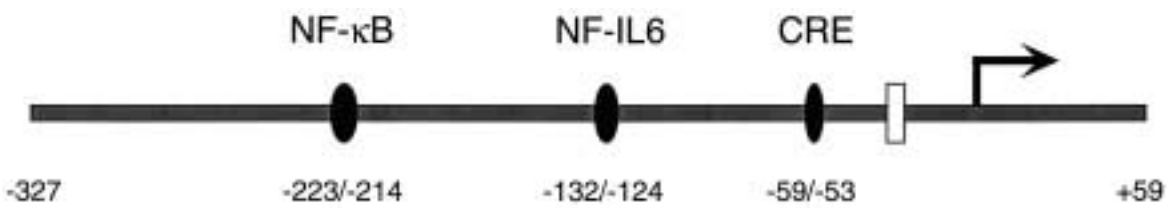

Figure 3 Human *COX-2* promoter schematic. The transcription start site is indicated by an arrow, the TATA box at –31/–25 is shown as a white rectangle, and three transcription factor binding sites lying between –327/+59 of the human *COX-2* promoter are depicted as black ovals.

gastrointestinal tumours, stimulate AP-1 activity and increase *COX-2* transcription and transcript stability (Zhang *et al.* 1998, Zhang *et al.* 2000*b*).

In colorectal cancer cells, constitutive *COX-2* expression is likely to result from a combination of transcriptional, post-transcriptional and translational effects (Hsi *et al.* 1999, Shao *et al.* 2000). Mutation of NF-IL6 and CRE elements has been shown to diminish *COX-2* promoter activity in two colorectal cancer cell lines (Shao *et al.* 2000), implicating these sites in transcriptional upregulation. Interestingly, we have recently detected a requirement for the NF-IL6 site for stimulation of the *COX-2* promoter by Ets factors of the PEA3 subfamily (Howe *et al.* 2001). PEA3 factors stimulate human *COX-2* promoter activity up to 20-fold when overexpressed in 293 human embryonic kidney cells. Since PEA3 factors are highly expressed in colorectal cancer cell lines, intestinal tumours, and *Wnt-1*-expressing mammary cell lines and tumours (Crawford *et al.* 2001, Howe *et al.* 2001), we speculate that PEA3 factors may contribute to *COX-2* induction during both intestinal and mammary tumorigenesis. Increased expression of c-*myb* may also contribute, since c-*myb* is upregulated in colon tumours and breast cancers (Guerin *et al.* 1990, Ramsay *et al.* 1992) and c-*myb* overexpression causes modest induction of *COX-2* promoter activity (Ramsay *et al.* 2000).

COX-2 expression can also be affected by dietary fat. Chemically induced mammary carcinogenesis is promoted by dietary n-6-PUFAs, which enhance tissue levels of arachidonic acid, and inhibited by n-3-PUFAs (reviewed by Rose & Connolly 1999). In experimental models, *COX-2* expression in mammary tissue and tumours is decreased in animals fed an n-3-PUFA-rich diet (i.e. menhaden oil) relative to those fed a diet high in n-6-PUFAs (i.e. corn or safflower oil) (Badawi *et al.* 1998, Hamid *et al.* 1999). Similarly, dietary fish oil decreased the expression of *COX-2* and the incidence of colorectal tumours in AOM-treated rats (Singh *et al.* 1997). This may help to explain epidemiological observations of decreased breast and colon cancer risk in populations with diets rich in fish oils (Rose & Connolly 1999).

Evidence from rodent models that COX-2 contributes to cancer

Genetic evidence for a role for COX-2 in tumour formation

Definitive evidence linking cyclooxygenases to tumorigenesis was first provided by studies using mice with targeted disruptions of the *COX-1* or *COX-2* genes. Oshima *et al.* (1996) pioneered these experiments by generating $Apc^{\Delta 716}$, *COX-2*-null mice. Intestinal adenoma incidence was reduced by 86% in *COX-2* knockout mice, and by 66% in *COX-2* heterozygotes, relative to *COX-2* wildtype mice carrying the *Apc* mutation (Oshima *et al.* 1996). Tumour size was also significantly reduced in *COX-2*-deficient mice. *COX-2* deficiency also protects against chemically induced papilloma formation in mouse skin (Tiano *et al.* 1997), and *COX-2*-null embryonic stem cells have a dramatically reduced ability to form teratomas when injected into syngeneic mice (Zhang *et al.* 2000*a*). Interestingly, disruption of either *COX-1* or *COX-2* caused similar reductions in tumour multiplicity in the Min mouse (Chulada *et al.* 2000), suggesting that both enzymes can impact on tumorigenesis. The results of similar studies to determine the effects of *COX-2* deficiency on the incidence of mammary cancer are eagerly awaited. However, results from the converse experiment designed to address the consequence of *COX-2* overexpression in mammary gland have recently been reported (Liu *et al.* 2001). Liu and colleagues overexpressed human *COX-2* from the mouse mammary tumor virus (MMTV) promoter, and demonstrated that *COX-2* overexpression was sufficient to cause breast tumour formation in more than 85% of multiparous mice. Virgin females did not develop tumours, but exhibited precocious lobuloalveolar differentiation and enhanced expression of the milk protein β-casein. MMTV-driven *COX-2* expression increased during pregnancy, suggesting a basis for the failure of virgin animals to develop tumours. Interestingly, mammary gland involution was delayed in *COX-2* transgenic mice, with a decrease in the apoptotic index of mammary epithelial cells, and *COX-2*-induced tumor tissue expressed

