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An Aspirin a Day: New Pharmacological Developments and Cancer Chemoprevention

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Abstract

Chemoprevention refers to the use of natural or synthetic agents to reverse, suppress, or prevent the progression or recurrence of cancer. A large body of preclinical and clinical data suggest the ability of aspirin to prevent precursor lesions and cancers, but much of the clinical data are inferential and based on descriptive epidemiology, case control, and cohort studies or studies designed to answer other questions (e.g., cardiovascular mortality). Multiple pharmacological, clinical, and epidemiologic studies suggest that aspirin can prevent certain cancers but may also cause other effects depending on the tissue or disease and organ site in question. The best-known biological targets of aspirin are cyclooxygenases, which drive a wide variety of functions, including hemostasis, inflammation, and immune modulation. Newly recognized molecular and cellular interactions suggest additional modifiable functional targets, and the existence of consensus molecular cancer subtypes suggests that aspirin may have differential effects based on tumor heterogeneity. This review focuses on new pharmacological developments and innovations in biopharmacology that clarify the potential role of aspirin in cancer chemoprevention.

INTRODUCTION

Long-term use of aspirin has been associated with a reduction in the incidence (and in some cases mortality) of a variety of types of cancer, especially gastrointestinal cancers (1). Much of these data are based on descriptive epidemiology and case-control and cohort studies. The evidence has been strongest for an effect on colorectal cancers (CRCs) (1, 2), especially in high-risk groups such as those with Lynch syndrome (3). Support has also come from cardiovascular disease (CVD) primary prevention trials where cancer reduction was assessed but was not the primary end point (1).

In 2016, the US Preventive Services Task Force (USPSTF) recommended low-dose aspirin for primary prevention of CVD and CRC in adults aged 50–59 years who have a 10% or greater 10-year cardiovascular risk, are not at increased risk of bleeding, have a life expectancy of at least 10 years, and are willing to take aspirin daily for at least 10 years (4). Based on a systematic review and a microsimulation model (5) acknowledging new data on benefit to risk, the USPSTF released a recent recommendation suggesting that use of low-dose aspirin for primary prevention of CVD in high-risk individuals ages 40–59 years be an individual one (6) since the net benefit is small. The USPSTF also recommends against initiating low-dose aspirin for this use in adults age 60 and older, concluding that the evidence is inadequate that low-dose aspirin use reduces CRC incidence or mortality in this group and that "initiating aspirin use for the primary prevention of CVD events in adults 60 years or older has no net benefit" (6, p. 1577). This modification acknowledges many of the uncertainties regarding the timing and long duration of exposure necessary for aspirin's chemopreventive effects and new data regarding how individual characteristics, lifestyle, and genetic background may impact aspirin's ability to prevent colorectal and other cancers. This recommendation is made despite a very large body of preclinical and epidemiological data suggesting that the use of aspirin is associated with a 13–18% reduction in the risk of recurrence of colorectal adenomas (a cancer precursor) after initial removal (7) and an overall 20–30% reduction in the risk of CRC with long-term use. Of substantial concern are the associated risks of gastrointestinal bleeding and hemorrhagic stroke associated with long-term aspirin use compared with the overall benefits. This review discusses new biopharmacological developments, the impact of patient and tumor heterogeneity, end points for efficacy, and how these factors may impact the potential role of aspirin in cancer chemoprevention.

ASPIRIN CHEMISTRY AND THE ROLE OF ACETYLATION

Aspirin is a highly reactive molecule that can acetylate a variety of macromolecules in biological systems, including proteins, nucleotides and lipids. The biological effects of acetylation reactions are most commonly associated with proteins. This is generally the result of the most common amino acids that tend to undergo acetylation by aspirin. There are chemical transacetylation reactions with the N-terminal amino groups of proteins as well as side-chain amino, hydroxyl, and sulfhydryl groups. Recent studies have described the real-time acetylation reaction by hyperpolarized imaging using ^{13}C-labeled stable isotopes to help understand the aspirin acetylation process in vivo (8). Reaction between ^{13}C hyperpolarized aspirin and Nα-acetyl lysine showed ^{13}C-Nε-diacetyl lysine 6 as well as ^{13}C-acetate and ^{13}C-salicylic acid. Many of the effects of aspirin have been attributed to cyclooxygenases (COXs), covered in detail in following sections, although enzyme-independent effects of aspirin also exist.

WHAT NEW INFORMATION CAN BE LEARNED FROM MOLECULAR PROFILING AND BIOINFORMATICS?

With the advent of modern sequencing coupled with bioinformatic analyses, we have learned much about normal tissues and tumors, primarily using whole-exome, genome, and single-cell

sequencing technologies. As we focus on platelets, these sequencing methods present a problem since platelets do not have nuclei and robust translational machinery that can maintain long-term and receptor feedback–related gene expression (9). A recent meta-analysis of 67 platelet proteomic analyses and pathway functions illustrated some of the technical challenges and biological effects of aspirin acetylation (10). This analysis evaluated current technology and molecular protein changes in ageing platelets, which have approximately a 10-day viability in circulation. This study also compared the complexity of platelet signaling pathways and functions in response to collagen, rhodocytin, thrombin, thromboxane A_2 (TxA_2), and adenosine diphosphate (ADP). Comparisons were also made regarding the effects of endothelial-derived mediators such as prostacyclin (PGI_2) and aspirin on the platelet proteome. It also evaluated the molecular protein changes in platelets from patients with congenital disorders or CVD (10).

Understanding the effects of aspirin on circulating platelets, in contrast to studies that compare tissues of interest using a large data set/bioinformatic approach, should therefore include protein sequencing or mass spectrometry to perform unsupervised analyses to reveal changes in response (10). One study using isolated platelets treated in vitro with aspirin found that the acetylated peptides that were discovered typically had tyrosine (Y), lysine (K), and arginine (R) amino acids present (11). This study also examined secreted proteins (11). Aspirin reduced the ability of platelets to secrete granular glycoproteins such as thrombospondin 1, TIMP1, and other platelet granule contents, many of which possess adhesive and angiogenic functions (11).

Another study focused on aspirin resistance syndrome, which results from inadequate platelet inhibition despite aspirin therapy (12). This study involved 51 clinically stable coronary ischemic patients taking aspirin (100 mg per day) who were divided into aspirin resistant ($n = 25$) and aspirin sensitive ($n = 26$) based on a platelet functionality test. Their 2D gel and mass spectrometry (MS-MS) studies revealed that two gelsolin precursor isotypes and one F-acting capping protein isotype were decreased in aspirin-resistant platelets ($p < 0.05$). The expression of glyceraldehyde 3-phosphate dehydrogenase was increased in the aspirin-resistant platelets and was accompanied by reduced expression and activity of 1,6-bisphosphate aldolase in platelets without changes in the content of pyruvate. Reduced expression of glutathione-*S*-transferase and the protein disulfide isomerase isotype 1 was found in aspirin-resistant platelets. Overall, aspirin-resistant and aspirin-sensitive platelets were different in terms of the level of expression of proteins associated with mechanisms such as energetic metabolism, the cytoskeleton, oxidative stress, and cell survival based on the differential response to aspirin. Overall, most of these aspirin studies generally involve small numbers of patients and reveal changes in the platelet proteome following acetylation by aspirin. Platelet proteomics and lipidomics have also been applied to better understand CVD (13). Ideally, what we learn bioinformatically from liquid chromatography mass spectrometry (LC-MS-MS) protein, lipidomic, and RNA sequencing (RNAseq) of isolated platelets with disease-affected tissues at the tissue and single-cell level will provide a fully informative picture of the biology and responses involved (9).

