



Edward Burgess Butler. *Summer Harvest, 1915*. Oil on canvas, 25" × 30".  
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*Current knowledge on the impact of cyclooxygenase and the effects of its inhibition in systemic cancers and in glioma is reviewed.*

# Cyclooxygenase in the Treatment of Glioma: Its Complex Role in Signal Transduction

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**Background:** High-grade glioma remains one of the most difficult cancers to treat. Recent studies in oncology have identified a role of the ubiquitous enzyme, cyclooxygenase (Cox), especially cyclooxygenase-2 (COX-2) in cell proliferation, and its inhibition in cancer control, apoptosis, as well as synergy with other forms of therapy. The inhibitors of the Cox enzyme are well known as members of the nonsteroidal anti-inflammatory drug (NSAID) class of pharmaceuticals.

**Methods:** *In vitro* and *in vivo* studies of different cancers expressing COX-2, including glioma studies, along with the few clinical trials that have been reported are reviewed to specifically identify the actions of these agents.

**Results:** The anticancer effect of the COX-2 inhibitors may occur irrelevant of COX-2 expression, and it appears to be drug-specific, as well as dose-specific in different cancers. In combination with chemotherapeutic agents, the COX-2 inhibitors may have an additive, synergistic, or inhibitory effect on tumor growth.

**Conclusions:** As evaluations of this class of drugs begin in glioma, *in vitro* and *in vivo* data should be acquired to accurately predict which compounds will have an effect in controlling tumor growth and at which doses these should be used. The actual expression and inhibition of COX-2 may not always be relevant to the effects on tumor growth.

## Introduction

The treatment of malignant glioma is multidisciplinary, involving surgery, radiotherapy, and chemotherapy/biologic therapy. With concerted effort of all three, small improvements are being seen in the quality of life and survival of many of the patients who suffer with this dismal disease. Treatment remains challenging, but advances in molecular biology, tissue microarray, imaging and surgical techniques, and radiobiology have been explosive, and

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knowledge concerning the growth characteristics of these tumors also continues to escalate. Because of the complexity of the molecular pathogenesis of this tumor, its therapy will most likely remain multifaceted. As signal transduction pathways are better defined, targeting specific steps in the transduction cascade has become the focus of many treatment trials. One of these steps that has gained interest is cyclooxygenase, particularly cyclooxygenase-2 (COX-2).

The expression and function of COX-2 has been studied in a number of different tumor tissues, but especially in those of the gastrointestinal (GI) tract. Inhibition of this enzyme is usually brought about through the use of agents known as nonsteroidal anti-inflammatory drugs (NSAIDs). Researchers have shown chemopreventive properties, direct antineoplastic activity, and synergistic or additive effects of these inhibitors of COX-2 in combination with various agents and with radiation. There are also reports of lack of synergy and occasional unexpected negative results as to the influence of COX-2 inhibitors in several *in vitro* and *in vivo* tumor models. Expression of COX-2 has been demonstrated in glioma, and effects of its inhibition have also been described. This article reviews the current knowledge of the influence of the COX-2 enzyme and its inhibition in the systemic cancers, alone and in combination with other therapies. A review of what is currently known in glioma is also presented, with a challenge to explore with caution what is becoming an exceptionally complex piece in the ever-growing puzzle of signal transduction.

## Cyclooxygenase: An Overview

The cyclooxygenase enzyme, also known as prostaglandin H synthase, is the rate-limiting enzyme that catalyses the first step in the synthesis of prostanoids by converting arachidonic acid into prostaglandin  $G_2$  (PG- $G_2$ ). Prostaglandin hydroperoxidase activity catalyzes the conversion of PG- $G_2$  to PG- $H_2$ , a common substrate for prostaglandin synthase and the first committed step in the metabolism of arachidonic acid into a cascade of signaling lipids, such as PG- $D_2$  (CNS), PG- $E_2$  (vascular beds), PG- $F_2\alpha$  (smooth muscle), PG- $I_2$  (vascular endothelium) and thromboxane (platelets) (Fig 1).<sup>1</sup> These metabolites affect cellular functions such as mitogenesis, cellular adhesion, invasion, and apoptosis.<sup>2</sup> Prostanoids coordinate signaling between the cell of origin and neighboring cells by binding to transmembrane G protein-coupled receptors. The formation of tissue specific prostanoids depends on tissue specific isomerases. In essence, more than one G protein-coupled receptor may promote different effects from the same prostanoid.<sup>3</sup> Also of importance is the fact that the prostanoids, including PG- $E_2$ , are released into the extracellular fluid space and can be measured.

Two distinct isoforms of the cyclooxygenase enzyme were recognized in the early 1990s: COX-1 and COX-2.

COX-1 mediates physiologic responses, including cytoprotection of the stomach and platelet aggregation. It is known as the so-called “constitutive” enzyme. COX-2 is involved in inflammation and is responsible for the synthesis of prostanoids. The gene locus for COX-1 is on chromosome 9 and for COX-2, on chromosome 1; the genes encoding the two enzymes reflect differences in the 5' flanking region. The COX-1 promoter site is an isoform present in most tissues where prostanoid synthesis is significant. On the other hand, the COX-2 promoter is induced by several stimuli, including lipopolysaccharides and pro-inflammatory cytokines (interleukin [IL]-1 $\beta$ , tumor necrosis factor) and other growth factors, whereas IL-4, IL-13, IL-10, and corticosteroids inhibit its expression. The promoter contains sites for transcription factors NF- $\kappa$ B, NF-IL-6, and cyclic adenosine monophosphate (cAMP) response element, regulated by tyrosine kinase growth factors as well as protein kinase C. The enzyme itself appears to be located in the wall of the endoplasmic reticulum (COX-1) and perinuclear envelope (COX-2) of the cell, where it is firmly bound.<sup>4</sup> The major metabolic product, PG- $E_2$ , has been shown to upregulate COX-2 in a variety of cell types by virtue of cAMP amplification. COX-2 is not detectable in most normal tissues.