reduced levels of the proapoptotic proteins Bax and Bcl-x_L. Together, these observations suggest that *COX-2* expression may contribute to tumorigenesis via a reduction in apoptosis, a result previously suggested by *in vitro* studies, as discussed below.

Pharmacological studies in rodent models of intestinal tumorigenesis

In addition to genetic evidence implicating cyclooxygenases in intestinal tumorigenesis, there are complementary pharmacological data. Many animal-based studies have been performed to investigate the utility of cyclooxygenase inhibitors for prevention or treatment of intestinal tumours. The prevention studies have predominantly examined either AOM-induced lesions in rat colon (aberrant crypt foci or carcinomas) or intestinal adenomas in *Apc*-deficient mice. A consistent finding has been that tumour incidence and multiplicity are reduced by both nonselective NSAIDs (Reddy *et al.* 1993, Rao *et al.* 1995, Boolbol *et al.* 1996, Jacoby *et al.* 1996, 2000*a*), and selective COX-2 inhibitors (Table 3). In addition, those tumours that do develop in drug-treated animals tend to be reduced in size relative to those in control animals (Nakatsugi *et al.* 1997, Fukutake *et al.* 1998, Jacoby *et al.* 2000*a*,*b*, Reddy *et al.* 2000*a*). It is notable that selective COX-2 inhibitors appear to be at least as effective in preventing tumours as nonselective NSAIDs. This result has important clinical implications, given the enhanced safety profile of selective COX-2 inhibitors versus traditional NSAIDs.

In addition to these prevention studies, cyclooxygenase inhibitors are also being evaluated as therapeutic agents for pre-existing tumours. Reduction in growth of colon cancer xenografts has been achieved by treatment with meloxicam, SC-58125 and celecoxib (Sheng *et al.* 1997, Goldman *et al.* 1998, Williams *et al.* 2000*b*). Celecoxib also decreased tumour multiplicity in Min mice by 52%, when administered after adenomas had been established (Jacoby *et al.* 2000*b*).

Pharmacological studies in rat breast cancer models

Carcinogen-induced rat mammary tumours have been used as a model system to test various NSAIDs and, more recently, selective COX-2 inhibitors for their chemopreventive potential (Table 4). In general, indomethacin was found to reduce the incidence and multiplicity of DMBA-induced tumours (Carter *et al.* 1983, 1989, McCormick *et al.* 1985, Noguchi *et al.* 1991). Because the incidence of breast cancer may be affected by dietary fat, some of these studies have compared NSAID effects in cohorts of animals fed low- versus high-fat diets. Carter *et al.* (1983) found that indomethacin reduced tumour incidence in DMBA-treated animals fed 18% corn oil to the level observed in DMBA-treated animals fed 5% corn oil, but did not see an effect on incidence in the low-fat cohort. In contrast, two other studies found that the inhibitory effect of indomethacin was not confined to rats fed high-fat diets (McCormick *et al.* 1985, Noguchi *et al.* 1991). Interestingly, McCormick *et al.* (1985) found that indomethacin treatment from 2 weeks before to 1 week after DMBA administration primarily targeted benign tumours. However, when treatment with indomethacin was initiated 1 week after DMBA and continued until the end of the trial, the multiplicity of malignant tumours was also significantly reduced. Conflicting data were obtained by Abou-el-Ela *et al.* (1989) who found no inhibition of mammary tumorigenesis by indomethacin. The basis for these discrepant observations is unclear. Two additional NSAIDs, flurbiprofen and aspirin, are also capable of reducing carcinogen-induced mammary tumorigenesis (McCormick & Moon 1983, Suzui *et al.* 1997, Mori *et al.* 1999), although piroxicam was not found to be effective in one study (Kitagawa & Noguchi 1994).

