'Angioprevention': angiogenesis is a common and key target for cancer chemopreventive agents

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ABSTRACT **The potential to block tumor growth by inhibition of the neoangiogenic process represents an intriguing approach to the treatment of solid tumors. The high proliferation rate in the tumor deprived of proper vascularization would be balanced by cell death due to lack of diffusion of nutrients and oxygen. Matrix metalloproteinases (MMPs), angiogenic growth factors, and their receptors are the main targets of an increasing number of clinical trials approved to test the tolerance and therapeutic efficacy of antiangiogenic agents. We observed that a series of substances proposed as possible cancer chemopreventive agents show antiangiogenic properties when tested in in vitro and in vivo angiogenesis models. We demonstrated that *N*-acetyl-L-cysteine is able to reduce the invasive and metastatic potential of melanoma cells, and to inhibit endothelial cell invasion by direct inhibition of MMP activity. We also showed that epigallocatechin gallate (EGCG), a flavonoid from green tea that possesses chemopreventive activity in experimental and epidemiological studies, is a potent inhibitor of MMP-2 and MMP-9. Angiogenesis has also been demonstrated to be a target for nonsteroidal anti-inflammatory drug chemopreventive activity. Based on these data, we hypothesize that other chemopreventive agents, including natural or synthetic retinoids, steroid hormone antagonists, peroxisome proliferator-activated receptor γ ligands, vitamin D, and protease inhibitors, might have antiangiogenesis as an important mechanism of action, a novel concept we will term 'angioprevention'. We analyze the mechanisms on how and why chemopreventive agents could exert antiangiogenic effects aimed at controlling tumor growth, and their potential use in the clinic.—Tosetti, F., Ferrari, N., De Flora, S., Albini, A. 'Angioprevention': angiogenesis is a common and key target for cancer chemopreventive agents. *FASEB J.* 16, 2–14 (2002)**

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FOREWORD: INHIBITION OF NEOANGIOGENESIS BY CHEMOPREVENTIVE AGENTS

FOR HALF A century biomedical research has established new tools for cancer diagnosis and treatment. Nevertheless, today when a solid tumor enters the metastatic phase, the chance of recovery for the patient still drops dramatically. Whereas evident tumor masses can be eradicated by surgical intervention, small primary tumors and micrometastases must be fought by noninvasive therapies. Many clinical protocols rely on very aggressive approaches based on high-dose irradiation or chemotherapy. However, both methods show only partial efficacy on certain tumor types, generally with severe side effects. The critical role of tumor angiogenesis in cancer progression was postulated ~30 years ago in pioneering studies by Folkman et al. (1). It is now accepted that a tumor mass cannot exceed ~1 mm^3 in an avascular state. However, only in recent years has the knowledge of endothelial cell physiology and tumor angiogenesis provided the necessary background to develop effective antiangiogenic strategies (reviewed in refs 2, 3). The endothelial cell represents a preferential target for therapy, as it is a cell type common to all solid tumors. Even though every cancer is virtually a unique disease, the tumor endothelium is a relatively uniform, normal cell type. Cancer cells are able to produce several angiogenic factors including basic-fibroblast-like growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), transforming growth factor-β (TGF-β), and others that cause endothelial cell recruitment and proliferation (reviewed in ref 4). These stimuli are constantly present so that the differentiation of the tumor endothelium into a mature vessel network is rarely complete, and tumor vessels show an abnormal morphology. These patterns suggest it may be possible to specifically target tumor angiogenesis by inhibiting endothelial cell recruitment by the tumor and its proliferation.

Another advantage of the antiangiogenic approach is the apparent inability by the endothelial cell to counteract therapy through development of multi-drug resistance mechanisms, due to the low mutagenesis rate of this normal cell type (5). The block of tumor growth requires a chronic inhibition of vascular recruitment, defined by Hanahan and Folkman as 'angiostasis' (6);

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thus, long-term treatment is necessary. Therefore, we need to identify and characterize angiostatic molecules endowed with low or no toxicity.

During the last 15 years substantial effort has been dedicated to identifying natural and synthetic compounds that can be used to either prevent insurgence of primary tumors in subjects at high risk to develop cancer or prevent tumor relapse after surgical removal. Cancer chemoprevention is the use of agents to slow the progression of carcinogenesis, reverse, or inhibit it, with the aim of lowering the risk of developing invasive or clinically significant disease (7, 8). Chemopreventive drugs must be devoid of toxicity and well tolerated since they must be used over extended periods. Most chemopreventive agents discussed here are either components of common food and beverages or are pharmacological agents used extensively for clinical applications other than cancer. It is therefore likely that these agents can elicit chemopreventive effects in a primary prevention setting, when small primary tumors tend to reach the critical size that requires neovascularization in apparently healthy individuals. Inhibition of neoangiogenesis is usually envisaged as a strategy either to block and reverse the progression of a diagnosed premalignant tumor in a secondary prevention setting, or to pursue the regression of the disease in cancer patients in a therapeutic setting. Antiangiogenic agents can prevent the further growth of micrometastases in a tertiary prevention setting. However, there is increasing evidence that the 'angiogenic switch', defined as the point at which a tumor induces angiogenesis, occurs very early in tumorigenesis in both murine models and human tumors (reviewed in refs 6, 9), and that early intervention can curtail tumor growth (see ref. 9). So we propose that antiangiogenesis also applies to primary prevention, limiting the expansion of hyperplastic foci and subsequent tumor development (**Fig. 1**).