NSAIDs affect cyclooxygenase in several ways. Aspirin, one of the oldest NSAIDs, irreversibly inactivates

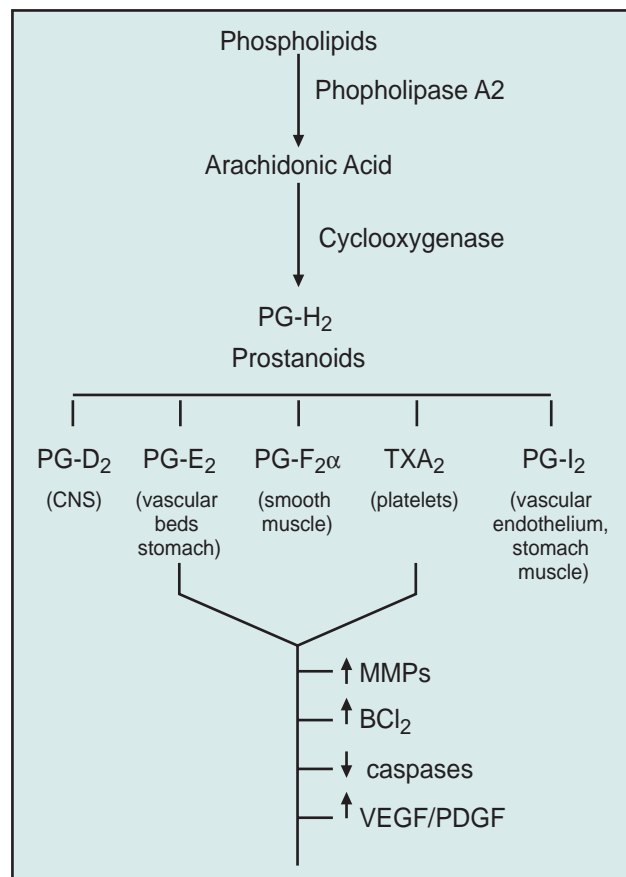


Fig 1. — Physiologic and pathologic roles of tissue isomerases, metabolites of arachidonic acid by cyclooxygenase.

COX-1 and COX-2 by acetylating a serine moiety at the active site. Ibuprofen is a reversible competitive inhibitor. Indomethacin is a time-dependent reversible inhibitor of COX-1 and COX-2. Salicylate alone does not inhibit COX-1 or COX-2 activity *in vitro*, but it is an effective inhibitor of prostaglandin at sites of inflammation. It is questioned whether there is an inhibitory effect on the NF- $\kappa$ B protein as salicylate seems to inhibit COX-2 *expression* at a target upstream. Metabolites of salicylic acid have been shown to inhibit COX-2-dependent synthesis of prostaglandins.

Cyclooxygenase specificity is determined by virtue of a single amino acid difference within the hydrophobic channel of the Cox enzyme at position 523 (isoleucine in COX-1, valine in COX-2). The smaller valine molecule in COX-2 gives access to a "side pocket" that has been proposed as the binding site of COX-2 selective substances. The total NSAID binding site is about 17% larger in COX-2 and can bind more bulky substrates than the COX-1 isoform. Celecoxib and rofecoxib are novel specific COX-2 inhibitors.<sup>5,7</sup> They are slow, time-dependent, irreversible inhibitors of COX-2. New compounds are in development that preferentially acetylate and irreversibly inactivate COX-2.<sup>8</sup>

COX-2 specificity can be determined by a whole blood assay system, which provides a direct indication of whether a test substance can inhibit the enzyme activity of COX-1 (thromboxane from platelets during blood clotting) or COX-2 (PG-E<sub>2</sub> synthesis) in lipopolysaccharide-stimulated monocytes. The assay system has shown no effective action on platelet COX-1 activity over the spectrum of clinically used doses of celecoxib and rofecoxib, thereby confirming their COX-2 specificity.<sup>1</sup>

Cyclooxygenase is expressed in the central nervous system (CNS), where it has a role in inflammation, fever, and pain. IL-1B is a major inducer of COX-2 upregulation in the CNS. Prostanoids are synthesized in the CNS and are neuroactive substances involved in the sleep-wake cycle, body temperature control, cerebral blood flow, and neuroendocrine function.<sup>2</sup> Normal astrocytes alone do not express COX-1, and COX-2 expression is minimal compared with astrocytoma cell lines. COX-1 and -2 expression is present in all grades of astrocytoma, with COX-2 more often expressed than COX-1. In addition to expression in tumor cells, the enzyme expression could be found in macrophages, microglial cells, neurons, and reactive astrocytes.<sup>9</sup>

There is also compelling evidence that this enzyme may have a role in carcinogenesis — in that COX-2-derived prostaglandins may modulate the production of angiogenic factors in colon cancer cells. Overexpression of COX-2 has been associated with resistance to apoptosis and induction of angiogenesis.<sup>10</sup> Also of interest is the observation that COX-1 may play a role in the modulation of angiogenesis through the production of prostaglandins in endothelial cells and therefore may be another target in tumors that do not express COX-2. Thus, NSAIDs may

inhibit angiogenesis by inhibition of COX-2 activity, down-regulation of angiogenic factors, induction of apoptosis, and inhibition of COX-1 in endothelial cells.<sup>1</sup>

The control of the cell cycle is influenced by a series of regulatory events involving proteins known as cyclins, cyclin-dependent kinases, tumor suppressor proteins, and transcription factors, which ultimately coordinate the expression of genes that regulate the S-phase transition. Alteration of expression of these cyclins and tumor suppressors results in dysregulation of cell proliferation.<sup>11</sup> The variable role of cyclooxygenase and effects of NSAIDs in this paradigm is complex, as shown in the studies reviewed.

## Cyclooxygenase Pathways and Cancer: What Have We Learned From Studies in Systemic Cancer?

The first reports to describe a role for the prostanoids in cancer arose in the 1970s, when Bennett and Del Tacca<sup>12</sup> reported that the concentration of PG-E<sub>2</sub> was greater in human colorectal tumor tissue than in normal colonic mucosa. This stimulated a flurry of research involving a chemically induced colorectal cancer model in mice and rats, as well as the effects of NSAIDs on this model.<sup>13-15</sup> The studies showed that aspirin and other NSAIDs (piroxicam, indomethacin, sulindac, ibuprofen, and ketoprofen) inhibit carcinogenesis in rats. Later studies showed that selective COX-2 inhibitors such as celecoxib and rofecoxib also inhibited chemically induced carcinogenesis.<sup>16,17</sup> The highest tolerated doses of NSAIDs reduced the number and size of tumors by 40% to 60%. High doses of celecoxib, however, inhibited 90% of tumors and were better tolerated than comparable nonselective NSAIDs. These experiments showed that both selective and nonselective NSAIDs are effective in inhibiting early stages of tumor development, but only COX-2 inhibitors were also able to reduce tumor incidence when treatment was delayed, while NSAID treatment needed to be continued without interruption in order to prevent recurrent tumor growth.<sup>13-15</sup>

Among the earliest human trials are those involving patients with the diagnosis of familial adenomatous polyposis (FAP). This is a rare condition resulting from a germline inactivation of one allele of the adenomatous polyposis coli (APC) gene, which has nearly 100% penetrance. This gene is located on chromosome 5q21 and is a tumor suppressor gene. The normal APC gene regulates proliferation, migration, differentiation, and apoptosis of epithelial cells, whereas the mutant protein is truncated and cannot regulate these processes. The mutation of APC is not confined to the FAP population; it occurs in 50% of the population with sporadic adenomatous polyps and 80% of those with carcinoma of the colon. Thus, it is an early molecular event in the development of adenoma and

carcinoma.<sup>18</sup> Multiple studies have reported a decreased incidence in the development of adenomatous polyps in the general population and a lower incidence of death from colon cancer in people who regularly use aspirin.<sup>19,23</sup>