EPIGENETICS AND THE ROLE OF ACETYLASES AND DEACETYLASES

Epigenetic alterations in DNA along with many posttranslational chemical reactions can impact gene expression, chromatin remodeling, and biological function (14, 15). Butyrate is an endogenous inhibitor of histone deacetylases in the gastrointestinal tract, and COX-2 overexpression makes intestinal epithelial cells resistant to butyrate-induced apoptosis (16). A key question related to aspirin use is whether aspirin acetylation can alter deacetylase function or potentially make alterations in acetylation-mediated changes in chromatin states. ATAC-seq (assay for transposase-accessible chromatin using sequencing) of esophageal squamous cell carcinoma indicated that

aspirin/cisplatin treatment results in remarkable epigenetic alterations on chromatin in cancer stem cells, suggesting that aspirin may serve as an adjuvant to therapy (17). In another study, the use of isotopically labeled aspirin (aspirin-d3), in combination with acetylated lysine purification and LC-MS-MS, revealed over 12,000 sites of lysine acetylation in cultured human cells (18). This study also found that aspirin amplifies endogenous acetylation signals at most detectable endogenous sites, stoichiometry correlates with biological relevance, and deacetylases act to minimize the biological consequences of nonspecific chemical acetylation (18). In support of this notion, one study showed that lysine acetylation of endothelial nitric oxide synthase (eNOS) is a posttranslational protein modification supporting low-dose aspirin-induced vasoprotection (19). Histone deacetylase 3 (HDAC3), by deacetylating aspirin-acetylated eNOS, antagonizes aspirin-stimulated endothelial production of NO (19). Aspirin-inspired acetyl-donating HDAC inhibitors, which promote alpha-tubulin and histone H3 acetylation by HDAC6 and HDAC1, are also under development (20). A separate study showed that global histone H3 acetylation and H3k9 acetylation were all downregulated during adipogenic differentiation, and aspirin can reverse any decreases (21). Ultimately, acetylation reactions driven by aspirin can have both selective and global effects on cells and tissues.

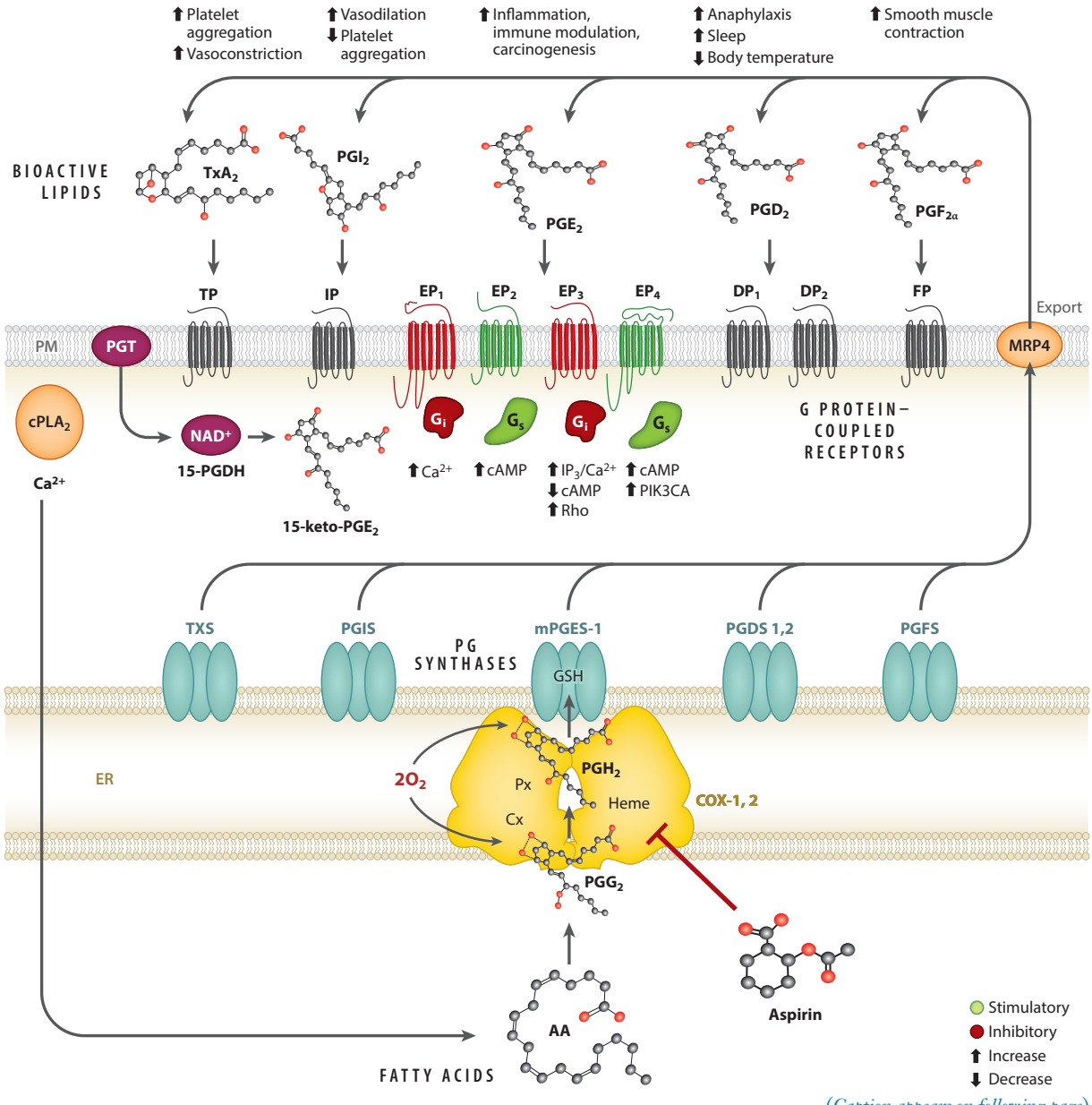
ASPIRIN COVALENTLY INACTIVATES CYCLOOXYGENASES: HOW ARE COX-1 AND COX-2 ISOFORMS AFFECTED?