Prominent chemopreventive drugs with well-established effects appear to aim at maintenance of the differentiated, nonproliferative state of the target cells, as is the case for the application of tamoxifen and/or the synthetic retinoid 4-hydroxyphenylretinamide (4-HPR) in the secondary prevention in women with breast cancer (10), or at anti-inflammation to reduce local proliferative stimuli, as it is the case for nonsteroidal anti-inflammatory drugs (NSAIDs) for colon cancer (11). However, the preventive effect may also be mediated by other, unknown actions of the drugs.

We have noticed that several agents shown to have chemopreventive activity in experimental test systems or clinical trials also show significant antiangiogenic activities. Similarly, Kerbel defined 'accidental' antiangiogenesis as the potential antiangiogenic properties of known chemotherapeutic agents or anti-oncoprotein signal transduction inhibitors such as Herceptin that were not originally intended to inhibit angiogenesis (12). *N*-acetyl-L-cysteine, epigallocatechin-3-gallate and flavonoids, NSAIDs, and retinoids such as 4-HPR all show evidence of direct antiangiogenic effects whereas other chemopreventive molecules such as tamoxifen or finasteride could indirectly interfere with angiogenesis by counteracting the proangiogenic effects of their hormonal counterparts. Preliminary studies have also indicated that diverse chemopreventive agents may interfere with common pathways in the angiogenic cascade, as is the case of NSAIDs and *N*-acetyl-L-cysteine (NAC) inhibition of cyclooxygenase-2 (COX-2) (11, 13). Inhibition of matrix metalloproteinases (MMPs), required for endothelial cell invasion, also appears to be a common target of active chemopreventive agents (14). Regulation of angiogenic growth factors or signal transduction mechanisms associated with inhibition of angiogenesis may also be involved (15).

We hypothesize that among the many mechanisms of inhibitors of carcinogenesis, the antiangiogenic activity of the chemopreventive compounds may actually be a common and critical effect for inhibition of cancer by these agents through blocking or retarding the development of the tumor vasculature. We thus propose the novel concept of 'angioprevention', assuming this is a common theme of many chemopreventive molecules. Although etymologically approximate, this term provides an immediate perception that inhibition of neoangiogenesis can be applied not only to cancer therapy but also to prevention of neoplastic mass progression.

This review analyzes the mechanisms of action of the individual chemopreventive agents more systematically, in an attempt to group them into classes based on their mechanism of action in an antiangiogenic setting, and

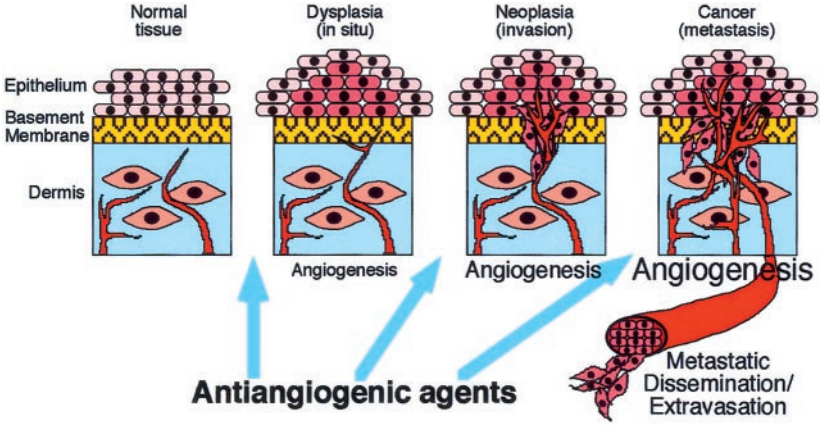

Figure 1. Chemopreventive agents can interfere with the angiogenic process at different stages of tumorigenesis. The 'angiogenic switch' that occurs early during the preneoplastic stages could be curtailed by 'angioprevention' agents before phenotypic and molecular changes result in the progression from dysplasia to invasive cancer.

to evaluate the hypothesis that common gene pathways are targeted by antiangiogenic chemopreventive drugs.

THIOLS

N-acetyl-L-cysteine

Modulation of extracellular and intracellular thiols is being investigated as a promising strategy in cancer prevention. Widely used in the clinic as a mucolytic, antioxidant, and antidote for acute intoxication, NAC is one of the most extensively studied modulators of thiol levels (13). The protective effects of NAC in preclinical studies of carcinogenesis have been reported to be linked to its antigenotoxic activity associated with nucleophilicity and antioxidant properties. It also possesses a very broad array of mechanisms working along all stages of the carcinogenesis process. The antioxidant activity of NAC is not limited to the ability of this molecule and its derivatives to act as scavengers of reactive oxygen species (ROS) (13), but includes inhibition of the COX-1-mediated activation of carcinogens (16) and inhibition of COX-2 expression (17). The role of COX activity in angiogenesis and carcinogenesis will be discussed in the sections on NSAIDs and retinoids.