The mechanism by which NSAIDs reduce colorectal neoplasia may be twofold, including the ability of these agents to restore apoptosis in APC-deficient cells and their capacity to inhibit angiogenesis. In this model, inhibition of apoptosis parallels COX-2 expression.<sup>24</sup> The signal transduction cascade has been implicated as one mechanism for this, as high doses of sulindac sulfone and sodium salicylate have been shown to effect either the *c-myc* oncogene,<sup>25</sup> NF- $\kappa$ B<sup>26,27</sup> or p-38, the mitogen-activated protein kinase.<sup>28,29</sup> The second mechanism, suppression of angiogenesis by COX-2 inhibition, has been demonstrated in laboratory models.<sup>30,31</sup> Migration of endothelial cells and tube formation is also blocked by these compounds.<sup>32,33</sup>

COX-2 activity produces prostaglandins that can have both autocrine and paracrine effects on proliferation and migration of endothelial cells. Cytokines derived from nonendothelial cells involved in angiogenesis stimulate the induction of COX-2 in vascular endothelial cells. An IL-6 cytokine secreted by macrophages, for example, has been shown to have a proliferative effect in association with induction of COX-2 in human microvascular endothelial cells.<sup>34</sup> COX-2 inhibition limits this effect. Prostaglandins derived from COX-2, TXA-2, and PG-F<sub>2</sub> induce endothelial cell migration as well. Leahy et al<sup>34</sup> demonstrated that neovascular cells associated with tumors consistently express COX-2 regardless of the Cox profile of their epithelial component.

Angiogenesis inhibitors target genetically stable host endothelial cells of tumor vasculature. Gradual loss of response and lack of acquired resistance have been described when these drugs are administered as monotherapy. Further studies of antiangiogenesis have shown that tumor cells deficient in p53 display a diminished rate of apoptosis under hypoxic conditions, rendering their survival independent of vascular supply and potentially resistant to antiangiogenic therapy.<sup>35</sup> These studies imply that selection and overgrowth of subpopulations of cancer cells with reduced dependence on blood vessels could occur over time in the face of antiangiogenic therapy.

A closer look at peroxisome proliferator-activated receptor (PPAR- $\gamma$ ), the member of the nuclear hormone receptor superfamily, is important since this protein not only can modulate gene expression upon ligand binding, but also is important in the signaling mechanism for COX-2 expression. Subbaramaiah et al<sup>36</sup> have studied the signaling pathways involved in COX-2 expression in a human epithelial cell line. They found that when PPAR- $\gamma$  is activated by ligand binding, it is able to heterodimerize with the retinoid X receptor and activate gene expression by binding to PPAR response elements. These ligands can also block activator protein-1 (AP-1) and NF- $\kappa$ B-mediated gene expression. Activation of AP-1 requires coactivation

of cyclic AMP response element binding protein (CBP/p300). The ligands of PPAR- $\gamma$  stimulate interaction between PPAR- $\gamma$  and CBP. Competition for limited amounts of these proteins is one of the mechanisms of repression by nuclear receptors including PPAR- $\gamma$ . Therefore, CBP is also important for regulating COX-2 gene expression.<sup>36</sup> Retinoids and dexamethasone, known ligands of nuclear receptors, block activation of COX-2 expression in this way. It would seem, then, that further development of PPAR- $\gamma$  ligands to modulate expression of COX-2 may be a reasonable focus of further development.

Cyclooxygenase inhibitors may also bring about apoptosis indirectly through modulation of intracellular arachidonic acid levels, which in turn activate sphingomyelinase and regulates the production of ceramide, a lipid second messenger. Ceramide can activate cellular apoptotic machinery<sup>37</sup> and provoke significant arrest in the cell cycle at the G<sub>0</sub>/G<sub>1</sub> phase. Subbaramaiah et al<sup>38</sup> have shown that ceramide can induce COX-2 in a human mammary epithelial cell line. The COX-2 promoter element that mediates this effect of c-Jun is the cAMP response element. A host of substances stimulate the hydrolysis of sphingomyelin and induce COX-2, including tumor necrosis factor alpha (TNF- $\alpha$ ), IL-1 $\beta$ , phorbol esters, interferon gamma (IFN- $\gamma$ ), and UV light. Despite the similar effects on cell cycle arrest, COX-2 and ceramide have opposite effects on apoptosis, the former being an inhibitor and the latter an inducing factor. The proapoptotic effects of ceramide may be counteracted by its ability to upregulate COX-2. Therefore, COX-2 inhibitors may exert their effect by potentiation of ceramide-mediated apoptosis. This pathway may provide another set of targets for transcriptional research.<sup>38</sup>

Dommels et al<sup>39</sup> studied cyclooxygenase and the role of its prostaglandin product, PG-E<sub>2</sub>, on the effects of n-6 and n-3 polyunsaturated fatty acids (PUFAs) in two colorectal cell lines, one that highly expressed COX-2 (Caco-2 cell line) and one that expressed low levels of COX-2 (HT-29 cell line). They reported unexpected and intriguing results. The long chain PUFAs, eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (AA; 20:4n-6), both inhibited cell proliferation in the Caco-2 line. Neither incubation with PG-E<sub>2</sub> nor reduction in PG-E<sub>2</sub> synthesis by EPA compared with AA affected cell proliferation, therefore suggesting that the effects of n-3 PUFA and n-6 PUFA are not mediated by PG-E<sub>2</sub>. There was no effect on the growth characteristics of the HT-29 cell line. However, upregulation of COX-2 activity by IL-1B resulted in a decrease in cell proliferation and the induction of cytotoxicity when AA or EPA was added. In this model, inhibition of the Cox pathway by indomethacin as well as inhibition of lipid peroxidation by antioxidants vitamin E and C surprisingly *diminished* the antiproliferative effects of EPA and AA. Both malondialdehyde, a product of lipid peroxidation, and Cox activity were decreased by vitamin E and indomethacin. The conclusion of the investigators

was that the cytotoxic effects of the PUFAs are most likely due to peroxidation products generated by lipid peroxidation and Cox activity, and therefore inhibiting the COX-2 enzyme in this model *enhanced* cell proliferation. Both EPA and AA are susceptible to enzymatic and nonenzymatic peroxidation of membrane lipids, thus generating large quantities of reactive cytotoxic products that then could control cell proliferation.<sup>39</sup>