The most profound biological effects observed from aspirin use result from acetylation and enzymatic inactivation of the COXs. Among the different COX or prostaglandin-endoperoxide synthase (PTGS) isoforms, COX-1 is considered to be constitutively expressed, whereas COX-2 was discovered based on its inducibility (22). COXs are mixed-function oxygenases, which require two oxygen molecules to form the rate-limiting substrate in prostaglandin (PG) synthesis (23). The IC_{50} values for purified ovine COX-1 and -2 are 0.75 and 1.25 mM, respectively (24). Aspirin is likely to have a greater effect on platelet and monocyte COX-1 in circulation compared to the differential effects of other nonsteroidal anti-inflammatory drugs (NSAIDs) (25). However, polymorphisms in COX-1 also have an impact on aspirin chemistry and biological effects (26). An R108Q variation exerts the largest functional effects, with evidence for impaired interactions with a COX substrate and inhibitors. As Arg108 is located on the protein surface and not in the active site, the effects of R108Q suggest a novel, unsuspected mechanism for the modulation of the prostaglandin endoperoxide synthase-1 (PGHS-1) active site structure. The lower intrinsic reactivity of the R108Q, V481I, and L237M isoforms with aspirin, together with the rapid hydrolysis of ASA in the blood, suggests that these variants may be associated with decreases in the antiplatelet effect of the drug (26).

Of the known isoforms, constitutively synthesized COX-1 was first concentrated from bovine vesicular microsomes in 1974 (27). It is the key enzyme in the synthesis of many different PGs from arachidonic acid (AA) and is enriched primarily in smooth muscle cells, monocytes, and platelets. Platelet activity is driven by COX-1, which is linked to TxA_2 production by thromboxane synthase (TXS) in circulation. When circulating platelets become activated, COX-1 synthesizes prostaglandin H_2 (PGH_2), which is then converted to TxA_2 by TXS (28). Aspirin irreversibly acetylates COX-1 at Ser530 (28), which eliminates PGH_2 biosynthesis and inhibits platelet function (29). Since PGH_2 serves as the rate-limiting substrate for all PG genesis, its inhibition impacts all PG bioactivities, whether they be hemostasis or immunomodulation, triggering proinflammatory responses and becoming pro-oncogenic or otherwise bioactive (**Figure 1**).

The existence of COX-2 remained unrecognized for some time after the discovery of COX-1. The existence of another isoform was initially suspected due to inducible properties (30), and the

inducible human COX-2 isoform was then cloned and characterized (31, 32). In contrast to COX-1, COX-2 inhibition by aspirin occurs predominantly through acetylation of the active site serine side chain (Ser516) and inhibition of PG biosynthesis at the level of PGH_2 substrate generation (33). Structurally, the active site pocket of COX-2 compared to COX-1 is much larger and was the key to developing COX-2-selective inhibitors (34). Better resolution of the COX-1 active site may also lead to more selective COX-1 inhibitors (35, 36). Similarly, the development of COX-2-selective inhibitors continues to evolve (36–44).



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Bioactive eicosanoid metabolism. Eicosanoid biology is complex. Eicosanoids have direct and indirect effects on platelet aggregation, inflammation, and immune modulation, which impact carcinogenesis and tumor progression. Bioactive lipids are derived from metabolic sources that arise from essential dietary fatty acids. These fatty acids, which are transported into cells, include arachidonic acid (AA), docosahexaenoic acid, and eicosapentaenoic acid. Acyl-coenzyme A is coupled to fatty acids by acyl-coenzyme A synthetases. Fatty acids like AA are then inserted as a storage source into membrane phospholipids by fatty acyltransferases. After platelet agonist stimulation, the cytoplasmic form of phospholipase A_2 ($cPLA_2$) catalyzes the release of AA from membrane phospholipids. Once membrane free, AA is enzymatically converted by cyclooxygenases (COXs) into prostaglandin G_2 (PGG_2), followed by prostaglandin H_2 (PGH_2). PGH_2 then serves as a substrate for multiple PG synthases. PG synthases occur in multiple forms, including thromboxane (TxA_2) synthase (TXS), prostacyclin (PGI_2) synthase (PGIS), prostaglandin E_2 (PGE_2) synthases (PGESs), prostaglandin D_2 synthases (PGDS), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthase (PGFS). Both TxA_2 and PGI_2 contain epoxide bonds that have significant chemical bond strain that leads to their rapid hydrolysis and short half-life, approaching 30 s. Bioactive lipids are exported outside the cell by multidrug resistance–associated protein 4 (MRP4) and other transport molecules. As PGs accumulate in the extracellular microenvironment, they bind to subtype-specific G protein–coupled receptors. These receptors include TxA_2 receptor (TP), PGI receptor I type (IP), PGE_2 receptor E types 1–4 (EP_{1-4}), PGD_2 receptor D types 1–2 (DP_{1-2}), and PGF receptor F type (FP). Depending on their function, various receptors interact with subtype-specific G-stimulatory (G_s) or G-inhibitory (G_i) proteins. Downstream signaling molecules stimulate cyclic adenosine monophosphate (cAMP), Ca^{2+}, inositol phosphates, or IP_3/Ca^{2+}, and Rho, among others. Metabolic breakdown relies on PG transporter (PGT), followed by inactivation involving NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). PGE_2 promotes cancer development and progression by multiple mechanisms. The roles of other prostanoids in cancer development is less clear but under active investigation. Abbreviations: Cx, cyclooxygenase; ER, endoplasmic reticulum; GHS, glutathione; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PM, plasma membrane; Px, PGH2 synthase.

ACETYLATABLE GAIN-OF-FUNCTION MUTATIONS: KEY ASPIRIN-SELECTIVE TARGETS

Gain-of-function mutations that result in amino acid substitutions that can undergo acetylation may be key aspirin-selective targets. As one example, a number of reports have linked aspirin treatment to the modulation of phosphatidylinositol-3-kinase (PI3K) activity as having a potentially direct effect on cancer (45). This direct effect of aspirin on PI3K may be related to the acetylation of E542K, E545K, or H1047R gain-of-function mutations due to lysine (K) and arginine (R) substitutions in the mutated PI3K molecules (46), which are most likely acetylated by aspirin, leading to enzyme inactivation. These types of *PIK3CA* mutations can also confer resistance to first-line therapy (47).

OMICS-BASED APPROACHES TO UNDERSTANDING ASPIRIN AND CANCER

Aspirin proteomics and lipidomics have also been applied to better understand cancer (48–50). This approach has also been applied to aspirin effects on early-stage cancers (51). One of the advantages of performing proteomic and lipidomic analyses of circulating platelets is that they are easy to isolate and perform liquid biopsies on to analyze drug responses. This also extends to circulating plasma proteins and vesicle-encapsulated or free macromolecules such as circulating cell-free tumor DNA. Platelet RNAseq has also been applied to circulating platelets that were isolated from cancer patients to identify tumor-educated platelets (52, 53). These studies also involved the isolation of platelets that have ingested RNAs, as they circulate through and are thereby educated by tumors. This process may involve uptake of macromolecules by the open canalicular system of platelets (9). Platelet coding and noncoding along with circular RNAs are thought to capture information from not only parent megakaryocytes and progenitor hematopoietic stem cells but also the bone marrow microenvironment and underlying disease states (54). Noncoding RNAs may also have an impact on tumors (55). Whether there are feedback loops to megakaryocytes or their progenitor cells during the evolution of various disease states that modify or refine platelet phenotypic genesis remains to be determined.