In preclinical studies, NAC has been shown to inhibit initial tumor take, tumor cell invasion in vitro, and metastasis formation in vivo (18). Different classes of proteases, including MMPs and their inhibitors, have been shown to be important in tumor invasion and angiogenesis (19). NAC is able to cause inhibition of secreted MMP-2 and MMP-9, the type IV collagenases typically overexpressed by tumors and activated endothelial cells involved in invasion. Recent studies have indicated these effects may be due to blocking angiogenesis, again through inhibition of MMPs (20). These same proteases are also expressed by activated endothelial cells, with the process of tumor cell invasion and activated endothelial cell invasion during angiogenesis being very similar (21). NAC indeed inhibited endothelial cell invasion via MMP inhibition at the protein level by directly blocking enzymatic activity in zymography assays and inhibiting activation of the MMP by removal of its propeptide (20). This block could also be caused by a competitive affinity of NAC for the zinc (Zn) ion necessary for enzymatic function. In fact, all MMPs have at least one Zn atom in their catalytic site, and NAC could sequester Zn by using its free sulfhydryl group. Moreover, NAC may also affect MMP transcription, as it has been shown to decrease c-*fos* and c-*jun* induction (22) and to inhibit activation and binding activity (23) of activator protein 1 (AP-1). AP-1 is a heterodimeric complex formed of c-*fos* and c-*jun* gene products that binds the promoter region of intermediate genes required for cell division and other cell functions, including MMP transcription.

As tumor endothelial cells need VEGF to survive, NAC could also affect angiogenesis by modulating VEGF expression by tumor cells. NAC has recently been shown to suppress VEGF release from ras-transformed tumor cells (24). We also have evidence that NAC alters the VEGF release by tumor cells (unpublished results). These effects may be due to NAC-promoted GSH scavenging of ROS that appear to act as oxygen tension sensors (25) and induce VEGF expression (26).

Down-regulation of VEGF by NAC has been shown in other laboratories (27, 28). Since NAC as a reducing agent modifies the intracellular redox state, its presence could affect mitochondrial activity and therefore oxygen sensitivity of endothelial and tumor cells (13). NAC has been shown to modulate a wide variety of gene expression and signal transduction pathways (reviewed in ref 13).

The observation that NAC may have an antiangiogenic activity suggested it may also be able to inhibit further growth and expansion of established tumors that depend on induction of vascularization for growth (6, 9).

POLYPHENOLIC COMPOUNDS

Flavonoids

Flavonoids are the most abundant polyphenols in our diet. They are natural estrogenic compounds derived from soybeans, tea, fruits, and vegetables and have been proposed to act as chemopreventive agents in Asian populations (29, 30).

Soy products reduced angiogenesis, increased apoptosis, and slightly reduced proliferation in transplantable murine bladder cancer in vivo (31). To determine whether prevention might be associated with dietary-derived angiogenesis inhibitors, Fotsis and coauthors fractionated urine samples of healthy human subjects consuming a plant-based diet and examined the fractions for their abilities to inhibit the proliferation of vascular endothelial cells (32). The most potent fractions contained several isoflavones, including genistein, which was the most effective inhibitor of endothelial cell proliferation and in vitro angiogenesis.

The isoflavone genistein is a potent inhibitor of tyrosine kinases (33) and, along with flavonoids such as kaempferol and apigenin, is an inhibitor of topoisomerases I and II, enzymes crucial to cellular proliferation (29). Shao and co-workers demonstrated that genistein inhibited invasion in vitro of MCF-7 and MDA-MB-231 breast carcinoma cells by down-regulating MMP-9 and up-regulating the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), and inhibited angiogenesis by decreasing vessel density and levels of VEGF and TGF-β1 (34). These in vitro findings were confirmed in vivo in nude mouse xenografts (34). Data in the literature report increased angiogenesis in the bone marrow of patients with acute myeloid leukemia (35) and B cell chronic lymphoblastic leukemia (36). When used in the clinic, treatment of therapy refractory B-lineage acute lymphoblastic leukemia with B43-genistein elicited objective responses at nontoxic dose levels (37).

Flavonoids are not the only active compounds with chemopreventive properties found in soy products. Several protease inhibitors derived from soybeans, including the Bowman-Birk serine protease inhibitor, possess anticarcinogenic and anti-inflammatory activity (reviewed in refs 38, 39). As will be discussed later, by inhibiting basement membrane invasion, protease inhibitors could synergize with the antiangiogenic potential of soy isoflavones.

Silymarin, another naturally occurring antioxidant flavonoid, exhibits anticancer effects against several epithelial cancers and has shown potential as an antiangiogenic agent in studies using HUVEC and human prostate or breast cancer epithelial cells (40). The inhibitory effects of silymarin on COX-2 and IL-1α should be explored to develop preventive strategies against those cancers in which these molecular targets play one of the causative roles, such as nonmelanoma skin, colon, and breast cancers in humans (41).

There is a strong possibility that prostatic intraepithelial neoplasia lesions that switch to the angiogenic phenotype eventually progress to cancer (42). In vitro studies demonstrated that genistein, silymarin, and epigallocatechin-3-gallate (EGCG) inhibit mitogenic signaling pathway(s) and alter cell cycle regulators, albeit at different levels, leading to growth inhibition and death of advanced and androgen-independent prostate carcinoma cells (43). Reduction in serum prostate-specific antigen (PSA) levels has been proposed as an end point biomarker for hormone refractory human prostate cancer intervention. However, PSA itself may represent an endogenous antiangiogenesis molecule (44) that can produce angiostatin (45).