Elevated COX-2 expression has been reported in pancreatic cancer,<sup>40,41</sup> esophageal cancer,<sup>42</sup> gastric cancer (in which its expression may be a predictor of survival),<sup>43</sup> skin cancers (including melanoma),<sup>44-47</sup> prostate cancer,<sup>48-55</sup> bladder cancer,<sup>56-58</sup> lung cancer (in which expression may also correlate with prognosis),<sup>59,60</sup> ovarian cancer (which also correlates with poor prognosis),<sup>61</sup> cervical cancer (COX-1 is expressed as well),<sup>62</sup> and B-cell lymphoma.<sup>63</sup> Expression in breast cancer has been reported in 36% to 79%.<sup>64-66</sup> The production of PG-E<sub>2</sub> in this tumor seems to be a potent angiogenic switch.<sup>67</sup> COX-2 expression in breast cancer has also been identified as a negative factor for disease-free survival and is correlated with hormone receptor status, expression of HER-2/*neu*, stage of disease, and lymph node status.<sup>68</sup>

In summary, studies in systemic cancer have shown that COX-2 inhibition itself is not the key to tumor control in all situations. The effects of COX-2 inhibitors and non-selective cyclooxygenase inhibitors on cell proliferation and the mechanisms by which they induce these effects vary in different tumor histopathologies. Likewise, different COX-2 inhibitors and different NSAIDs may influence tumor growth by various mechanisms independent of their effect on cyclooxygenase. The question remains as to the importance of COX-2 in the center of these manipulations. What is its actual role in the tumor pathologies in which it is expressed, and how should it be utilized as a marker or a target in the treatment of cancer? How does it function in relation to other enzymes, their products, growth factors, receptors, and signals in the cell cycle cascade?

## COX-2 Expression and Inhibition in Glioma

How have studies of cyclooxygenase and its metabolites and inhibitors in glioma compared with those of systemic cancer? The reported studies are not as numerous, yet those that have been reported have shed further light on potential usefulness and mechanisms in this tumor setting. Even more importantly, in addition to the studies reviewed above, they may guide us in important research directions.

Studies by Wilson et al<sup>69</sup> in 1990 showed that lipoxigenase/cyclooxygenase inhibitors inhibited the growth of a human glioblastoma cell line and provided astrocytic differentiation. Joki et al<sup>70</sup> evaluated COX-2 expression in 50 glioma samples. All specimens revealed the presence of COX-2 protein. High-grade gliomas expressed a higher

level of COX-2 than did lower-grade tumors. High-grade tumors also showed marked intratumoral heterogeneity. In a similar fashion, Prayson et al<sup>71</sup> evaluated 47 glioblastoma samples by immunohistochemistry using COX-2 antibody and correlated data with MIB-1 immunostaining. As a whole, tumors with a higher proliferation rate seemed to have an increased expression of COX-2.

Joki et al<sup>70</sup> also looked at two glioma cell lines in culture, U-87 with strong COX-2 expression, and U-251 with weaker expression of COX-2. The selective COX-2 inhibitor NS-398 suppressed growth proliferation in both cell lines. It inhibited both growth proliferation and migration in a tumor spheroid culture system of U-87 MG and U-251 MG. No inhibitory effect was observed on tumor cell invasion, as noted by previous investigators.

A retrospective study by Shono et al<sup>72</sup> compared the expression of COX-2 with survival in glioma, as well as the expression of various molecular markers. Sixty-six tumor specimens were analyzed, including 31 glioblastomas, 25 anaplastic astrocytomas, and 10 low-grade astrocytomas. They demonstrated that higher-grade tumors correlated with higher expression of COX-2 but also that higher expression of COX-2 correlated with poorer prognosis. They did not see a relationship with p53, bcl-2 expression, retinoblastoma (Rb) gene, or p16 expression or with MIB-1 staining. As in other reports, COX-2 staining was closest to areas of necrosis but was also seen in the nuclei of tumor cells in a subset of specimens. Similar to previous reports, the investigators found that the expression of COX-2 was 71% in glioblastomas and lowest (30%) in low-grade astrocytomas.<sup>72</sup>

Glioma-infiltrating microglia are a major source of PG-E<sub>2</sub> through the COX-2 pathway. Both PD-E<sub>2</sub> and PG-F<sub>2</sub>α can increase the permeability of peripheral microvessels and are linked to changes in the blood-brain barrier in CNS inflammation. PG-E<sub>2</sub> has also been shown to be responsible for increasing brain edema. Microglia and not glioma cells produce high levels of PG-E<sub>2</sub>. These cells are essentially CNS macrophages. This would explain why glioma cells in culture may not express COX-2. In vivo models may be necessary to demonstrate the presence of inflammatory cells, especially in areas of edema. Microglia are the immune effectors of the brain. When activated, they release oxygen intermediates and become capable of phagocytosis, antigen presentation, and lymphocyte activation. They are a major component of the population of infiltrating cells in malignant brain tumors and account for approximately 20% of the cells in intracranial rat glioma models. In an intracranial C-6 rat glioma model, rofecoxib inhibited PG-E<sub>2</sub> production by approximately 80% and at the same time abolished the diffusion of contrast material into the brain parenchyma.<sup>73</sup> This decrease in blood-brain permeability was approximately equivalent to that produced by dexamethasone and supports the role of microglia in the edema formation of glioma. PG-E<sub>2</sub> production has also been shown to cause brain microvessels

to become more permeable, and therefore the production of PG-E<sub>2</sub> by microglia may be responsible for enhancing endothelial cell permeability within glial tumors. COX-2 expression may be important for the formation of new vessels, especially leaky capillaries. Studies by Badie et al<sup>73</sup> suggest that intracranial tumors express more COX-2 than models of subcutaneous C-6 glioma, which supports the suggestion that as CNS-specific cells, microglia may be a significant source of COX-2 expression. Deininger et al<sup>74</sup> also reported the presence of COX-1+ macrophages and microglia along with COX-2+ endothelial cells in tumor parenchyma and in the surrounding areas of infiltrative tumor growth, using a rat C6 glioblastoma and a 9L gliosarcoma model.

TNF- $\alpha$  is a cytokine that elicits cell responses by activation of MAP-kinase and NF- $\kappa$ B. Hernandez et al<sup>75</sup> evaluated the timing of these molecular events in 1,321 N1 astrocytoma cells in culture. They reported that TNF- $\alpha$  produced the phosphorylation of cytosolic phospholipase A2 (cPLA2), which was preceded by the activation of both c-Jun N-terminal kinase (JNK) and p38-MAP kinase and associated with the release of AA. Analysis of the AA metabolites showed that all of the AA released within the first hour after addition of TNF- $\alpha$  was pure AA, whereas in samples obtained at 24 hours after the addition of TNF- $\alpha$ , 25% of the AA had been converted to Cox products. Also TNF- $\alpha$  produced an increase in COX-2 expression and a long-lasting activation of NF- $\kappa$ B. These results would suggest that TNF- $\alpha$  produces in astrocytoma cells an early activation of both p38-MAP kinase and JNK, followed by phosphorylation of cPLA2 and release of AA. Also, the activation of NF- $\kappa$ B may explain the induction of expression of COX-2 and delayed production of prostanoids.<sup>75</sup> Further studies by the same investigators<sup>76</sup> have evaluated the effects of NSAIDs and COX-2 inhibition on signal transduction in astrocytoma cells.