Two recent studies evaluated the effects of selective COX-2 inhibitors on mammary tumorigenesis. As shown in Fig. 4A, treatment with celecoxib significantly delayed tumour onset in DMBA-treated rats, and was more effective than ibuprofen (Harris *et al.* 2000). Dietary administration

Table 3 Chemoprevention of intestinal tumorigenesis in rodents using selective COX-2 inhibitors.

Reference	Animal	Model	Tumour type	Drug	Effect on tumour multiplicity
Oshima *et al.* (1996)	Mouse	$Apc^{\Delta 716}$	Adenoma	MF tricyclic	62% inhibition
Nakatsugi *et al.* (1997)	Mouse	Apc Min	Adenoma	Nimesulide	48% inhibition
Kawamori *et al.* (1998)	Rat	AOM	Colon carcinoma	Celecoxib	97% inhibition
Fukutake *et al.* (1998)	Mouse	AOM	Colon carcinoma	Nimesulide	81% inhibition
Reddy *et al.* (2000*a*)	Rat	AOM	Colon carcinoma	Celecoxib	84% inhibition
Sasai *et al.* (2000)	Mouse	$Apc^{\Delta 474}$	Adenoma	JTE-522	32% inhibition
Jacoby *et al.* (2000*b*)	Mouse	Apc Min	Adenoma	Celecoxib	71% inhibition

Several of these studies tested a range of drug concentrations. The effect on tumour multiplicity (number of tumours per animal) reported in this table was that achieved at the highest drug dose tested. In addition to inhibition of tumour multiplicity, these agents also caused reduced tumour incidence (proportion of animals with tumours). Note that individual studies examined different endpoints – carcinomas or adenomas.

Table 4 Chemoprevention of mammary tumorigenesis in rats using cyclooxygenase inhibitors

Reference	Tumour induction	Drug	Effects
Carter *et al.* (1983)	DMBA/18% corn oil	Indomethacin	54% inhibition of tumour multiplicity; reduction in tumour incidence
McCormick & Moon (1983)	NMU	Flurbiprofen	Reduction in tumour incidence and multiplicity at low NMU dose
McCormick *et al.* (1985)	DMBA	Indomethacin	Reduction in benign or malignant tumours according to period of drug administration
Abou-el-Ela *et al.* (1989)	DMBA	Indomethacin	No inhibition
Carter *et al.* (1989)	DMBA/20% fat	Indomethacin	Inhibition of tumorigenesis in animals fed 4 or 12% linoleate
Noguchi *et al.* (1991)	DMBA/20% corn oil	Indomethacin	61% inhibition of tumour multiplicity; reduction in tumour incidence
Kitagawa & Noguchi (1994)	DMBA/20% soybean oil	Piroxicam	No inhibition
Suzui *et al.* (1997)	PhIP/high corn oil	Aspirin	44% inhibition of tumour multiplicity
Mori *et al.* (1999)	PhIP/high fat	Aspirin	Inhibited tumour multiplicity
Harris *et al.* (2000)	DMBA	Celecoxib	86% inhibition of tumour multiplicity; 68% reduction in tumour incidence
Nakatsugi *et al.* (2000)	PhIP/24% corn oil	Nimesulide	54% inhibition of tumour multiplicity; 28% reduction in tumour incidence

of celecoxib reduced incidence, multiplicity and volume of malignant breast tumours by 68%, 86% and 81% respectively relative to the control group. The chemopreventive properties of another COX-2 inhibitor, nimesulide, was tested in rats in which the environmental carcinogen PhIP, together with a 24% corn oil diet, was used to induce *COX-2* expression and mammary tumours (Nakatsugi *et al.* 2000). A small reduction in tumour incidence was achieved by administration of 400 parts per million nimesulide (Table 5). In addition, both size and multiplicity of tumours were significantly reduced in the nimesulide-treated animals. Together, these studies represent the first direct evidence that selective COX-2 inhibitors can protect against experimental breast cancer.

Two additional studies suggest that cyclooxygenase inhibition may be a useful strategy for treating breast cancer. Robertson *et al.* (1998) measured tumour size in rats that were maintained for 100 days post DMBA treatment then fed a control or an ibuprofen-containing diet for an additional 5 weeks prior to necropsy. Tumours from the control animals increased in volume by approximately 180%. In contrast, those from the ibuprofen-treated cohort decreased in volume by almost 40%. More recently, a similar study was conducted in which the effects of the selective COX-2 inhibitor celecoxib were investigated (Alshafie *et al.* 2000). In this study, rats were maintained for 4 months post DMBA treatment to induce tumours. Subsequently, the rats were given a control or a celecoxib-containing diet for an additional 6 weeks. The mean tumour volume increased by 518% in control animals, but decreased by 32% in the group fed celecoxib (Fig. 4B). In addition, the total tumour number continued to increase in the control animals, but was reduced in the celecoxib cohort. This report of regression of mammary tumours *in vivo* by a selective COX-2 inhibitor is consistent with earlier studies showing that various NSAIDs reduced the growth of mammary tumour xenografts (Fulton 1984, Karmali & Marsh 1986). Together, these observations suggest that COX-2 inhibition may represent a strategy not only for prevention but also for treatment of human breast cancer.