EGCG

Epidemiological studies have shown a lower incidence of esophageal (46) and breast cancer (47) among regular drinkers of green tea. Recently, the clinical efficacy of green tea in preventing gastric cancer, though suggested by preclinical and case control studies (48), has been questioned by a prospective cohort study conducted in Japan that showed no association between green tea consumption and the risk of gastric cancer (49), indicating that the effect might be relevant on specific neoplasms. Nevertheless, the beneficial effects of green tea and its active components have been abundantly documented in the literature and include cancer chemoprevention, inhibition of tumor cell growth, invasion, and metastasis, and antiviral and anti-inflammatory activities (50). Green tea contains numerous polyphenols, most of which are flavonols commonly known as catechins, that show a strong antioxidant activity. Polyphenols are mostly lost through oxidation during the production of black tea. Polyphenols interact with ROS and have high complexation affinity to some metal ions and biological molecules (51). Generation of the serine proteinase plasmin can be catalyzed by two serine proteinases: the urokinase- and tissue-type plasminogen activators (uPA and tPA). uPA, one of the hydrolases implicated in degradation of extracellular matrix and tumor invasion, is directly inhibited by EGCG, the main flavonol found in green tea extracts (52). However, EGCG plasma concentrations (0.1–0.3 μM) after moderate green tea consumption (two or three cups) are much lower than those required for uPA inhibition (IC_{50}=4 mM). Recent in vivo experiments show that these plasma levels are instead sufficient to inhibit angiogenesis (53). We have in fact demonstrated that EGCG appears to act as a direct inhibitor of MMP-2 and, with slightly lower efficacy, of MMP-9 (14, 54). This activity of EGCG is observed at very low doses corresponding to plasma levels detected in green tea drinkers (0.1–0.3 μM). Further evidence to support the antiangiogenic activity of EGCG emerged in a recent study demonstrating that this compound also inhibits in vivo growth and angiogenesis of tumors derived by the colon carcinoma HT29 cell line by blocking the induction of VEGF (55). EGCG has recently been shown to act as a potent inhibitor of the chymotrypsin-like activity of the proteasome, which could further contribute to its preventive anticancer activity (56). Another effect of EGCG, which has been tested in a mouse model of UVB-induced photocarcinogenesis (57), is the elevation of IL-12 in draining lymph nodes. IL-12 is an antitumoral and antiangiogenic molecule that is capable of activating an antiangiogenic program in mouse spleen and human peripheral blood mononuclear cells (58) and to block neovascularization in human Burkitt's lymphoma, colon carcinoma, and ovarian carcinoma (59). When administered to NOD/SCID mice transplanted intraperitoneally with human non-Hodgkin's lymphoma (NHL) cell lines, green tea inhibited by 50% tumor growth by inducing apoptotic tumor and endothelial cell death (60).

Various cell signaling pathways involving mitogenic signals, cell cycle regulation, and modulation of the activity of transcription factors, including AP-1, NF-κB, and STAT1, are targeted by EGCG and, as mentioned above, by other polyphenols (61). Relevant in the context of the antiangiogenic properties of EGCG is its selective inhibitory effect on the activity of receptor tyrosine kinases that play a regulatory role in angiogenesis, including EGF-R (62), PDGF-receptor beta, and phosphatidylinositol-3 (PI-3) kinase, and on the downstream p44/p42 MAP kinase pathway in vascular smooth muscle cells (63). Heregulin β1, the ligand of the erbB-3 and erbB-4 tyrosine kinase receptors, has been demonstrated to increase VEGF expression and secretion in cancer cells and to stimulate angiogenesis (64).

NSAIDs

NSAIDs are effective colon cancer chemopreventive agents that might also be useful in preventing other types of cancer (65). Recent reports indicate that NSAIDs inhibit tube formation by endothelial cells in

in vitro models of angiogenesis (11, 66). The mitogen/cytokine-inducible cyclooxygenase isoenzyme COX-2, involved in prostaglandin (PGE) generation from arachidonic acid, has been shown to regulate angiogenesis induced by colon cancer cells in a coculture model of colon carcinoma and endothelial cells, where the selective COX-2 inhibitor NS-398 inhibited the production of angiogenic factors by colon carcinoma cells (66). In a study by Jones et al. (11), the most effective antiangiogenic compound was again NS-398. NS-398 also inhibited angiogenesis and in vivo growth of tumors derived by the PC-3 human prostate cancer cell line (67).

The antiangiogenic effect of the selective COX-2 inhibitor celecoxib has been demonstrated in a rat model of angiogenesis (68). COX-2 overexpression seems to occur first in stromal cells, and results in angiogenic growth factor release that is capable of inducing endothelial cell proliferation in a paracrine fashion (69). COX also provides a superoxide radical-generating pathway (70) and could contribute in part to the establishment of a more oxidized intracellular environment, which in turn can stimulate proliferation of tumor cells (71). Inhibition of angiogenesis by NSAIDs apparently follows more than one pathway, prostaglandin dependent and independent. All types of NSAIDs inhibit the mitogen-activated protein (MAP) kinase Erk2 (11). Early angiogenic stimuli also use the MAP kinase pathway (72), which in turn can lead to activation of nitric oxide synthase (NOS) (73). Although the inhibitory effects of NO on tumorigenesis have been associated with an antiangiogenic effect (74), the importance of the different isoforms of NOS for tumor vascularization is not yet clear. Many angiogenic molecules also stimulate NOS activity (75); endothelial NOS has been shown to play an essential role in VEGF-induced angiogenesis (76).