Salicylate seems to act as a competitive inhibitor of I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ) by inhibiting the binding of adenosine triphosphate (ATP). This seems to be the main biochemical mechanism that explains its pharmacologic effect on NF- $\kappa$ B activation.<sup>77</sup> Others have shown that IKK $\beta$  is a key regulator of NF- $\kappa$ B in human glial cells.<sup>78</sup> On the other hand, glucocorticoids inhibit the expression of the adhesion molecules IL-8, VCAM-1, and ICAM-1 in the absence of inhibition of nuclear translocation of NF- $\kappa$ B proteins and the degradation of I $\kappa$ B $\alpha$ . Again, this suggests other pathways as yet undefined that may influence the inhibition of these tumor-associated molecules. Other investigators<sup>79-81</sup> studied the effects of NSAIDs on PPAR and showed that they may inhibit cytokine-induced COX-2 expression, yet they may have a stimulatory effect at certain concentrations. The net effect of PPAR agonists on COX-2 expression induced by the cytokines seems to be inhibitory. Certainly macrophages (and microglia) can induce cytokines, which in turn stimulate tumor cells to induce expression of COX-2. NSAIDs then can inhibit the production of

cytokines by macrophages and the induction of COX-2 by tumor cells in response to the cytokines.

With this body of data, other investigations have been initiated to evaluate therapy with COX-2 inhibitors in glioma. Preclinical studies have reported variable responses to concentration and dosage of NSAIDs in this tumor. One such report by Gati et al,<sup>82</sup> who evaluated the effects of cyclooxygenase and lipoxygenase inhibitors in human glioma cell lines cultured as spheroids and in monolayers, also showed that indomethacin actually enhanced growth at intermediate concentrations but reduced growth when the dose was increased by a factor of 10. Ketoprofen, which inhibits both cyclooxygenase and lipoxygenase, also had an inhibitory effect on growth and cell proliferation. The weak lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) (quinone form) decreased growth rate, whereas the stronger form of the drug (a hydroquinone form) did not reduce growth rate but did significantly decrease cell proliferation. Dexamethasone had no effect on this model. Aas and colleagues<sup>83</sup> studied the effects of aspirin, salicylic acid, piroxicam, and indomethacin on the growth of rat glioma in vitro and in vivo. The in vitro studies revealed that aspirin and salicylic acid strongly inhibited growth of the rat glioma cells at standard doses used to treat rheumatic conditions. Indomethacin and piroxicam had no effect in this model. They noted that the synthesis of ATP was markedly reduced and protein synthesis was slightly more inhibited than cell growth. Their in vivo studies involved the administration of aspirin alone to Fischer 344 rats with RG2 glioma cells inoculated into the caudate nucleus. Growth inhibition was seen when aspirin was administered on the day before inoculation of tumor cells and at 5 days following inoculation.<sup>83</sup>

As early as 1988, Farrell et al<sup>84</sup> reported an effect of ibuprofen on tumor weight at high and low doses in a C6 spheroid glioma implantation model. Arrieta et al<sup>85</sup> studied multiple doses of acetylsalicylic acid (ASA) ranging from 12.5 to 400 mg/kg per day on the growth of C6 glioma implanted subcutaneously. A paradoxical result was observed. When high doses of ASA (200 mg or 400 mg/kg per day) were given, tumor volume, cell proliferation, vascular density, and mitotic index *increased*, whereas when lower doses were administered (12.5 or 25 mg/kg per day), the tumor size *diminished*. In another experiment, they induced tumor growth in rats exposed in utero to ethylnitrosourea and treated the experimental group with 70 mg/kg per day from day 50 of age until the end of the experiment at day 400. Incidence, localization, histologic diagnosis, and time until tumor development were compared with matched controls. In this experiment, localization and incidence of tumors were the same for both groups, but in the rats treated with ASA, the time to tumor development was actually shortened. The authors concluded that at higher doses, ASA may block

certain autocrine inhibitory factors dependent on the cyclooxygenase pathway, whereas at lower doses, mechanisms of apoptosis may be induced.<sup>85</sup>

Malignant glioma also expresses an increased amount of thromboxane and prostaglandin. The capacity of arachidonic acid synthesis has been shown to directly correlate with the percentage of cells in the S phase in culture. Inhibition of glial cell migration by specific thromboxane inhibitors has also been described. The antimigratory effect appears to be associated with caspase activation and is followed by DNA fragmentation and apoptotic cell death.<sup>2</sup> Kurzel et al<sup>2</sup> investigated 10 human glioma cell lines and found that thromboxane synthase inhibitors strongly inhibited cell migration in a dose dependent manner. Also, sulindac produced dose-dependent growth inhibition. Interestingly, cell lines with greater COX-2 expression were more resistant to treatment. Cell death took several days after inhibition of thromboxane synthase. Both furegrelate and sulindac produced DNA fragmentation. Aspirin did not have a similar effect. Intracellular DNA fragmentation in apoptosis, being an active cellular process, requires protein synthesis and metabolism. Cell death associated with the breakdown of membrane function may follow hours to days after DNA fragmentation. The absence of DNA fragments in the supernatant implies that subsequent cell death occurs due to early loss of membrane function as detected in necrotic cell death. Kurzel et al<sup>2</sup> detected DNA fragmentation at 16 hours after treatment with inhibitors of COX-2 and thromboxane synthase and detection increased up to 48 hours. No synergistic effects of furegrelate and sulindac were reported by the investigators when these agents were combined. The same investigators showed that levels of thromboxane-2 formation correlate with glioma migration rates and inhibitors of thromboxane synthase block glioma migration. The antimigratory effect of specific thromboxane inhibitors appears to be associated with caspase-3 activation and is followed by DNA fragmentation and apoptotic death of glioma cells. Thromboxane synthase inhibition-induced DNA fragmentation could be blocked by caspase-9 inhibitors, suggesting a mitochondrial pathway for apoptosis. Thromboxane synthase in these experiments seems to be an intersecting point of diverging signaling cascades regulating motility and apoptosis in glioma cells. The synthesis of thromboxanes is in turn dependent on cyclooxygenase activity, the rate-limiting step of this pathway.

Cell migration and apoptosis seem to be interrelated cellular phenomena in this model. Both are related to the proliferative activity of the cell population. The proapoptotic disposition of a cell is correlated to the proliferative rate of the cell population, which is also important to invasive cells. Migration and proliferation may be antagonistic. The invasive phenotype may be less proliferative and may actually protect tumor cells from apoptosis.