Although these proteomic and lipidomic approaches have been applied to circulating platelets, there do not seem to be many studies that have identified platelet-specific signatures found in tissues affected by CVD or cancers (56). These studies showed that aspirin treatment of normal colon organoids diminished the transit-amplifying cell population, inhibited PG synthesis, and dysregulated expression of novel genes implicated in colon tumorigenesis (56). Finding concordant signatures between circulating platelets and any associated tissue responses or plasma changes would be very informative when coupled with bioinformatic analyses. This would be important in a prospective context in the study of aspirin. Identifying the key signatures of patients that benefit from aspirin use versus those who do not, in either a cardiovascular or cancer disease setting, would help to improve the efficacy of treatment regimens and help eliminate toxicity in those who fail to show an improved aspirin response. Data and insight gained from unsupervised -omics-based analyses can then be applied to targeted evaluations using polymerase chain reactions, enzyme-linked immunosorbent assays, or microfluidics. Galectin-3 is an example of such a protein being successfully employed as a single or a multiplexed circulating biomarker for early and advanced cancers (57, 58).

ASPIRIN AND CYCLOOXYGENASE BIOLOGY

COXs and PGs play a critical role in the microenvironmental influences and immunobiology of gastrointestinal cancers (59, 60). These influences may also extend to platelet function in CRC metastasis and potentially the establishment of minimal residual disease as defined by circulating tumor DNA (61). In early mouse models, PG synthesis involving membrane-derived AA by COX-1 and COX-2 enzymatic activity was shown to decrease systemic arterial pressure and increase pulmonary arterial pressure, and this observation was further supported in knockout mouse studies (62). Mouse models have also revealed the role of PG genesis in cancer progression and metastasis. In one mouse model, direct evidence was provided with a truncation mutation in adenomatous polyposis coli at amino acid 716 ($Apc^{\Delta 716}$), which predisposes them to adenoma formation in the small intestine (63). In another mouse model strain, genetic knockout of COX-2 and pharmaceutical blockage approaches both led to polyp reduction. In this case, both COX-1-null/$Apc^{Min/+}$ and COX-2-null/$Apc^{Min/+}$ mice had decreased numbers of intestinal polyps (64). Genetically engineered and carcinogen-induced animal models consistently show the importance of the COX-1/2 pathways in a variety of organ systems (65). Improvements have been made in aspirin and sulindac delivery using phosphatidylcholine (PC) to treat C57B/6 $Apc^{Min/+}$ mice (66). Both sulindac and sulindac-PC treatments outperformed aspirin and resulted in significantly reduced polyp burden and decreased urinary PGs, but sulindac-PC treatment also resulted in the reduction of gastric lesions compared to sulindac alone (66).

Eberhart et al. (67) first reported the upregulation of *COX-2* gene expression in human colorectal adenomas and adenocarcinomas. These findings lent support for a celecoxib clinical trial in patients with familial adenomatous polyposis (FAP) that has resulted in significantly reduced adenomas (68). These COX-2-specific inhibitor-related outcomes led the US Food and Drug Administration to approve celecoxib for use in FAP patients as an adjunct to surgery. Subsequently, individuals who were previously diagnosed with adenomas also showed reductions in adenoma recurrence in similar cyclooxygenase inhibitor (COXIB) trials, and the recurrence was reduced following treatment with celecoxib, particularly in patients with advanced adenomas.

As metabolically produced bioactive lipids, PGs are derived from AA, which is mobilized from membrane phospholipids. Any given PG pathway subtype activation varies depending on the cell type, tissue involved, and the expression of surface receptors present on target cells. Proinflammatory stimuli mobilize bioactive lipid genesis by catalyzing the release of AA from membrane phospholipids via phospholipase A_2 (69). AA released from the phospholipid layer is converted

into bioactive lipids by a series of enzymatic reactions. AA is first converted to PGH_2 with the incorporation of two oxygen molecules by the COX enzymes. This substrate generated by COX enzymes is used by a number of enzymes downstream of COXs that generate a variety of PGs, each with a specific mode of action and a different biological function. COX enzymes catalyze the rate-limiting reactions within this pathway and thereby serve as targets in limiting all PG production.

By targeting the rate-limiting COX activity, both PGE_2 proinflammatory effects and those of other critical PGs that influence hemostasis would be attenuated. One key PG that regulates homeostasis is PGI_2, which is continuously produced in nucleated vascular endothelial cells by prostacyclin synthase (PGIS) downstream of COX-2. Vascular endothelial cell PGIS synthesizes PGI_2, which is transported to the bloodstream. PGI_2 has a very short half-life (seconds in solution) and acts locally on blood vessels by inducing vasodilation and inhibiting platelet aggregation. Because endothelial cells are nucleated and contain all of the gene expression machinery, PGIS is constantly turned over and replaced. Compared to aspirin, many NSAIDs and COXIBs are typically competitive inhibitors that occupy the catalytic site of COX enzymes. Proinflammatory and pro-oncogenic stimuli stimulate COX-2 synthesis and enzymatic activation.

TOWARD MORE SELECTIVE INHIBITION

Selective inhibition of targets downstream of COX-1 and COX-2 could potentially avoid certain toxic effects associated with eliminating both beneficial and harmful downstream bioactive metabolites. In the case of platelet biology, TxA_2 is synthesized by thromboxane synthase (TXAS), which primarily lies downstream of platelet COX-1 and may not rely on COX-2 (70). More selective TXAS inhibitors and platelet TXAS have been studied since the 1970s (71). In particular, Upjohn Company chemists produced a synthetic PG analog: the 9,11-azoprosta-5,13-dienoic acid inhibitor. This nitrogen-substituted azo analog structurally resembled PGH_2 and was shown to be particularly potent at inhibiting oxygen-based endoperoxide containing PGH_2 as well as ADP-, epinephrine-, and collagen-induced platelet aggregation (71).

TUMOR CELL–INDUCED PLATELET AGGREGATION: A POTENTIAL TARGET FOR CANCER THERAPY

The importance of tumor cell–induced platelet aggregation (TCIPA) to the cancer and metastasis field was first reported in the proceedings of the first international conference on prostaglandins and cancer (72). Additional efforts revealed that platelet COX (TxA_2 production) or 12-lipoxygenase (12-HETE production) enzyme inhibitors were unable to inhibit TCIPA alone but when combined inhibited TCIPA even at higher concentrations of tumor cells (73). In other studies, a novel TxA_2 modulator, BM-567 (II/II), inhibited platelet function (74) along with TCIPA and TxA_2 release. Similarly, 1-alkyl (*N*-alkyl)-imidazole derivatives such as OKY-046 (Ozagrel) are TXAS inhibitors, especially in human platelet–tumor cell interactions that have been studied since the early 1980s (75, 76), and have been shown to inhibit platelet function, TCIPA (77), and hepatic metastasis (78). Likewise, R-68070 (Ridogrel) is a combination TXAS inhibitor–TxA_2 receptor antagonist (79, 80) that prevents platelet aggregation. Similarly, various natural compounds can also inhibit platelet TXAS function and aggregation (81).