Nuclear localization of MAP kinase is essential for its function, and NSAIDs interfere with ERK-2 nuclear translocation (11). However, the antiangiogenic effect of NSAIDs may not to be limited to inhibition of COX-2 and PGE synthesis. Future detailed analysis of the antiangiogenic effects of NSAIDs could be interesting for chemoprevention of those cancers in which COX-2 overexpression is observed.

NATURAL AND SYNTHETIC STEROID RECEPTOR SUPERFAMILY LIGANDS

Retinoids

Like steroids and vitamin D, retinoids exert most of their effects by regulating gene expression through specific receptors belonging to the steroid/thyroid hormone superfamily. Chemopreventive protocols using these drugs target tissues that express the corresponding receptors.

Carotenoids, retinol, and retinoids are fundamental regulators of cell growth, differentiation, and development. Retinoids bind and activate their nuclear receptors, the RARs and RXRs. They enhance apoptosis and prevent the conversion of in situ cancer to locally invasive malignancy by suppressing the invasive process (77). Retinoids have been reported to be active in treating specific premalignant lesions and reducing the incidence of second primary tumors in patients with prior head and neck, lung, or liver cancer (8). However, a carotenoid (β-carotene) was shown to significantly increase the mortality for primary lung tumors in smokers and asbestos-exposed individuals (78). This underscores the need for relevant experimental models to identify pathways signaling chemopreventive effects.

All-*trans*-retinoic acid (RA) has shown antiangiogenic effects in several systems. In one study, it caused the endothelial cells of large and small vessels to become refractory to stimulation of migration by tumor-conditioned media or purified angiogenic factors (α-fibroblast growth factor, vascular endothelial growth factor, TGFβ-1, and IL-8) without affecting cell proliferation (79). Rats given all-*trans*-RA were unable to mount an angiogenic response to tumors implanted in their corneas (80). These results indicated that all-*trans*-RA can directly affect both tumor and endothelial cells and thereby suppress the formation of new blood vessels in vivo.

13-*cis*-RA was found to synergize with interferons in the inhibition of angiogenesis in experimental models of Kaposi's sarcoma tumors (81) and breast and vulval carcinomas (82).

9-*cis*-RA, which binds and activates RARs and RXRs, showed antiangiogenic activity synergistic with interferon α in a experimental mouse model of tumor-induced angiogenesis (83).

Retinoid receptors can regulate gene expression by interacting directly with cognate response element in target gene promoters, but they can also cross-talk with other signaling pathways. All-*trans*-RA interferes with AP-1 signaling, a key regulatory pathway in angiogenesis. Transcriptional interference is not restricted to AP-1 but can involve other transcription factors such as NF-κB (84) and CCAT/enhancer binding protein beta (85). Examples of genes that have been shown to be suppressed by all-*trans*-RA through antagonism of AP-1 include VEGF (86), stromelysin (87), collagenases (88), and TGF-β (89). Retinoids also exert transcriptional repression on interstitial collagenase (90). Although retinoids are inhibitors of in vitro angiogenesis, they have distinct effects on the plasminogen-dependent proteolytic system: all-*trans*-RA and 9-*cis*-RA increase u-PA activity in human microvascular endothelial cells (91), and t-PA synthesis is induced in the absence of altered PAI-1 synthesis in HUVEC cells exposed to *all-trans* RA (92). In a defined cell-free system, plasminogen activators (uPA and t-PA) have been shown to generate angiostatin, a naturally occurring inhibitor of angiogenesis, from plasminogen (93). TIMPs are also angiogenesis inhibitors: retinoids positively modulate endothelial cell production of TIMP-1 and TIMP-2 (94). COX-2-derived PGEs contribute to tumor growth

by inducing newly formed blood vessels; retinoids suppress the basal expression and EGF- or TPA-mediated induction of COX-2 in human oral squamous carcinoma cells (95). The recent discovery of STAT1 as a novel retinoid-regulated gene (96) supports further evidence for the interference of retinoids with signaling pathways involved in angiogenesis.

The future in this area will depend on the development of new agents whose mechanism of action is well understood and that show increased preventive or therapeutic efficacy, and less toxicity, than the parent natural compounds.

Rexinoids such as SR11246, LG100268, and LGD1069 (Targretin or bexarotene) are RXR nuclear receptor selective ligands. Different from RARs, RXR can heterodimerize with partners of the steroid receptor superfamily, including vitamin D receptor (VDR) and peroxisome proliferator-activated receptor (PPAR). In preclinical studies of breast cancer chemoprevention, the RXR selective ligand LGD1069 showed evidence of activity on tamoxifen-resistant cancer (97), and a less toxic profile compared to RAR agonists in the *N*-methylnitrosourea (NMNU) -induced rat mammary carcinogenesis model, where it strongly inhibited tumor burden and tumor incidence (98). The antitumoral effects of LGD1069 have been related to inhibition of tumor angiogenesis (99). LGD1069 (bexarotene) is under evaluation in a phase II trial for the treatment of the highly vascular AIDS-associated Kaposi sarcoma. RXR synergistic or modulatory activity on other hormone receptors can influence the activity and toxicity of therapeutic ligands relevant in cancer prevention and cure.