Studies have shown that invasive cells are resistant to radiation and chemotherapy.<sup>86</sup> Microarray analyses have shown that glial cells, which tend to be invasive, have apoptotic gene downregulation as well as cellular proliferation gene expression. In summary, invasive glioma cells overexpress genes associated with migration and show less apoptotic predisposition compared to a proliferating mass. Thromboxane synthase inhibition decreases cell migration and may shift the cellular program to susceptibility towards apoptosis. Thromboxane synthase inhibitors may block the invasive phenotype of glioma cells, increase apoptotic predisposition, and sensitize glioma cells to standard chemotherapy.<sup>2</sup>

Kardosh et al<sup>87</sup> evaluated several glioblastoma cell lines as to the effect of COX-2 inhibition on cell proliferation. The investigators found that celecoxib inhibited the proliferation of various glioblastoma cell lines in vitro more potently than did traditional NSAIDs. Also, although several different selective COX-2 inhibitors reduced PG-E<sub>2</sub> levels in these cells, none exerted antiproliferative effects comparable to celecoxib. The addition of external PG-E<sub>2</sub> to the cells treated with celecoxib did not restore proliferation, suggesting that growth inhibition by celecoxib was not mediated by blockage of PG-E<sub>2</sub> production. The molecular processes involved in celecoxib-mediated antiproliferative effects included a loss of the activity of cyclin-dependent kinases, the essential regulators of cell proliferation, due to transcriptional downregulation of cyclin A and cyclin B expression, two important components of the cell cycle machinery. Again, results implied that celecoxib exerted antiproliferative effects independent of COX-2 inhibition and that these were more potent than other selective COX-2 inhibitors or traditional NSAIDs.

Drug trials in human glioma are just beginning. Several of these will be studying the effect of COX-2 inhibition in combination with other antitumor agents and in combination with radiotherapy.

## Effects of Cyclooxygenase Inhibition in Combination With Other Anticancer Agents

The effects of inhibiting the cyclooxygenase enzyme selectively and nonselectively have been described. A review of combination studies of COX-2 inhibitors and chemotherapeutic agents predominantly in the laboratory setting will follow. As targeted therapy has been the new focus of drug development, COX-2 is an attractive candidate for several reasons: the enzyme can be inhibited with available drugs, seems to have a major influence on cancer biology, is applicable to many tumor types, is involved in many aspects of carcinogenesis, and seemingly can be combined with other agents. Other specific targets for therapy similarly have included vascular endothelial growth factor (VEGF), epidermal growth factor receptor

(EGFR), Ras, p53, and tumor angiogenesis. Chronic myelogenous leukemia and GI stromal tumors are solely dependent on one specific kinase, bcr-abl and c-kit, and can be inhibited by ST1571 (imatinib mesylate; Gleevec). However, most other cancers have multiple pathways and aberrations during their neoplastic development and progression. Overexpression of COX-2 has been correlated with invasive phenotype, loss of cell-to-cell adhesion molecules, and an increase in matrix metalloproteinase-2 (MMP-2).<sup>88,89</sup> Its expression is also linked to VEGF expression. For all of these reasons, COX-2 manipulation is a logical approach to combination anticancer therapy.

An interesting situation exists concerning the microtubule-inhibiting drugs, paclitaxel and docetaxel. Two investigations<sup>90,91</sup> reported that paclitaxel actually upregulated COX-2 and PG-E<sub>2</sub> synthesis. Subbaramaiah et al<sup>92</sup> verified this effect of paclitaxel as well as other microtubule inhibiting agents (MIAs) including vincristine, colchicine, and vinblastine in a human mammary epithelial cell line. These chemotherapeutic agents have been found to stimulate p38, JNK, extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinase

(MAPK) activities. Inhibitors of p38 and ERK/MAPK blocked the induction of COX-2 by the MIAs. The investigators also found that MIAs stimulated the binding of AP-1 transcription factor complex to the cAMP response element in the COX-2 promoter (Fig 2). AP-1 is a collection of dimers of members of the Jun, Fos, and ATF/cAMP response element binding protein families. MAPKs regulate AP-1 by increasing the abundance of AP-1 components and their activity. ERK1/2 MAPK regulates c-Fos transcription, whereas p38 MAPK phosphorylates and activates ATF-2. It is this transcription factor complex that mediates the induction of COX-2 by MIAs as well as actin interfering agents such as cytochalasin D. These agents increase binding of the COX-2 promoter to cAMP response element. The components, c-Jun, c-Fos, and ATF-2, can be identified within the DNA binding complex.<sup>92</sup> The authors postulate that the effectiveness of these drugs might be compromised by the fact that they do upregulate COX-2 activity and that adding a COX-2 inhibitor to the treatment regimens that incorporate these agents might further improve antitumor activity as well as the arthralgia/myalgia side effects caused by them.