The ability of COX-2 inhibitors to significantly reduce tumour multiplicity strongly suggests that COX-2 contributes to tumorigenesis. However, COX-independent effects of NSAIDs have also been described (see below), raising the possibility that the observed inhibition may not necessarily be ascribed solely to effects on COX-2. Nevertheless, taken together the pharmacological and genetic studies provide overwhelming support for a role for COX-2 in tumorigenesis. Definitive evidence has now been provided by the recent demonstration that *COX-2* overexpression is sufficient to induce mammary tumor formation in transgenic mice (Liu *et al.* 2001).

How does COX-2 contribute to cancer?

Prostaglandins stimulate proliferation and mediate immune suppression

Since COX-2 is a PG synthase, the most obvious consequence of *COX-2* overexpression is increased PG production, and indeed high PG levels have been detected in many cancers. Enhanced PG synthesis may contribute to carcinogenesis in several ways, including direct stimulation of cell growth. $PGE_{2\alpha}$ and $PGF_{2\alpha}$ can both stimulate mitogenesis in Balb/c 3T3 fibroblasts in synergy with epidermal growth factor (EGF) (Nolan *et al.* 1988), and $PGF_{2\alpha}$ is also mitogenic for Swiss 3T3 cells and osteoblasts

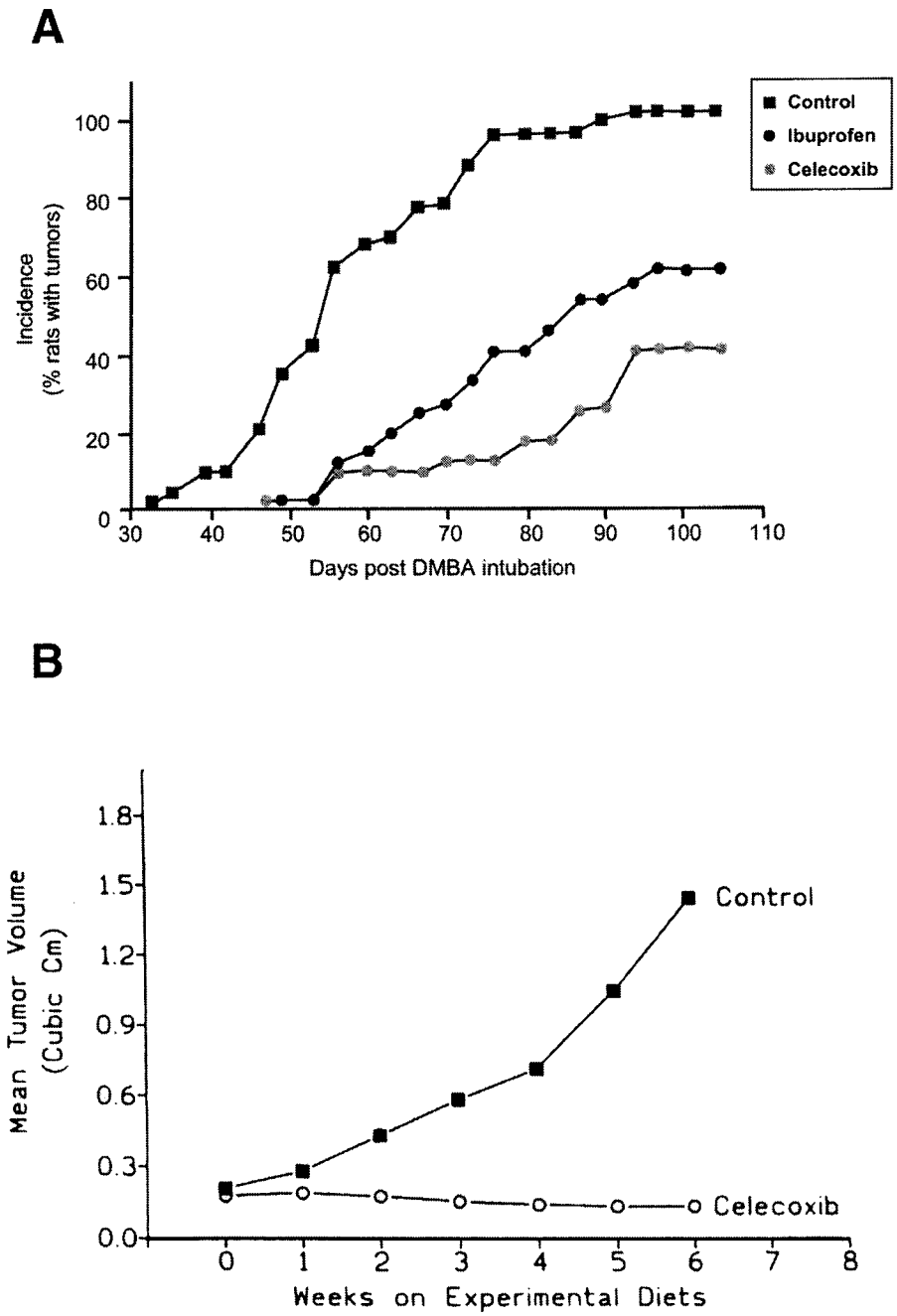

Figure 4 Celecoxib is effective for breast cancer prevention and treatment. (A) Rats were assigned to a control diet, or a diet containing 1500 ppm ibuprofen or 1500 ppm celecoxib 7 days prior to a single intragastric dose of DMBA, and tumour incidence was measured for 16 weeks. This figure is reproduced with permission from Harris *et al.* (2000). (B) Rats were maintained for four months after a single intragastric dose of DMBA to allow palpable tumour development, then assigned to a control diet or a diet containing 1500 ppm celecoxib, and tumour size was monitored for 6 weeks. This figure is reproduced with permission from Alshafie *et al.* (2000).

Table 5 Effects of nimesulide on the incidence, multiplicity and volume of mammary carcinomas induced by PhIP in Sprague-Dawley rats. Results are means ± S.E.

	Control diet	400 ppm nimesulide
Tumour incidence (% rats with cancers)	30/42 (71%)	19/37 (51%)
Multiplicity (no. of cancers/rat)	2.6 ± 0.5	1.2 ± 0.2*
Cancer volume/rat (cm^3)	4.1 ± 1.3	1.1 ± 0.4*