The synthetic retinoid 4-HPR, or fenretinide, has been shown to prevent breast, prostate, and ovarian cancer in preclinical models and has been evaluated in clinical trials of cancer prevention (100, 101). Pienta et al. (102) using three different angiogenesis inhibition assays, demonstrated that 4-HPR inhibits angiogenesis as well as endothelial cell motility and tubule formation, thus providing a putative mechanism responsible for the proven chemopreventive effect of 4-HPR on prostate cancer development.

Cell–matrix interactions have been investigated in 4-HPR-treated BALB/c 3T3 cells whose invasive potential is lowered by 4-HPR (103). Preliminary experiments obtained in our laboratory in an in vivo angiogenesis assay show that 4-HPR significantly inhibits angiogenic growth factor-stimulated neovascularization. Based on these data, we hypothesized that the antitumor effect of 4-HPR could be due at least in part to its inhibitory effect on endothelial cell growth and tubular morphogenesis, thus preventing neoangiogenesis at an early stage of tumor development. 4-HPR acts through a mechanism involving ceramide biosynthesis in neuroblastoma cells (104) and, more generally, by mitochondrial damage in several tumor cell types. C2 ceramide has been demonstrated to be involved in detachment-induced endothelial cell apoptosis derived by αVβ3 and αVβ5 RGD binding integrins blockade, with consequent c-*jun* N-terminal kinase activation (105). 4-HPR action on endothelial cells has not been investigated at the molecular level, but 4-HPR-dependent long-term production of ROS in endothelial cells might also inhibit endothelial cell proliferation and neoangiogenesis. 4-HPR decreases cellular release of insulin-like growth factors (IGFI and II) and enhances insulin-like growth factor binding protein synthesis and secretion, thus reducing the circulating levels of the biologically active molecule (106), a potent proangiogenic growth factor (107). The networking pathways of IGFs and VEGF signaling, as shown by recent findings on IGF-I-regulated VEGF mRNA expression in NIH3T3 and endometrial adenocarcinoma cells (108), indirectly support a role for 4-HPR as an antiangiogenic agent.

PPARγ LIGANDS

Another member of the steroid hormone receptor superfamily, PPARγ, is activated by eicosanoids, including the natural ligand 15-deoxy-delta12, 14-prostaglandin J2 (15D-PGJ2), a prostanoid derived from the cyclooxygenase product PGD2, and by antidiabetic agents such as thiazoldinediones. PPARγ is a key transcription factor involved in adipogenesis and monocyte differentiation. The role of PPARs in cancer chemoprevention has recently been reviewed (see ref 109). PPARγ, activated by 15D-PGJ2 or by new antidiabetic agents (BRL49653 and ciglitazone) showed a potent antiangiogenic activity by inhibiting differentiation of HUVEC cells into tube-like structures in a tridimensional collagen matrix (110). VEGF receptors and uPA mRNA were decreased in the same cells whereas PAI levels were elevated. 15D-PGJ2 has been shown to induce endothelial cell apoptosis via a PPAR-dependent pathway involving nuclear translocation of PPAR as well as caspase activation (111). PPARγ ligands also inhibited choroidal neovascularization in response to VEGF (112). In addition, 15D-PGJ2 and cyclopentenone prostaglandins have been shown to inhibit the NF-κB-dependent transcription of target genes, including COX-2, by directly blocking IkappaB kinase in a PPARγ-independent manner (113, 114). Again, blockade of NF-κB signaling inhibits angiogenesis of ovarian cancer by suppressing the expression of VEGF and IL-8 (115). As the NF-κB signaling pathway appears to be a key regulatory pathway in inflammation and angiogenesis, this novel mechanism could enhance the antiangiogenic activity of COX-2 inhibitors.

Ligands for PPARγ have been shown to have marked synergism with rexinoids in the treatment of experimental diabetes. It seems reasonable to speculate that a similar synergism between the two classes of agents could be also found in chemoprevention and angioprevention of cancer.

STEROIDS

Angiogenic activity has been reported for ligands of the nuclear hormone receptor superfamily such as androgens and estrogens. Inhibition of the proangiogenic effects of estrogens could underlie the chemopreventive action of tamoxifen and selective estrogen receptor antagonists on mammary carcinogenesis. Recently, the molecular mechanism underlying angiogenic growth factors regulation by estrogens in the female reproductive tract has been elucidated by the finding that a functional estrogen responsive element is present in the promoter region of VEGF (116), and that estrogens regulate VEGF mRNA and protein expression in human breast cancer cells (117).

Tamoxifen and raloxifene both bind to the estrogen receptors ER-α and ER-β and can act as estrogen antagonists or agonists, depending on the target tissue, thus promoting the beneficial effects of estrogens on bone or suppressing cancer-promoting effects in the breast (8). Tissue-specific antiangiogenic/angiogenic effects of estrogen antagonists could also explain the increased incidence of endometrial cancer in tamoxifen-treated women. Further development of steroid receptor modulators devoid of angiogenic effects would be crucial to avoid the insurgence of secondary neoplasms in treated patients.