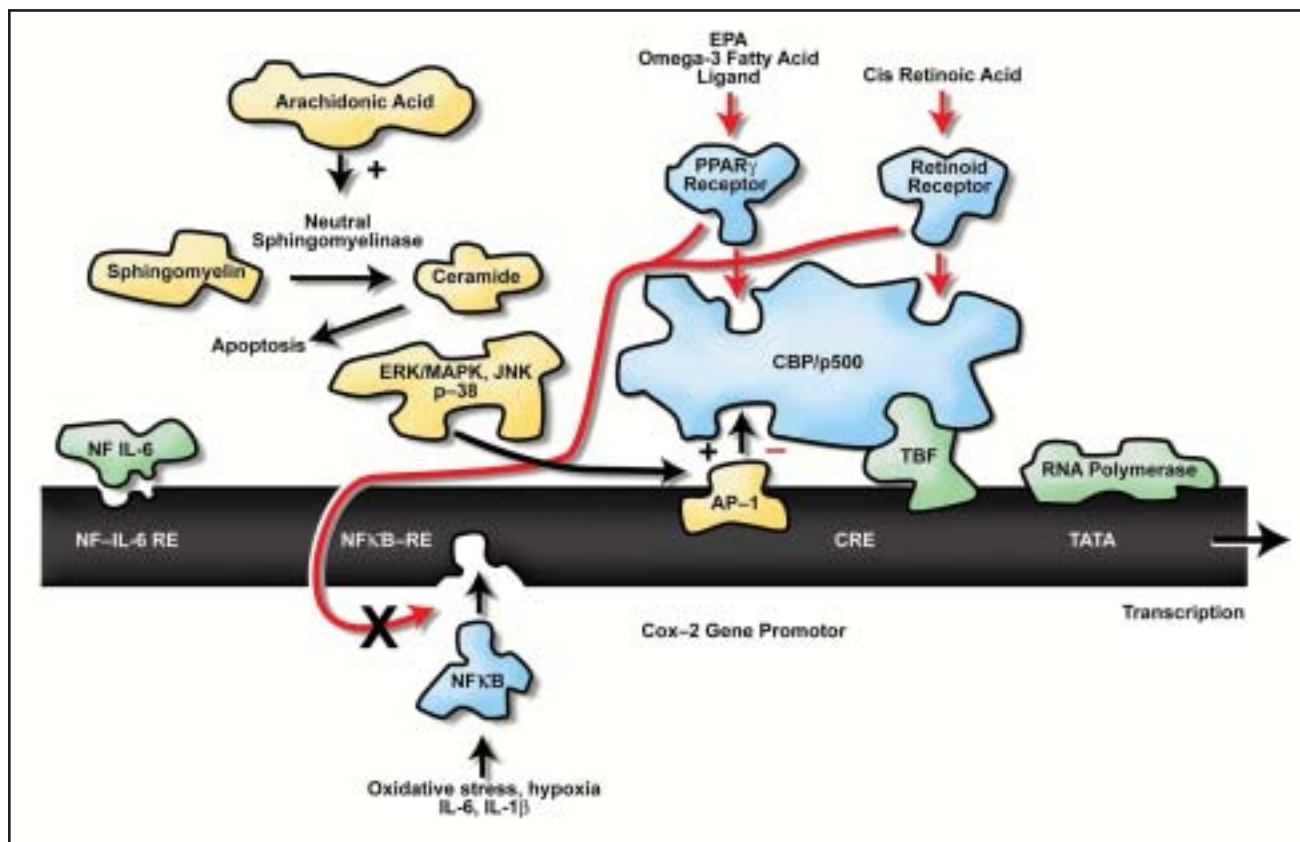


Fig 2. — Effects of PPAR- $\gamma$  ligand, retinoid receptor binding, ceramide, NF- $\kappa$ B and IL-6 activation on COX-2 transcription. CBP/p300 links AP-1 to components of the basal transcription mechanism. Binding of the PPAR receptor and the retinoid receptor by their respective stimuli increases the binding of the receptors to the CBP/p300, preventing AP-1 binding to CBP/p300 and preventing activation of COX-2 gene expression. Activation by oxidative stress and hypoxia stimulates the binding of NF- $\kappa$ B to its response element and likewise enhancing gene transcription. Excess arachidonic acid stimulates sphingomyelinase to metabolize sphingomyelin to ceramide which in turn acts thru the MAPK pathway to stimulate AP-1 binding to the CRE site of the COX-2 promoter (AP-1 = activator protein-1, CRE = cAMP response element, CBP = CRE binding protein, PPAR = peroxisome proliferator-activated receptor, PG = prostaglandin, MAPK = mitogen-activated protein kinase, ERK = extracellular signal-regulated kinase, JNK = c-Jun *N*-terminal kinase, TBF = TATA binding factor, NF- $\kappa$ B = nuclear factor  $\kappa$ B, EPA = eicosapentaenoic acid).

Munkarah et al<sup>93</sup> addressed this issue in a study that evaluated the effects of combining paclitaxel and the selective COX-2 inhibitor NS398 in two ovarian cancer cell lines, which in previous experiments had been shown to express COX-2. Neither cell line showed an increase in apoptosis with exposure to NS398 alone. They were sensitive to paclitaxel-induced apoptosis. When cells were treated concomitantly with NS398 and paclitaxel, the rate of apoptosis actually *reduced* when compared to paclitaxel alone, and the same results occurred when the drugs were given in sequence. These unexpected results, especially the negative effect of the combination of COX-2 inhibition and MIA, question whether the upregulation of COX-2 with certain agents may be beneficial or even necessary for antitumor activity, as was described in the colon cancer experiments of Dommels et al.<sup>39</sup>

Becerra et al<sup>94</sup> developed a protocol incorporating COX-2 inhibition with rofecoxib in patients with metastatic colorectal carcinoma using a standard regimen of 5-fluorouracil (5-FU) (450 mg/m<sup>2</sup>) and leucovorin (20 mg/m<sup>2</sup>) daily for 5 days, repeated at 4 weeks, followed by 5-week intervals. Tumor samples from all patients exhibited evidence of moderate COX-2 overexpression. Rofecoxib was begun on day 1 of chemotherapy and continued for the duration of treatment. Ten patients were entered on this trial; performance status on entry was 0-2. No responses were seen, and toxicities included grade 3 upper GI bleeding in 3 patients that prompted early termination of the study. The median progression-free survival for the 10 patients was 2 months, while that reported for the 5-FU/leucovorin regimen alone was 3 to 4 months, with a 30% response rate. The investigators questioned the potential influence of rofecoxib on the pharmacokinetics of the regimen. The issue of GI bleeding was also discussed. Although the incidence of this complication is less with the COX-2 specific inhibitors, it is recognized that a subset of patients with pre-existing risk factors, such as a history of peptic ulcer disease or previous GI bleed, have a 5% per year risk of complicated ulcer compared with 0.4% per year risk with no such history.<sup>95</sup> The combined use of chemotherapeutic agents, which can increase the risk of mucosal injury, as in this trial, also needs to be recognized and evaluated carefully. A possible compounding effect of *Helicobacter pylori* infection may need to be considered as an added risk factor in the GI toxicity of such combined therapies.

In glioma, one combination study involving COX-2 inhibition and chemotherapy in vitro has been reported by Roller et al,<sup>96</sup> who examined 5 glioma cell lines: T-98G, A172, LN-18, LN-229, and LN-308. T-98G alone expressed COX-1. COX-2 expression was minimal in all cell lines. The investigators showed that despite this lack of COX-2 expression, NSAIDs enhanced the cytotoxicity of doxorubicin and vincristine in the T-98G cell line alone. All 5 glioma cell lines expressed the multidrug resistance protein (MRP), which was particularly high in the T-98G line.