Thromboxane A_2 receptors (TPs) found in platelets are among the family of eicosanoid G protein–coupled receptors designated by their PG ligand molecular subclass. In the case of platelets, surface receptors also facilitate tumor cell–platelet (TC-platelet) cross talk (9). Identifying critical receptor-ligand interactions that mediate TC-platelet activation could reveal effective therapeutic targets.

TPs play a key role in the early and fast platelet-activation events. Since TxA_2 is produced locally during platelet aggregate formation, its chemical half-life is critical (82). TxA_2 is rapidly hydrolyzed in solution to inactive thromboxane B2 (TxB2) in approximately 30 seconds by epoxide ring opening. TxA_2 interactions with TPs rapidly stimulate platelet responses (73, 83). TxA_2 pathway activation is a major stimulus and amplification trigger of heterotypic aggregate formation during TCIPA (73, 83). TP-mediated platelet activation signals through $G_{12/13}$ and guanine nucleotide exchange factor Rho (Rho-GEF), followed by its downstream Rho-associated kinase (ROCK), which activates LIM domain kinase (LIMK) and stimulates actin reorganization (84). Additional downstream signaling from the TP-G_{13} interactions includes those with myosin light-chain kinase leading to platelet cytoskeletal changes (84). Selective TP antagonists continue to emerge as options for cancer treatment and include CAY10535, ifetroban, SQ 29,548, BM 567 pinane, terutroban, daltroban, picotamide, and sulotroban (85).

The initiation of TCIPA also depends on platelet surface glycoprotein $\alpha_{IIb}\beta_3$ (GPIIb/IIIa), which plays an important role in platelet aggregation and surface expression. This platelet integrin serves in the adhesion of tumor cells to platelets and may promote tumor metastasis. Inhibiting this platelet GPIIb/IIIa-mediated interaction with heparin (modified heparins), peptides, or blocking antibodies could prevent TCIPA (86). During inflammatory and TCIPA reactions, platelets serve as first responders and, once activated, can recruit polymorphonuclear leukocytes or macrophages to the sites of inflammation (87).

PROSTAGLANDIN E_2–RELATED MECHANISMS AND CANCER

PGE_2, the most common PG, is present at high levels in a variety of cancers (88) and serves as a primary driver of inflammation and carcinogenesis. PGE_2 regulates a tumor cell's prosurvival and antiapoptotic pathways by acting on the four E-type prostanoid receptors (EPs) highlighted in later sections. Decreasing PGE_2 levels in the tumor microenvironment by various mechanisms can reduce its protumorigenic effects. Anti-inflammatory drugs such as steroids and NSAIDs have cancer-preventive functions as they reduce PGE_2 levels. The production of PGE_2 is dependent on PGE_2 synthases, of which microsomal prostaglandin E_2 synthase (mPGES) seems to be key and is upregulated along with COX-2. In the case of COX-2-linked biology, the two enzymes are closely associated and are now gaining importance as more viable target enzymes downstream of COXs that do not trigger major side effects (89). The use of microsomal preparations of mPGES1 may be critical to inhibitor development when compared to the low activity found when using the purified enzyme (D.G. Menter & P. Marie, unpublished results). The importance of mPGES1 in cancer progression and immunosuppression highlights the need for further inhibitor-targeted development (90, 91).

PROSTAGLANDIN E RECEPTOR ANTAGONISTS AS POTENTIAL THERAPEUTIC AGENTS

EP biology is complex and involves both inhibitory and stimulatory G-coupled proteins. Some of these EPs mediate the proinflammatory/tumorigenic effects of PGE_2, which can include direct effects on precancerous and cancer cells as well as immune cells. Four different EPs ($EP_{1–4}$) exist that respond through either G-stimulatory (G_s) or G-inhibitory (G_i) protein coupling and can activate second-messenger signaling such as cAMP, Ca^{2+}, or inositol phosphates. Within the EP subclasses, EP_1 signals by regulating Ca^{2+} flux, EP_2 and EP_4 increase cAMP levels by coupling to G_s, and EP_3 has three known isoforms generated by alternative splicing that regulate cAMP levels by being bound to G_i or G_s. The various EP_3 isoforms function differently from each other and can also increase IP_3/Ca^{2+} and activate Rho (92). Platelets express $EP_{2–4}$ (93) and upon PGE_2

binding activate downstream signaling pathways, depending on the receptor type (94). Clinically, EP_2 and EP_4 antagonists continue in their development (95), including the first-in-human Phase I study of immunomodulatory E7046, an antagonist of EP_4, in patients with advanced cancers (96).

OTHER EFFECTS OF ASPIRIN ACETYLATION THAT MAY AFFECT CANCER PROGRESSION

Aspirin can also affect other lipid-metabolizing enzymes. As a monooxygenase-based effect, aspirin acetylation of COX converts the COX into a lipoxygenase that catalyzes the formation of 15-HETE and 11-HETE from AA (97). In contrast, acetylation of COX-1 renders it inactive. A shunting mechanism also exists following the inhibition of COX, which shifts the AA pathway to the 5-LOX pathway and leukotriene B4 (LTB4) production. Additionally, in several cancers, including colon, lung, breast, and head and neck squamous cell carcinoma, 5-LOX overexpression is also reported (98). Thus, monooxygenase activities can play an important role in cancer progression and metastasis. LOX activation, which could be enhanced with aspirin, results in leukotriene and lipoxin synthesis.

