Review: Molecular Pathology of Cyclooxygenase-2 in Cancer-induced Angiogenesis

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Abstract. Cancer-induced angiogenesis is the result of increased expression of angiogenic factors, or decreased expression of anti-angiogenic factors, or a combination of both events. For instance, in colon cancer, the malignant cells, the stromal fibroblasts, and the endothelial cells all exhibit strong staining for cyclooxygenase-2 (COX-2), the rate-controlling enzyme in prostaglandin (PG) synthesis. In various cancer tissues, vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) co-localize with COX-2. Strong COX-2 and VEGF expression is highly correlated with increased tumor microvascular density (MCD); new vessels proliferate in areas of the tumor that express COX-2. Moreover, high MVD is a predictor of poor prognosis in breast and cervical cancers. COX-2 and VEGF expression are elevated in breast and prostate cancer tissues and their cell-lines. In vitro, PGE2 induces VEGF. Supernatants of cultured cells from breast, prostate, and squamous cell cancers contain angiogenic proteins such as COX-2 and VEGF that induce in vitro angiogenesis. A selective COX-2 inhibitor, NS-398, restores tumor cell apoptosis, reduces microvascular density, and reduces tumor growth of PC-3 prostate carcinoma cells xenografted into nude mice. The COX-2 produced by a malignant tumor and COX-2 produced by the surrounding host tissue both contribute to new vessel formation, which explains how selective COX-2 inhibition reduces tumor growth where the tumor COX-2 gene has been silenced by methylation. (received 6 June 2001, accepted 8 July 2001)

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Introduction

Angiogenesis, the formation of new vessels from existing vessels, is an important feature of embryogenesis, inflammation, and cancer growth and metastasis [1]. Chronic inflammation is a risk factor for cancer [2], but the exact reason why is unknown. At the site of inflammation, cyclooxygenase-2 (COX-2) is the rate-limiting enzyme in the synthesis of pro-inflammatory and angiogenic prostaglandins (PG) such as PGE2, which induces metalloproteinases (MMP) and vascular endothelial growth factor (VEGF) [3,4]. The antiphlogistic and analgesic effects of nonsteroidal anti-inflammatory drugs (NSAIDs) are largely due to their inhibition of COX-2 [5].

A variety of human malignancies overexpress COX-2 and prostaglandin [6] along with VEGF and transforming growth factor-beta (TGF-β). For instance, in colorectal carcinoma strong COX-2 expression, as evidenced by immunostaining, is highly correlated with tumor microvascular density: a large number of small vessels form around the areas that express COX-2 [7]. Furthermore, the expression of COX-2, TGF-β, and VEGF in the same areas of the tumor suggests their coordinated expression in the cancer-induced angiogenesis.

Tumor invasion into the local tissue and tumor growth at the site of metastasis are preceded by tumor-induced proliferation of an abundantly vascular stroma, for instance in breast cancer [8]. For such tumors, anti-angiogenesis therapy is an encouraging new approach. COX-2, the angiogenic
molecules associated with it, and their interrelated signaling pathways in malignant tumors are therefore of major interest in understanding the tumor-induced angiogenesis. Studying the expression of COX-2 in cancer tissues and its role in the growth of malignant tumors is important because NSAIDs might help to prevent cancer [9]. Furthermore, selective COX-2 inhibitors are available that block the effects of COX-2 expression but spare the expression of COX-1 [5,10].

Vasculogenesis and Angiogenesis

A. The yolk sac
During implantation, trophoblast invasion, vasculogenesis, and angiogenesis are essential for the formation of the placenta. The human trophoblast secretes metalloproteinases, and metalloproteinase inhibitors can abort in vitro invasion. In contrast to the invasion by a malignant tumor, the invasion by the trophoblast is stringently controlled. However, the controlling factors are unknown [11]. Many angiogenic factors that are highly expressed in malignant tumors are essential for blastocyst implantation and yolk sac vasculogenesis, for formation of new vessels from stem cells, and for yolk sac angiogenesis (Fig. 1) [12,13]. Vasculogenesis requires the development of the hemangioblast, the precursor for hematopoiesis and vessel development, into the endothelial cell. A multitude of endothelial cells form

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**Fig. 1.** Cyclooxygenase (COX-2), prostaglandin (PG), and transforming growth factor (TGF)-β are involved in vascular development and vascular pathology from vasculogenesis and angiogenesis, to atherosclerosis and cancer. Lack of maternal COX-2 or its inhibition by a nonsteroidal anti-inflammatory drug (NSAID) prevents implantation. Lack of TGF-β1 is lethal due to faulty vasculogenesis and angiogenesis during embryogenesis with a penetration of about 50%. Lack of the TGF-β receptor component endoglin (ENG) prevents proper angiogenesis. Disruption of the PGE2 receptor EP4 gene prevents closure of the ductus arteriosus. COX-2 and TGF-β co-localize in atherosclerotic lesions and in carcinogen-induced colon carcinomas, suggesting coordinated expression. In the cancer, COX-2 induces new vessel formation via PGE2 that induces vascular endothelial growth factor (VEGF). In some poorly differentiated malignancies, vasculogenesis is also induced. A human cancer tissue may overexpress one or more of the proteins indicated in the highlighted boxes. Text in italics indicates an agent used in vitro.
the capillaries, which mature into veins and arteries during angiogenesis.

Expression of COX-2 and TGF-β and its receptors is fundamental for blastocyst implantation [14] and yolk sac vascularization. Gene knockout experiments have shown that expression of COX-2, prostaglandin and prostaglandin receptors, and TGF-β and TGF-β receptors are essential for embryonic vasculogenesis and angiogenesis.