2-Methoxyestradiol (2-ME), an endogenous estrogen metabolite that disrupts microtubule formation, induces apoptosis in endothelial cells and inhibits angiogenesis by a mechanism involving stress-activated protein kinase signaling and modulation of Fas expression (118). In an animal model, 2-ME inhibited estrogen-induced pituitary tumor growth and angiogenesis, probably by down-regulating VEGF expression (119).

Hormonally regulated tissues such as the prostate are angiogenesis-dependent, and androgens have been studied extensively as regulators of prostatic angiogenesis (120). The ongoing large-scale Prostate Cancer Prevention Trial begun in 1997 is using finasteride as a chemopreventive agent (121). Finasteride, a testosterone analog, competitively inhibits the enzyme 5α reductase that converts testosterone into the more potent dihydrotestosterone. The use of finasteride in prostate cancer chemoprevention is based on the rationale that androgens promote prostate tumorigenesis (122). Several authors have reported studies of the effect of androgens on VEGF release. Recent work from the group of Folkman (123) reports that androgens modulate the angiogenic activity of hormone responsive human prostate cancer by regulating VEGF mRNA and protein expression. Androgen withdrawal also inhibits hypoxic induction of VEGF mRNA. In the case of prostate cancer, antioxidants such as vitamin E and selenium protect from tumor insurgence, suggesting that a combination of hormonal therapy and antioxidants could synergistically contribute to the antitumoral and antiangiogenic effect of the chemopreventive treatment (124).

VITAMIN D

Vitamin D (calciferol) and its analogs (deltanoids) are well-recognized regulators of cell proliferation and differentiation besides their classic function in mineral homeostasis. The active form of vitamin D, 1α,25-dihydroxy vitamin D_3, binds to the nuclear receptor VDR belonging to the steroid/thyroid hormone receptor superfamily, which can heterodimerize with the rexinoid RXR receptor.

The antitumoral effect of vitamin D has been studied in prostate (125) and breast cancer cells (126), where it induces cell death. Vitamin D_3 receptors have been identified on bovine aortic endothelial cells and human capillaries (127), where a 4.5-fold up-regulation of VDR is detectable in activated proliferating cells. Vitamin D_3 showed antiangiogenic properties in several cellular contexts, interfering with the action of angiogenic molecules. 1α,25-Dihydroxy vitamin D_3 has been shown to inhibit VEGF-induced endothelial cell sprouting and elongation in vitro due to induction of apoptosis and to reduce in vivo vascularization of tumors derived by MCF-7 breast carcinoma cells overexpressing VEGF (128). Angiogenesis was also inhibited by vitamin D_3 in a transgenic murine model of retinoblastoma, with the tumors from treated animals showing significantly lower vessel counts than controls (129). 1α,25-Dihydroxy vitamin D_3 has been shown to inhibit Kaposi's sarcoma (KS) cell growth in vitro by reducing the production of the angiogenic cytokines IL-6 and IL-8, which are autocrine growth factors for the highly vascular KS (130). This effect of vitamin D_3 is particularly relevant in that tumor cells in KS, which is a strong inflammatory disease, display the phenotypic and morphological characteristics of activated endothelial cells. A DNA sequence mediating this repression was localized in the 5′ flanking region of the IL-6 gene. In the same study, a synthetic VDR agonist, calcipotriol, showed antitumor effects in KS patients (130). The potential clinical use and possible toxic side effects of less calcemic vitamin D analogs are under evaluation to prevent prostate, colon, and breast cancer.

PROTEASE INHIBITORS (PIs)

Since an altered equilibrium of the protease/PI activity ratio is at the base of tumor invasion and extravasation (19) and is associated with other diseases characterized by excessive angiogenesis, an increasing number of selective PIs, including synthetic MMP inhibitors, are currently under clinical investigation. Molecular targets for PIs involve proteinases of almost all the known classes, including serine, cysteine, and metalloproteinases. The role of NAC and EGCG as MMP inhibitors has been discussed. Among the first PIs to be investigated, the BBI family (Bowman-Birke inhibitor) of plant serine PIs derived from legume seeds and beans (soybean, chickpea, peanuts), has been studied for preclinical cancer chemoprevention of oral cavity and colon

carcinogenesis, the former being one of the best models for clinical studies of chemoprevention (131). The chemopreventive effect of BBI concentrate in oral leukoplakia has been tested in a phase IIa trial (132). However, the difficulty of isolating the active compound in this extract, whose activity is expressed as general chymotrypsin inhibitory units, and to provide a reproducible formulation for clinical trials are limiting factors for the clinical use of BBI. Phase III clinical trials targeted to advanced stage cancers with second generation MMP inhibitors Marimastat and Batimastat led to disappointing results (reviewed in ref 38) whereas a phase I clinical trial for the treatment of refractory solid tumors with the MMP inhibitor COL-3, a chemically modified nonantibiotic tetracycline derivative, induced disease stabilization in patients affected by a nonepithelial type of malignancy, including the vascular neoplasm hemangioendothelioma (133). Taken together, these results indicate a role for PIs mostly in prevention rather than in the cure of the established tumor phenotype.