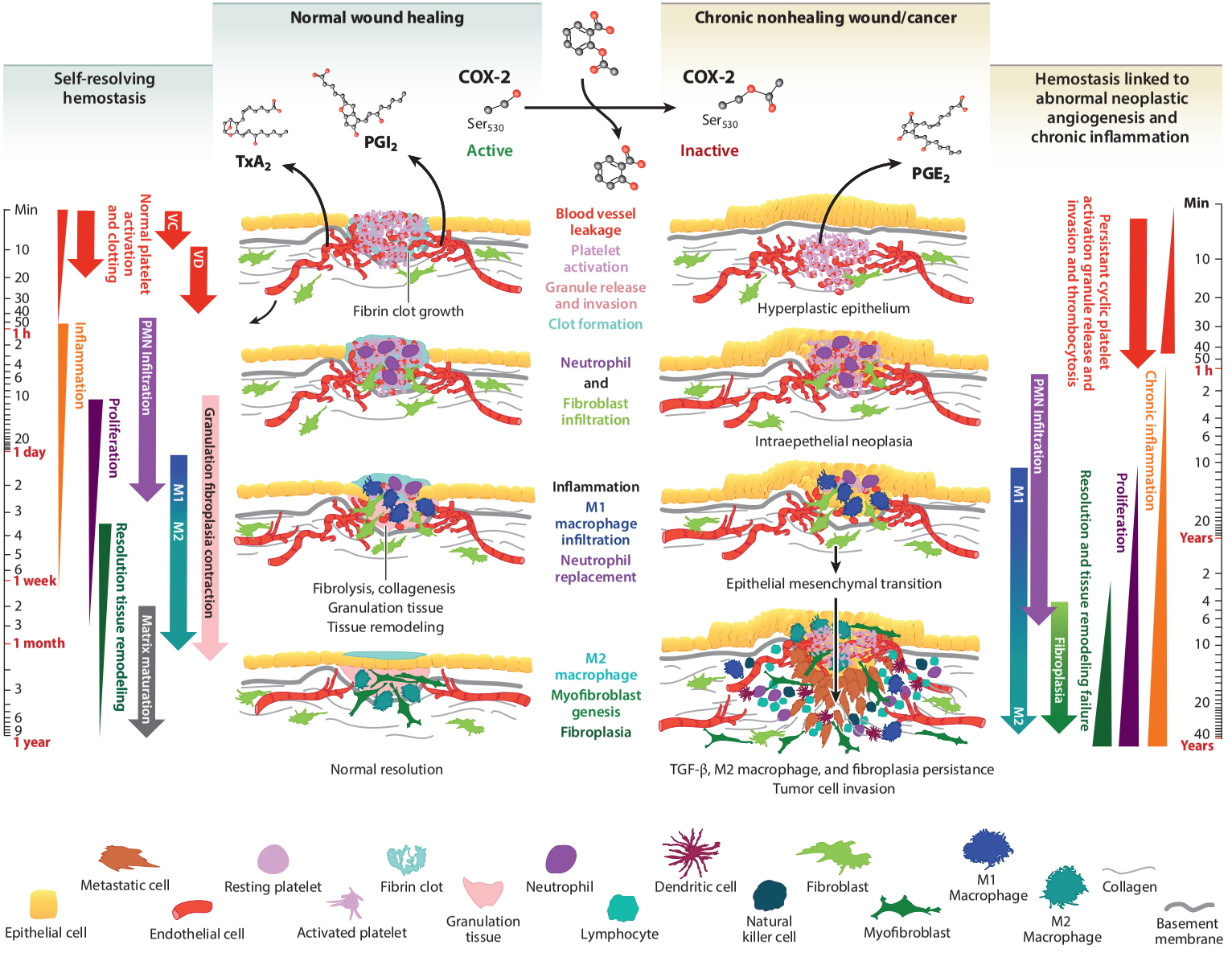
The resolution of inflammation via resolvins and maresins may also play a role in aspirin responses. Acetylated COX-2 can also act on eicosapentaenoic acid and docosahexaenoic acid, resulting in the synthesis of resolvins, maresins, and protectins (99). As the name reflects, resolvins initiate the resolution phase of inflammation and can suppress cytokine generation. Together, these resolvins, protectins, and maresins can clear inflammatory signatures, promote homeostasis, and return tissue to normalcy.

The exact mechanisms of action for NSAIDs' and aspirin's cancer-preventive efficacy are likely to involve a variety of eicosanoid metabolites. These likely include not only TxA_2, PGI_2, and PGE_2 but also other bioactive lipids along with a variety of altered proteins. As new molecular mechanisms emerge, the overall benefit of these drugs may involve multiple targets and microenvironmental influences, not the least of which will include platelets and other immune factors during multiple stages of carcinogenesis.

In normal wound healing, platelets and the coagulation system are activated in response to an insult that, when left unchecked, can promote inflammation and carcinogenesis. Aspirin acetylates Ser530 on COX-2 and chemically inactivates enzymatic activity, eliminating PG production along with its downstream effects on coagulation, inflammation, and immune biology. Immunosuppressive pathways persist during carcinogenesis and promote a loss of control over cancer-related proinflammation and immunosuppression. More invasive and angiogenic tumors recruit platelets as an unintended consequence of the hyperinflammatory process. Platelets can initiate and coordinate the normal wound healing response, but they also play unintended roles in carcinogenesis. Tumors that upregulate complement, angiogenesis, adhesion molecules, epithelial-mesenchymal transformation (EMT), and stromal pathways trigger inadvertent carcinogenesis associated with chronic inflammation that can be prevented by aspirin-mediated elimination of PG synthesis (**Figure 2**).

PROSTAGLANDIN INFLUENCES ON IMMUNE RESPONSE MODULATION

NSAIDs' and COXIBs' impact on cancer prevention and mortality reduction results not from a single enzyme inhibition but from a combination of pathway interference leading to a complex interaction within the tumor microenvironment. Among the key immunomodulatory events, the role of platelets has often gone unnoticed for reasons already discussed (9, 100). As an example of the importance of the immune microenvironmental production of eicosanoids, cancer



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Aspirin effects on wound healing, inflammation, precancers, and carcinogenesis. In normal wound healing, platelets and the coagulation system are activated in response to an insult that, when left unchecked, can promote inflammation and carcinogenesis. Aspirin acetylates Ser530 on COX-2 and chemically inactivates enzymatic activity, eliminating PG production along with its downstream effects on coagulation, inflammation, and immune biology. Complement, platelet, and local inflammatory signals switch on widespread changes in endothelial surface markers, vascular permeability, and release of cytokines and chemokines. Aspirin-based inhibition can potentially limit PG-driven influx of neutrophils and later macrophages, in addition to the induction of fibroblasts, and limit early precarcinogenic stimuli and promote resolution to a normal immune state. Within a matter of days, this process is replaced by the proliferation of fibroblasts and connective tissue as immature granulation tissue is replaced by more mature forms of secreted extracellular matrix. Inflammatory and fibroblastic cells are eventually programmed to die and switch off these processes during the remodeling phase. Precancerous microenvironments differ in that the stimulus for wound healing is not resolved. Immunosuppressive pathways persist during carcinogenesis and promote a loss of control over cancer-related proinflammation and immunosuppression. More invasive and angiogenic tumors recruit platelets as an unintended consequence of the hyperinflammatory process. Platelets initiate and coordinate the normal wound healing responses, but they also play unintended roles in carcinogenesis. Tumors that upregulate complement, angiogenesis, adhesion molecules, EMT, and stromal pathways trigger inadvertent carcinogenesis associated with chronic inflammation that can be prevented by aspirin-mediated elimination of PG synthesis. M1/M2 (previously designated N1/N2) describes the two major and opposing activities of macrophages. M1 activity inhibits cell proliferation and causes tissue damage while M2 activity promotes cell proliferation and tissue repair. Abbreviations: COX-2, cyclooxygenase 2; EMT, epithelial mesenchymal transition; PG, prostaglandin; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; PMN, polymorphonuclear leukocyte; TGF-β, transforming growth factor-β; TxA_2, thromboxane; VC, vessel contraction; VD, vessel dilation. Figure adapted with permission from Reference 102.

can be heavily influenced by platelets since it is often thought of as a wound that does not heal (101). Eicosanoid metabolism is dynamic and often provides acute triggers during the wounding response (102). This is particularly true of platelets coupled with tumor-intrinsic responses (85, 102). Rapid eicosanoid release and cognate receptor engagement have a profound effect on not only the spread of cancer but also immune responses that enable a cancerous lesion to heal or remain in a wounded or immunosuppressive state (84, 102).