Effective no. of rats	42	37

*Significantly different from the control diet group by Welch's *t* test (P >0.05).
This table is reproduced with permission from Nakatsugi *et al.* (2000).

(Goin *et al.* 1993, Quarles *et al.* 1993). Both PGE_1 and PGE_2 stimulate proliferation of mammary epithelial cells in the presence of EGF (Bandyopadhyay *et al.* 1987). Thus, inappropriate stimulation of cellular proliferation by PGs may contribute to tumorigenesis. However, PGs do not act as mitogens for all cell types, and in fact depress proliferation of some cells, particularly those of the immune system (Marnett 1992).

Antiproliferative effects may contribute to the immune suppression associated with PGs. PGE_2 inhibits T and B cell proliferation and cytokine synthesis, and diminishes the cytotoxic activity of natural killer cells. PGE_2 also inhibits the production of TNFα while inducing interleukin-10 production, which itself has immunosuppressive effects (Huang *et al.* 1996). PGs may also inhibit antigen processing by dendritic cells (Stolina *et al.* 2000). Thus, PG-mediated immune suppression may contribute to tumorigenesis, since this may allow tumours to avoid immune surveillance that might otherwise limit their growth.

In breast tissue, PGs may also stimulate proliferation indirectly by increasing oestrogen biosynthesis (Harris *et al.* 1999). The aromatase gene *CYP19*, which is responsible for oestrogen synthesis, has three promoters, I.4, I.3 and II, from which distinct transcripts are generated. In adipose tissue, aromatase is normally expressed from promoter I.4. However, in adipose tissue adjacent to breast tumours, *CYP19* tends to be expressed from promoter II. Recently, PGE_2 has been demonstrated to increase aromatase activity (Zhao *et al.* 1996, Purohit *et al.* 1999) and cause *CYP19* promoter switching to promoter II in adipose stromal cells (Zhao *et al.* 1996). These data suggest that PG overproduction can induce aromatase, leading to increased oestrogen synthesis. Consistent with this, a positive correlation has been observed between *CYP19* and *COX* expression in human breast cancer specimens (Brueggemeier *et al.* 1999). Thus it is possible that PG-mediated oestrogen overproduction may be an important organ site-specific consequence of *COX-2* upregulation in breast cancer.