CONCLUSIONS: COMMON MOLECULAR PATHWAYS FOR ANTIANGIOGENESIS BY CANCER CHEMOPREVENTIVE AGENTS

Diverse chemically unrelated chemopreventive agents apparently share common mechanisms to exert antiangiogenic activity.

It is evident that several molecular mediators of the inflammatory process and related signaling pathways are involved in tumorigenesis and angiogenesis. Antiinflammatory drugs show evidence for antitumoral activity when administered in the early stages of carcinogenesis.

As a general mechanism, oxidative stress is a common hallmark of inflammation and the tumoral phenotype. Tumor growth produces large amounts of ROS (134), which can activate tumor-infiltrating leukocytes to induce the angiogenic response (135). One effect of antioxidant chemopreventive agents is alteration of the redox equilibrium in target cells. They can act directly on endothelial cells, but their influence on the production of extracellular matrix molecules and angiogenic growth factors by tumor cells or surrounding stromal cells can contribute as well to their antitumor activity. Interference with endogenous ROS generation can lead to opposite effects in endothelial and tumor cells, depending on the extent of the stimulus. ROS can modulate signal transduction pathways as do other second messengers (136). Although a moderate subtoxic increase in intracellular ROS can induce a defense response by activating a cell survival response and endothelial cell proliferation, disruption of the intracellular redox balance by oxidative stress exceeding the defense mechanisms can lead to endothelial and tumor cell death (137).

Sometimes the same signaling pathway triggered by stimuli of different intensity can drive opposite responses depending on the ultimate transducing molecule of the cascade. This is the case for the AP-1 transcription complex, a key element in the angiogenic response that can be activated by apoptotic or proliferative stimuli (138). Along with p53 and NF-κB, AP-1 is one of the known transcription factors whose activity can be post-translationally modified by redox regulation (139). AP-1 activity is inhibited by NAC (23, 140), retinoic acid (141), and 4-HPR (142); one of its components, c-*jun*, is also the mediator of 4-HPR induced cell death in prostate cancer cells (143). AP-1 inhibition by aspirin has been directly linked to its antineoplastic activity (144). AP-1 binding sites are present in the promoters of potent angiogenic growth factors such as VEGF (145) and IL-8 (146), and regulate stromelysin basal level of transcription (147) or repression (148).

Another key element of the angiogenic response (again networked with other complex regulatory pathways involved in inflammation and tumorigenesis) is the MAP kinase pathway, recently reviewed in the context of angiogenesis by Pages and co-workers (15). VEGF-stimulated KDR receptor signals a proliferative response through ERK-1/2 in endothelial cells (149). ERK2 has been considered a chief target of NSAIDs inhibitory action on HMVEC angiogenesis (11); this signaling pathway is also modulated by NAC (150), polyphenolic compounds such as EGCG (151), and 1α,25-dihydroxy vitamin D_3 (152).

The chemopreventive agents considered here also affect other signaling pathways critically involved in the regulation of angiogenesis. NAC induces gene expression of the CDK inhibitor p21(WAF/CIP1) (153). In vitro and in vivo data show that p21(WAF/CIP1) is required for the activity of the fumagillin derivative TNP-470 (one of the first antiangiogenic agent entering clinical trials) to specifically induce endothelial cell growth arrest (154). p21(WAF/CIP1) expression is induced by the green tea polyphenol EGCG (155), whereas breast cancer cell growth arrest induced by 1,25-dihydroxyvitamin D_3 strongly correlates with p21(WAF/CIP1) induction (156).

Finally, several chemopreventive agents such as NAC, EGCG, and retinoids work as protease inhibitors, exerting their action on MMPs and uPA. Although clinical trials with synthetic inhibitors of MMPs have yielded limited results in the tertiary prevention setting (38), the possible use of less toxic, naturally derived compounds could provide more encouraging results in secondary and primary prevention settings.

What emerges from an examination of the data is that apparently the same molecular processes that regulate cellular homeostasis in tissues, involving hormonal balance and intracellular defense mechanisms are partly devoted to the control of angiogenesis. The tumor should be considered in its microenvironment, where endothelial cells, stromal cells, and the immune system are not simple bystanders of tumor cell growth. Results from COX-2 overexpression in colon cancer, for instance, which takes place first in the stromal cells (157), indicate that defective epithelial-stromal com-

munications may be at the base of the carcinogenic process (158). Although this review focuses on the convergence of diverse chemopreventive agents on common regulatory mechanisms governing angiogenesis, an evaluation of the overall biological effects of the molecules examined here should be carefully considered. When planning a long-term chemopreventive clinical intervention, it is necessary to preserve the internal equilibrium of healthy cells in the organ and the body from exposure to harmful compounds. Indeed, some angiogenic growth factors and angiogenic regulatory molecules are also implicated in the vital processes of tissue regeneration and healing. For example, VEGF and IGFs are survival factors for endothelial cells and neural cells, respectively, and COX-2 is essential for normal maintenance and healing of the gastric mucosa. The chemopreventive agents that selectively interfere with particular biochemical alterations occurring in tumor cells or those acting on the highly specialized biology of endothelial cells during neovascularization deserve special attention. FJ

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