MRP transport activity was most modulated in T-98G cells by indomethacin. The investigators suggested that NSAIDs may enhance the cytotoxic effects of specific drugs subject to MRP-dependent transport in MRP-expressing tumors.<sup>4</sup>

## Cyclooxygenase Inhibition in Combination With Radiation

The end result of therapeutic radiation is to inhibit the ability of cells to proliferate, the principal target being DNA. Genes that are modulated following exposure to radiation have an effect on cell cycle signaling pathways that allow survival or promote cell death, and it is the balance between these that determines the fate of the cell.<sup>97</sup> Specific genes activated by radiation include those that encode the various growth factors including VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor 1 (PDGF1), those that control cell cycling (p27 and cyclins), and those involved in production of signaling proteins and regulation of gene transcription. Indirectly, radiation produces an effect on membrane lipids through oxygen-containing free radicals that lead to radiation-induced edema and cell death apart from DNA damage. Mechanisms involved in these cellular membrane events induced by radiation include the activation of phospholipase A-2, triggering release of arachidonic acid, and production of eicosanoids through cyclooxygenase and lipoxygenase pathways. Cyclooxygenase inhibition, then, would seem to enhance radioresponse by direct actions on tumor cells, but it has also been shown to act indirectly by affecting tumor vasculature.<sup>98</sup>

Eicosanoid production is increased in many tissues after exposure to radiation. Within hours after irradiation, increased levels of prostaglandins and thromboxanes, including PG-E<sub>2</sub>, PG-E<sub>1</sub>, PG-F<sub>2</sub>α, PG-I<sub>2</sub> (prostacyclin, produced by endothelial cells), TXA-2, and TXB-2, are detectable and the increased levels may persist for days to weeks. Prostaglandins are essentially short-lived, locally acting autocooids on the cells that produce them (autocrine activity) and on neighboring cells (paracrine activity). To induce a cellular response, prostacyclins bind to membrane receptors that in turn couple to G (guanine nucleotide)-binding proteins and generate second messengers, particularly cAMP, calcium, and inositol triphosphate. Protein kinases are then activated, especially protein kinase C, A, and likely tyrosine kinase.<sup>99</sup>

A number of these natural prostaglandins (PG-E<sub>1</sub>, PG-E<sub>2</sub>, PG-F<sub>2</sub>α, and PG-I<sub>2</sub>) and their synthetic analogues (misoprostol and iloprost) have shown the ability to protect cells and tissue from radiation injury, in particular bone marrow, hair follicles, testes, and jejunum.<sup>100</sup> They have been shown to be radioprotective when administered before irradiation in experiments performed in vitro and in vivo.<sup>101-103</sup> Ligand receptor binding is important in this situation as it has also been shown that radioprotection cannot be achieved if

cells lack prostaglandin receptors or if these receptors are blocked. The production of prostaglandin by tumor was found to be associated with tumor radioresistance. If production of prostaglandin is suppressed, these tumors are more radioresponsive and NSAIDs increase the in vitro radiosensitivity of cancer cells.<sup>104</sup>

In as early as 1988, researchers reported that indomethacin (a nonselective cyclooxygenase inhibitor) improved radiation response in mouse fibrosarcoma and had minimal effect on radiation sensitivity of normal tissues. They demonstrated that cells after treatment with indomethacin accumulated in the G<sub>2</sub>-M phase of the cell cycle, which is considered to be the most sensitive to ionizing radiation.<sup>104</sup> These investigators also reported that in murine tumor models, the tumor growth inhibiting mechanisms were seen only in prostaglandin-producing tumors, indicating that inhibition of prostaglandin synthesis was related to the antitumor activity in this setting.<sup>105</sup> Ibuprofen in high concentrations was also found to be cytotoxic alone and to improve radiation response in 2 human prostate carcinoma cell lines.<sup>106</sup> Kishi et al<sup>107</sup> investigated this effect using SC-'236, a selective COX-2 inhibitor, and evaluated the effect on radiation response of another in vivo sarcoma model, FSA, a tumor that had previously been shown to be resistant to apoptosis induced by radiotherapy or chemotherapy. They also evaluated the response of normal tissues to the combination of drug and radiotherapy. Results indicated that the drug increased tumor growth delay and tumor curability to a greater degree than had indomethacin with radiation, and it had minimal effect on normal tissue. Thus, a decrease in PG-E<sub>2</sub> levels may correlate with a loss of this protective effect in tumor cells and increase their susceptibility to radiation. The mechanism of apoptosis vs antiangiogenesis and potency of the NSAIDs is still in question.<sup>107-110</sup>

Petersen et al<sup>111</sup> evaluated the combined effect of SC-'236 and radiotherapy on a human glioma cell line, U251, which moderately expresses COX-2, in monolayer culture as well as tumor xenografts. Exposure of U251 cells to SC-'236 results in a significant reduction in cell survival (approximately 90%). COX-2 inhibition of cell cycling, mediated indirectly through effects on regulatory cyclins and cyclin-dependent kinases, may cause cells to arrest in G<sub>2</sub>-M. This mechanism of cell cycle arrest seems to be operative in some cell systems but not in others,<sup>112</sup> and like the effects described of COX-2 inhibition on various tumor types and in the combination drug trials, there may be variability among different types of cells/tumors in the mechanisms of radiation sensitization.

After radiation damage, COX-2-derived PG-E<sub>2</sub> may be a critical survival factor for aberrant or immature tumor neovasculature. Rofecoxib, another specific COX-2 inhibitor, has been reported also to reduce endothelial tube formation when combined with radiation.<sup>113</sup> Using a high-molecular-weight contrast dye and magnetic resonance imaging, Davis et al<sup>114</sup> then produced the visual

effects of radiation and COX-2 inhibition on tumor neovasculature, with the intent to evaluate integrity and function. The combination of celecoxib and radiation led to a marked increase in vascular permeability by 24 hours after treatment, whereas neither radiation nor celecoxib achieved this parameter alone. Also, the subsequent maintenance of inhibition of COX-2 activity and PG-E<sub>2</sub> synthesis after acute therapy may impair neovascularization and the regrowth of tumor.<sup>115</sup> Thus, COX-2-derived PG-E<sub>2</sub> seems to have an important role in tumor survival after radiation damage, and these effects appear to be most important in angiogenesis. COX-2 inhibition is a logical method to manipulate these changes and improve the impact of radiotherapy.

## Conclusions

This review summarizes knowledge of multiple preclinical and clinical studies in oncology involving cyclooxygenase and its inhibition, as well as the puzzling and unexpected results investigators have reported over the last few years. Cyclooxygenase is a complex enzyme, regulated by numerous tissue and growth factors, autocrine factors, and its own metabolites. COX-2 inhibitors often function as antitumor agents regardless of their effect on cyclooxygenase. They produce effects at multiple areas of signal transduction and apoptotic pathways, and they influence other growth factors.

Inhibition of COX-2 may also be detrimental to tumor control and may result in cell proliferation. Reports of combinations of a COX-2 inhibitor with chemotherapeutic agents resulting in an unexpected proliferative effect are disturbing. The responses to upregulation and downregulation of cyclooxygenase in different cancers need further exploration as trials in human brain tumors are beginning.

The function of COX-2 in combination with radiotherapy is mainly through production of prostanoids and their effects in radioprotection of tumor cells. Chemoradiation trials with the addition of COX-2 inhibition warrant in vitro and in vivo investigation due to reported toxicity. COX-2 inhibitors may be of benefit in our chemotherapeutic armamentarium. It will take the collaborated efforts of many investigators to isolate the best drugs, the best combination, the optimum dose, the best timing, and the best schedule for individual drugs in combination. This is especially important in the setting of glioma, a tumor in which subtle changes in invasiveness and proliferative rate may result in a major impact in overall tumor growth and host prognosis.

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