Cyclooxygenase-mediated production of mutagens

Thus far, the potential contributions of PG overproduction to tumorigenesis, including increased cellular proliferation and diminished immune surveillance, have been discussed. However, *COX-2* overexpression may also have PG-independent consequences. In particular, *COX-2* overexpression may result in increased production of mutagens. Malondialdehyde (MDA) can be produced by isomerisation of PGH_2 both enzymatically and non-enzymatically (Fig. 1). Therefore, MDA production may be elevated due to increased availability of the precursor molecule PGH_2. MDA forms adducts with deoxynucleosides and induces frame-shifts and base-pair substitutions, and thus has potent mutagenic activity (Marnett 1992). Additional carcinogens can be formed by oxidation of aromatic amines, heterocyclic amines, and dihydrodiol derivatives of polycyclic hydrocarbons (Wiese *et al.* 2001). This oxidation step is catalysed by the peroxidase activity of cyclooxygenase, which requires a reductant to convert PGG_2 to PGH_2. Thus, *COX-2* overexpression may lead to DNA damage, thereby contributing to carcinogenesis. Consistent with this hypothesis, the selective COX-2 inhibitor nimesulide decreases formation of the mutagen 8-oxo-7,8-dihydro-2′-deoxyguanosine in the colonic mucosa (Tardieu *et al.* 2000).

Effects on angiogenesis

Recently, it has become apparent that cyclooxygenases are involved in angiogenesis (reviewed by Gately 2000). This is a crucial facet of tumorigenesis since neovascularisation is required for tumours to grow beyond 2–3 mm in size. Experiments in the 1980s showed that xenograft vascularisation was significantly reduced by the NSAIDs indomethacin, diclofenac and aspirin (Peterson 1983). More recently, COX-2 has been specifically implicated. *In vitro*, selective COX-2 inhibitors decrease endothelial tubule formation (Tsujii *et al.* 1998, Jones *et al.* 1999), while, *in vivo*, selective COX-2 inhibitors reduce angiogenesis in several models (Majima *et al.* 1997, Daniel *et al.* 1999, Sawaoka *et al.* 1999, Yamada *et al.* 1999, Masferrer *et al.* 2000). A representative illustration of celecoxib-mediated inhibition of corneal angiogenesis is shown in Fig. 5.

In an interesting corollary, Lewis lung carcinoma xenografts showed marked attenuation of growth when implanted in *COX-2*-null mice, but grew normally in *COX-1*-deficient mice (Williams *et al.* 2000*a*). The tumours from *COX-2* knockout mice exhibited 30% decreased vascular density, implicating host COX-2 in tumour neovascularisation. It seems likely that COX-2 in epithelial cells, endothelial cells and fibroblasts may all contribute to the angiogenic process, although there are some