These eicosanoid microenvironmental immune changes can extend to colitis, particularly when there are adverse microbiome influences (103). Interestingly, platelet-specific deletion of COX-1 in megakaryocytes/platelets recapitulates the human pharmacodynamics of low-dose aspirin and ameliorates dextran sulfate sodium–induced colitis in mice (104). In another mouse study, aspirin also downregulated the expression of the checkpoint protein programmed cell death protein-1 in macrophages and $CD8^+$ T cells from the colonic mucosa (105). This study also showed that aspirin treatment can activate resolution pathways to reprogram T cell and macrophage responses in colitis-associated CRC (105). In a chemically induced model of colitis, PGE_2-EP_2 signaling functions to promote chronic inflammation, which shapes the tumor microenvironment in CRC (106). Another study showed that COX-2, inducible nitric oxide synthase (iNOS), the active form of Yes-associated protein 1 (YAP1), and cytosolic high mobility group box 1 (HMGB1) were strongly expressed at colorectal tumor sites and clearly suppressed by aspirin (107). As part of the Nurses' Health Study, frequent use of NSAIDs but not aspirin seemed to be associated with increased absolute incidence of Crohn's disease and ulcerative colitis (108). In a separate single-center analysis of 174 patients, aspirin use did not impact major clinical outcomes in patients with inflammatory bowel disease (109). Although the effect of aspirin use on mucosal inflammation was not directly assessed in this study, the findings support the safety of daily aspirin use in this population (109). PGE_2 served as a biomarker of response to aspirin in individuals with colorectal adenomas in a randomized clinical trial (110). In the case of Lynch syndrome, naproxen chemoprevention was shown to promote immune activation in the colorectal mucosa (111). Extending these immune activation efforts, transcriptomic-assisted immune and neoantigen profiling was performed in premalignancy as a key step toward better targeting and vaccine synthesis (112).

From a tumor immune microenvironment perspective, PGE_2 helps to influence the local immunosuppressive tumor microenvironment along with systemic responses (84, 102). PGE_2 has an

effect on all of the critical cells found in the tumor immune microenvironment, including various immunosuppressive cells, T cells, antigen-presenting cells, and innate immune cells (59). There may also be key contributions of any resident immune cells, depending on whether a lesion is in the primary or metastatic site, which can have a critical impact on initiating a localized primary response or systemic recruitment response (61). Considering the influence of PGs on the immune system along with the direct effects on tumor cells and platelets sheds more light on the potential depth of bioactive lipid-driven mechanisms. When PGE_2 is released, it can recruit myeloid-derived suppressor cells (MDSCs) into the tumors by activating CXC chemokine receptor 2 (CXCR2) signaling (59). Upon infiltration, MDSCs participate in cancer progression, as these cells can inhibit $CD8^+$ T cell–mediated cytotoxicity once within the tumor (59). PGE_2, as a mediator of both inflammation and cancer, also suppresses dendritic cell (DC) differentiation, and this, combined with inducing MDSC function, promotes tumorigenesis. In other mouse models, the use of COX-2 inhibitors modulated MDSC functions, blocked tumor growth (59), inhibited PGE_2 synthesis, and delayed tumor progression (113). More selective EP_2 and EP_4 antagonists were shown to prevent the differentiation of MDSCs and inhibit tumor progression (114). PGE_2 also suppresses $CD8^+$ T cell proliferation, cytotoxicity, and interferon-γ release. PGE_2 initiates DNA methyltransferase alpha (DNMT3A)-dependent tolerogenic functions in human MDSCs in tumors (59).

The effects of PGE_2 are based on the function of various immune cells (59). PGE_2 is inhibitory toward immature B cells, induces apoptosis in immature thymocytes, and promotes regulatory T cell development that selectively alters immune cell populations (59). Elevated circulatory PGE_2 levels can also affect T cell signaling responses. Activation of EP_2 and EP_4 signaling pathways by PGE_2 was shown to result in an increase in programmed cell death protein-1 (PD-1)-mediated immune tolerance in the tumor microenvironment. Similarly, PGE_2 increased programmed cell death ligand 1 (PD-L1) in tumor-infiltrating myeloid cells and immune evasion.

When natural killer (NK) cell function was examined, tumor-derived PGE_2 blocked early activation of NK cells and interfered with subsequent adaptive immune cell recruitment to the tumor (115). PGE_2 also caused cross talk with cytotoxic $CD8^+$ T cells and other immune-suppressive cells (116). PGE_2 can also directly suppress NK cell function (117). NK cell suppression was mediated by EP_2 and EP_4, suggesting that NK cell activity can be reestablished by specific receptor antagonists.

Regulatory T cells (Tregs) ensure the control of self-tolerance and are currently used in clinical trials to alleviate autoimmune diseases and graft-versus-host disease after hematopoietic stem cell transfer (118). Based on CD39/CD26 markers, blood natural Treg analysis revealed the presence of five different cell subsets, each representing a distinct stage of maturation when used to monitor chronic inflammatory diseases (59). PGE_2 also modulates Treg activity, leading to immunosuppression (59). One group found that transforming growth factor-β (TGF-β)-induced Foxp3 expression and inhibitory Treg differentiation were significantly inhibited by PGE_2 activation of EP_2 and EP_4 (74). Furthermore, the infiltration of immunosuppressive Tregs can be reduced by COX-2 inhibitors or EP_1, EP_2, and EP_4 antagonists (59).

DC function is complex and can be regulated by a variety of factors. Immature DCs and tolerogenic DCs are essential for the induction and maintenance of peripheral tolerance. Immunomodulatory factors, including PGs, help to imprint a protolerogenic, maturation-resistant state in DCs (59). The behavior of DCs can be altered by a variety of PGs. The PG I2 analog, iloprost, for example, when used to treat DCs, promotes antigen-specific Treg differentiation in mice (59). Similarly, PGE_2 can be used as part of an IL-6-, IL-10-, TGF-β-, and glucocorticoid-containing cocktail to differentiate peripheral blood mononuclear cell–derived monocytes (59). Aspirin alters the induction of tolerance by DCs in not only tumor tissues but also vascular

tissue autoimmune responses in atherosclerosis (119). Aspirin can also modulate DC activity and recruitment into inflamed tissue via prostaglandin D2 (PGD_2) (120). PGE_2 modulates DC functions along with their differentiation, maturation, and ability to secrete cytokines, which could be reduced with aspirin (121). In the case of macrophages, plasticity is altered by PGE_2 (59, 74). The conversion of M1 (inflammatory macrophages) to M2 (immunosuppressive macrophages) has been observed in various tumor types (59). Aspirin has been shown to alter the M2 macrophage status in a variety of tumors and preclinical models (122). Aspirin can also induce lipoxins and their 15-epimers (59). Aspirin-triggered lipoxins trigger potent anti-inflammatory and pro-resolution effects in tumors (123). Aspirin-triggered lipoxin A4 selectively programs the formation of M2 tumor-associated macrophages and thereby controls tumor progression (124).