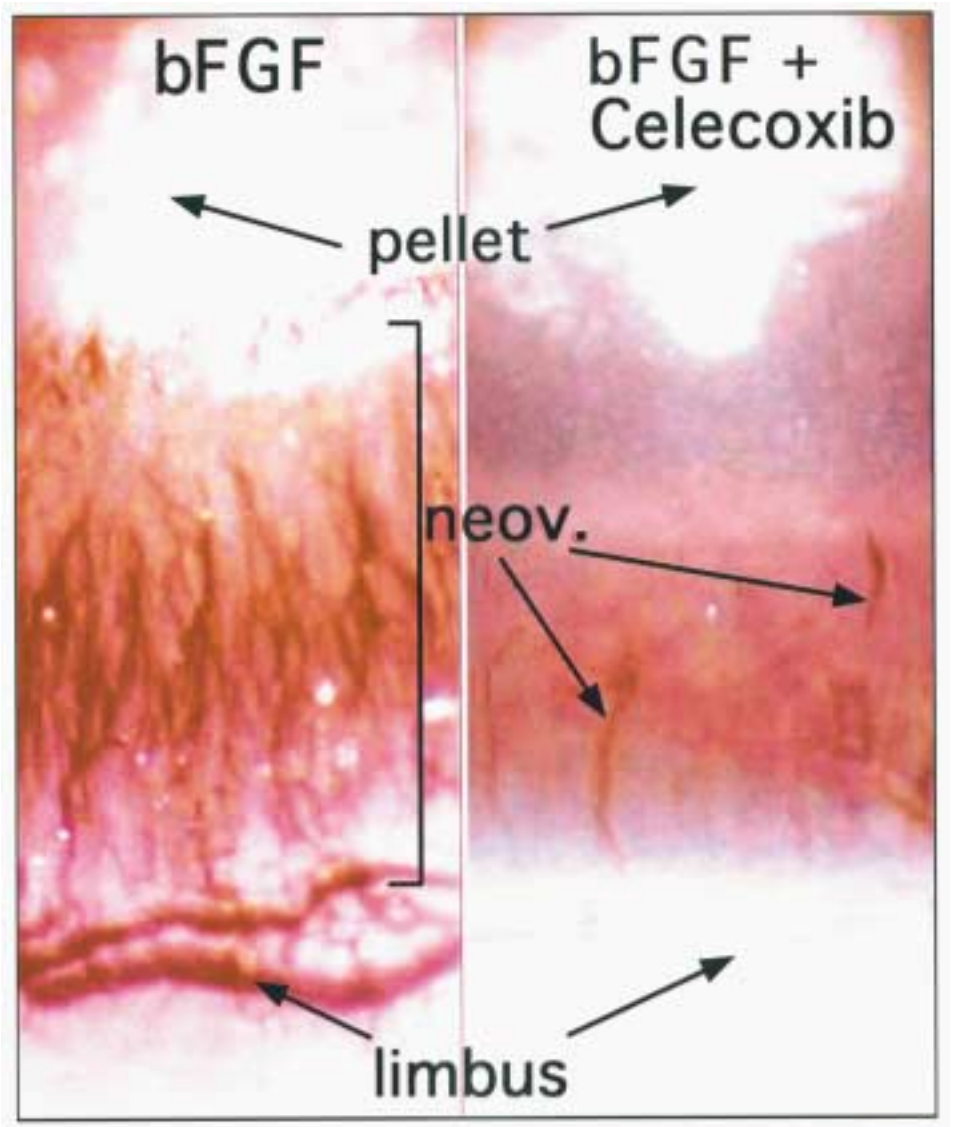

Figure 5 Celecoxib inhibits corneal angiogenesis induced by bFGF. The contribution of COX-2 to angiogenesis was evaluated in an *in vivo* rat corneal model. Implanted bFGF induced neovascularisation (neov.; left panel) accompanied by corneal thickening. Celecoxib caused a substantial reduction in the number and length of sprouting capillaries (right panel). This figure is reproduced with permission from Masferrer *et al.* (2000).

discrepancies between observations made *in vivo* and *in vitro* (Majima *et al.* 1997, Tsujii *et al.* 1998, Daniel *et al.* 1999, Masferrer *et al.* 2000, Williams *et al.* 2000*a*). COX-2 apparently contributes to the production of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-1, PDGF, and endothelin-1. NS-398 treatment of a *COX-2*-overexpressing colorectal cancer cell line diminishes secretion of these factors (Tsujii *et al.* 1998), and *COX-2* (–/–) fibroblasts have a 94% reduction in the ability to produce VEGF relative to wild-type fibroblasts (Williams *et al.* 2000*a*). However, the molecular mechanisms underlying COX-2-mediated production of pro-angiogenic factors remain to be defined.

COX-1 can also contribute to angiogenesis. Nonselective NSAIDs decrease the vascularisation of xenografts comprised of cells not expressing *COX-2* (Sawaoka *et al.* 1999). Moreover, NSAIDs inhibit endothelial tubule formation even when cells do not express *COX-2* (Tsujii *et al.* 1998, Jones *et al.* 1999). Thus both COX-1 and COX-2 are likely to contribute to tumour vascularisation. The possibility that COX-2 inhibitors diminish tumorigenesis

partly by preventing angiogenesis further enhances their attractiveness as potential anti-cancer agents.

Effects of *COX-2* overexpression on cell invasiveness and apoptosis

The potential consequences of *COX-2* overexpression have been addressed *in vitro* by generation of cell lines overexpressing *COX-2*. In particular, rat intestinal epithelial cells stably overexpressing *COX-2* show several altered characteristics, including increased adhesion to extracellular matrix, resistance to butyrate-induced apoptosis and a delayed transit through the G1 phase of the cell cycle (Tsujii & DuBois 1995, DuBois *et al.* 1996*b*). Additionally, stable *COX-2* expression in Caco-2 cells or in the breast cancer cell line Hs578T increases expression or activity of enzymes capable of digesting the basement membrane, presumably contributing to the observed increase in ability to invade through a layer of Matrigel (Tsujii *et al.* 1997, Takahashi *et al.* 1999). All of these characteristics may contribute to tumorigenicity, although the molecular mechanism(s) by which COX-2 causes these effects is unknown.

Much interest has centred on the ability of COX-2 to suppress apoptosis. Diminished apoptosis is thought to favour carcinogenesis by permitting survival of cells that have acquired mutations, and thus is viewed as one of the central mechanisms of tumorigenesis. Conversely, many NSAIDs enhance apoptotic cell death, although this is unlikely to be solely due to inhibition of cyclooxygenase activity (see below). Several hypotheses have been advanced to account for suppression of apoptosis in response to *COX-2* overexpression. The ability of PGE_2 to inhibit apoptosis caused by a selective COX-2 inhibitor, and concomitantly to induce *Bcl-2*, suggests that PG-mediated upregulation of *Bcl-2* may suppress apoptosis (Sheng *et al.* 1998*a*). Alternatively, since arachidonic acid stimulates apoptosis, enhanced *COX-2* expression could inhibit apoptosis by increasing the conversion of arachidonic acid to PG (Chan *et al.* 1998, Cao *et al.* 2000). Kinzler and colleagues propose that arachidonic acid stimulates the conversion of sphingomyelin to ceramide, which then causes apoptosis (Chan *et al.* 1998). They further suggest that the apoptosis-promoting effect of NSAIDs such as sulindac is due to NSAID-induced accumulation of arachidonic acid. In contrast, although Prescott and co-workers also consider arachidonate to be a key determinant of apoptosis, they do not observe increased levels of ceramide in response to exogenous administration of arachidonic acid (Cao *et al.* 2000).

Clearly, the suppression of apoptosis associated with *COX-2* overexpression could be an important factor in tumorigenesis, although the precise mechanistic basis remains uncertain. Interestingly, an apoptosis-related protein was found in a two-hybrid screen designed to identify proteins that interact with cyclooxygenases (Ballif *et al.* 1996). Nucleobindin associates with DNA from apoptotic cells, and can itself promote apoptosis. The interaction of COX-1 and COX-2 with nucleobindin may contribute to COX-mediated suppression of apoptosis, potentially via sequestration of nucleobindin, but further studies are required to fully understand the significance of the interaction.

As mentioned above, multiple NSAIDs, including selective COX-2 inhibitors, induce apoptosis in a variety of cells (Lu *et al.* 1995, Hara *et al.* 1997, Sheng *et al.* 1998*a*, Ding *et al.* 2000, Hida *et al.* 2000, Li *et al.* 2000). The simplest interpretation of this phenomenon is that, since *COX-2* overexpression suppresses apoptosis, inhibition of COX-2 activity is sufficient to induce apoptosis. However, NSAID-induced apoptosis has also been demonstrated in cell lines that do not express *COX-2*, including *COX-2*-null mouse embryo fibroblasts (Hanif *et al.* 1996, Elder *et al.* 1997, Zhang *et al.* 1999). Additionally, non-cyclooxygenase-inhibiting sulindac metabolites such as sulindac sulphone retain the ability to induce apoptosis (Piazza *et al.* 1997, Lim *et al.* 1999*b*). Thus, NSAIDs most likely stimulate apoptosis via both COX-dependent and -independent mechanisms (Rigas & Shiff 2000), including inhibition of the protein kinase Akt (Hsu *et al.* 2000) and suppression of NF-κB activation (Kopp & Ghosh 1994, Grilli *et al.* 1996, Yin *et al.* 1998, Yamamoto *et al.* 1999).

Clinical prospects for COX-2 inhibitors and breast cancer

The weight of evidence implicating COX-2 in colorectal cancer has stimulated clinical trials to investigate the efficacy of selective COX-2 inhibitors in individuals at risk for colorectal cancer. Treatment with celecoxib has been shown to reduce the size and number of polyps in FAP patients (Steinbach *et al.* 2000), and is currently being evaluated for efficacy in preventing sporadic colorectal adenomas. Undoubtedly the potential use of selective COX-2 inhibitors for the treatment of colorectal cancer will also be investigated.

Here we have reviewed evidence that aberrant *COX-2* expression is also associated with breast cancer, both in rodent models and in the human disease. Selective COX-2 inhibitors have proved effective in preventing experimental breast cancer (Harris *et al.* 2000, Nakatsugi *et al.* 2000). Whether COX-2 inhibitors will also be useful for preventing breast cancer in high-risk individuals needs to be investigated. In addition, selective COX-2 inhibitors may have a role in the treatment of breast cancer (Alshafie *et al.* 2000). Since *COX-2* is overexpressed in HER-2/neu-positive breast cancers (Subbaramaiah *et al.* 1999*b*), selective COX-2 inhibitors should be evaluated as therapy in this patient population. Because COX-2-derived PGs may enhance aromatase activity, a therapeutic regimen combining a selective COX-2 inhibitor with an aromatase inhibitor should be considered. There is also recent evidence that

microtubule-interfering agents, including taxol, stimulate *COX-2* transcription (Subbaramaiah *et al.* 2000). This could decrease the efficacy of this class of drugs. Thus, coadministration of a selective COX-2 inhibitor with drugs such as taxol might enhance their anti-cancer activity. Finally, a number of natural substances have been identified that inhibit the transcriptional activation of *COX-2*. Examples include retinoids, triterpenoids, antioxidants and resorcins (Mestre *et al.* 1997*a*,*b*, Chinery *et al.* 1998, Subbaramaiah *et al.* 1998*b*, Suh *et al.* 1998, Mutoh *et al.* 2000). Some of these compounds also inhibit experimental breast cancer. Hence, it is possible that studies of COX-2 will provide insights that will prove useful in developing dietary recommendations to decrease cancer risk.

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