It is clear that aspirin has multiple effects on the biologic and immune tumor microenvironment. With the advent of leading-edge sequencing technologies, new insights are emerging regarding the immune biologic changes caused by aspirin-mediated acetylation reactions (56). New technologies include single-cell sequencing, positional transcriptomics, and modern multiplex immunohistochemistry.

PHARMACOLOGY, BIOLOGY, AND ASPIRIN CHEMOPREVENTION

The summary provided above emphasizes the complexity of aspirin biochemistry and its effects on pathways that may impact cancer at various stages, including initiation, promotion, and progression. New clinical data emphasize this complexity, and the key question regarding aspirin use may not be whether the literature supports its role in cancer chemoprevention but how to best select those who will most benefit from aspirin use with the least harm. Newly modified recommendations from the USPSTF (see above) translate ambiguities and unanswered questions related to impact dose, timing, and duration of use necessary for chemoprevention into the recommendation that the evidence is currently inadequate that low-dose aspirin use reduces CRC mortality (5, 6). This does not mean that aspirin is not an effective chemopreventive agent but that, if this is offset by substantial risks, overall mortality may be unaffected or even higher in some individuals. A risk-stratified approach is therefore warranted (125).

Several studies indicate that the chemopreventive effects of aspirin do not become evident for at least 10 years after initiation, while others suggest that, at least in some individuals, an effect may be evident sooner. Remote use and use within the previous 10 years appear to contribute independently to decreased risk (126). Benefit and risk may depend, for example, on the timing of aspirin use based on age. The ASPirin in Reducing Events in the Elderly (ASPREE) randomized controlled trial in healthy older adults reported that low-dose (100 mg) aspirin may be associated with increased all-cause mortality and had an adverse effect on later stages of cancer evolution (127). This trial also highlighted the risk of hemorrhage in this older age group (HR = 1.38, 95% confidence interval 1.18–1.62) (128). Another analysis of individual patient data from randomized trials also suggested that aspirin use may be associated with an increase in cancer incidence in subjects at least 70 years of age (129). In a recent clinical practice update and expert review of chemoprevention for colorectal neoplasia (130), the American Gastroenterological Association advised that individuals at average-risk for CRC use low-dose aspirin to reduce CRC incidence and mortality if they are younger than 70 years, have a life expectancy of at least 10 years, have a high CVD risk (10-year risk of at least 10%), and are not at high risk for bleeding. Individuals with a history of CRC were also advised to consider aspirin to prevent recurrent colorectal neoplasia. It should be noted that these recommendations preceded new recommendations from the USPSTF.

The benefits and risks of aspirin use according to dose have also been debated. Several trials suggest that low-dose aspirin (81–100 mg) may be sufficient for chemoprevention, but other trials utilized a standard aspirin dose (325 mg). Few compare different doses. At least one trial with

adenoma recurrence as the end point found an effect with low- but not standard-dose aspirin use (7). Recent data suggest that factors such as height, weight, and body mass index (BMI) may impact the bioavailability of aspirin, and that BMI may have a modifying effect on efficacy (129, 131). Increased platelet turnover associated with diabetes in high-BMI individuals may lead to unblocked platelets in the circulation when a once-daily aspirin is initiated (132).

The end point in chemoprevention trials is also important to consider. Most short-term colorectal neoplasia trials, for example, use new adenoma formation after previous polypectomy in adenoma-bearing subjects or those with CRC (7) as the end point. The recently published Systematic Evaluation of Aspirin and Fish Oil (Seafood) Polyp Prevention trial found that aspirin use alone or in combination with eicosapentaenoic acid was not associated with a reduction in adenoma detection rate in subjects with a previous history of high-risk adenomas but did result in a statistically significant decrease in the number of conventional colorectal adenomas and a decrease in right-sided and serrated lesions (133).

Aspirin's beneficial effects likely result from multiple interrelated mechanisms discussed above, including effects on COX-1 and COX-2, 15-PGDH, WNT-β-catenin signaling, inflammatory and immune responses, and platelet-mediated effects. Indeed, these pathways may provide markers for risk stratification of who will most benefit from aspirin use. ASPIRED is a prospective, double blind, randomized trial of aspirin usage in individuals previously diagnosed with colorectal adenoma that will examine a variety of related pathways and investigate putative mechanistically based risk-stratification biomarkers (134). This study demonstrated that the major urinary metabolite of PGE_2, PGE-M, is a biomarker for CRC that is modifiable by aspirin (110). Aspirin confers a significant reduction in the risk of cancers that overexpress COX-2 but not in those with negative or low expression (135). Advances in understanding how the COX-2-PGE_2 pathway effects tumor immune evasion, and how this may be affected by aspirin, is also an area of active investigation (59). One area deserving investigation is the possible role of the gut microbiome, which may indirectly effect PGE-mediated effects such as proliferation and stem cell renewal (136).

Aspirin benefit may also depend on a variety of other genetic factors. Variations in the molecular properties and biological influences of various drugs may be the result of significant differences in the consensus molecular subtype properties associated with a given precancer or cancer (137–139). In the case of colorectal precancers and cancers, four primary molecular subtypes exist: CMS1 (microsatellite instability immune, 14%) is hypermutated, is microsatellite unstable, and has strong immune activation; CMS2 (canonical, 37%) is epithelial and has marked WNT and MYC signaling activation; CMS3 (metabolic, 13%) is epithelial and has evident metabolic dysregulation; and CMS4 (mesenchymal, 23%) has prominent TGF-β activation, stromal invasion, and angiogenesis. Aspirin use is associated with a lower risk of *BRAF* (a proto-oncogene in the MAPK signaling cascade) wild-type CRC but not *BRAF* mutant CRC (140, 141). Aspirin use is also associated with improved survival among individuals whose established tumors have mutations in *PI3CA* (encoding the PI3K catalytic subunit-α) but not in those with wild-type *PI3CA* cancers (142).

The weight of the evidence discussed above suggests that aspirin is indeed a promising chemopreventive agent for at least some types of cancer and that we should not throw the baby out with the bath water. A rational approach to the use of aspirin for chemoprevention requires an understanding of a complex matrix of benefits and risks that is evidence-based and may be incorporated into personalized care.

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