For successful implantation, the blastocyst must induce the expression of maternal COX-2 in the uterus [15]. Studies of the COX-2 null mouse prove the vital importance of the COX-2 expression; complete failure of implantation renders the mice sterile. Half of the COX-2 knockout mice develop cardiac fibrosis and all develop renal dysplasia [16].

In the mouse, disruption of the gene for TGF-β1, a strong inducer of COX-2, causes defective vessel formation and may cause death. However, there are dosage and mouse-strain effects [17,18] that modulate the severity of this gene disruption. One-half of TGF-β1(-/-) and one quarter of TGF-ββ(+-) mice die at about 1.5 wk post-conception because of failing endothelial cell differentiation [17].

VEGF can regulate vascular permeability and is an important mediator of vasculogenesis and angiogenesis [19]. VEGF signals are conveyed via the VEGF receptors Fli-1 and Flk-1. Fli-1 is expressed in the hemangioblast. Disruption of the Flk-1 gene is lethal in mice at about day 9 of gestation, due to lack of hematopoiesis and vasculogenesis. Normally, Fli-1 is strongly expressed during physiological vasculogenesis and angiogenesis in embryos and is re-expressed during tumor-induced angiogenesis [20].

B. Vascular remodeling

COX-2 and prostaglandins are evidently involved in vascular remodeling. For example, rapid and selective physiological vascular remodeling occurs during closure of the ductus arteriosus and there may be a difference in response of the prenatal and postnatal ductus.

Firstly, a lack of the PGE2 receptor EP4 in the EP4-null mice causes the ductus to remain open. By comparison, indomethacin administered during pregnancy may cause premature closure of the ductus arteriosus. In fetal lambs, the COX-2 is expressed in the endothelium of the ductus arteriosus, while COX-1 is expressed in both the endothelium and the muscular layers [21].

Secondly, the inhibition of prostaglandin synthesis by indomethacin constricts the fetal ductus arteriosus in normal mice, but not in EP4(-/-) mice, which do not express the prostaglandin EP4 receptor. Such knockout mice generally die shortly after birth. They (as well as the few that survive for a longer time) have a patent ductus arteriosus (Fig. 1) [22].

In mice, a lack of either COX-1 or COX-2 or both does not cause premature closure of the ductus arteriosus in utero. Furthermore, lack of COX-1 does not affect postnatal life. In contrast, about one third of COX-2 null mice and almost 80% of COX-2(-/-) + COX-1(+/-) mice die within 48 hr after birth. Importantly, the absence of both COX isoforms causes postnatal death within 12 hr [23]. This suggests that maternal prostaglandins alone can maintain intrauterine patency of the ductus arteriosus. During closure of the ductus arteriosus, the expression of the 3 TGF-β isoforms increases significantly. Over the 10 postnatal days, it increases from the very low levels before birth. The highest levels are seen in the neo-intima and the outer muscle media, but TGF-β is not detected in vascular endothelial cells [24].

With advancing age, there is diffuse intimal thickening (DIT) of the elastic arteries (Fig. 1) [25,26] and there is evidence that TGF-β is involved in vascular remodeling. A comparison of 30-mo-old versus 6-mo-old Fisher rats revealed significant increases of TGF-β and MMP-2 in the intima, which was 5-fold thicker in the older rats [27].

C. Inflammation

Chronic inflammation is a risk factor for a variety of human malignancies [2]; many of the angiogenic proteins expressed in cancer tissues are the same as those expressed at sites of inflammation [5]. In vitro studies and animal models of inflammatory angiogenesis show that COX-2 plays a pivotal role in new vessel formation at sites of inflammation. For instance, COX-2 is strongly expressed and produces pro-inflammatory prostaglandins such as PGE2. Besides, PGE2 can induce VEGF, for instance in
synovial fibroblasts in vitro. The signaling occurs via the prostaglandin EP2 receptor and protein kinase A pathways [4].

Angiogenesis is an essential part of the formation of granulation tissue [28]. In an animal model of wound healing, application of neutralizing anti-VEGF antibody to a post-surgical wound demonstrated that VEGF is an essential angiogenic signal transducer in the formation of wound granulation tissue. Neutralizing anti-VEGF antibody reduced granulation tissue VEGF levels and new vessel formation [29].

COX-2 and VEGF are both expressed in rat sponge-implant-angiogenesis [30]. COX-2 is located in the endothelial cells of the new vessels that are formed in the sponge-granuloma tissue [31]. The experimental angiogenesis can be blocked by either a non-selective NSAID, indomethacin, or by selective COX-2 inhibitors, NS-398 or JTE-522, as well as by VEGF anti-sense oligonucleotides [30]. VEGF-induced angiogenesis in a mouse cornea model of angiogenesis can be inhibited by NS-398 or by cyclosporin-A administered systemically. Importantly, PGE2 restores corneal angiogenesis even in the presence of these inhibitors [32].

In a rat model of pleurisy, carrageenan-treated rats express elevated levels of COX-2 in the lung. Treatment of rats with ip melatonin prior to the carrageenan administration inhibits the COX-2 expression [33]. High dosages of melatonin were used in this study, ranging from 12.5 to 50 mg/kg. However, it is remarkable that melatonin can inhibit COX-2 expression.

The Malignant Tumor

A. Cancer-induced angiogenesis

The pathological growth of human cancers resembles in several ways the physiological growth of the implanting blastocyst. Both need to evade the immune defenses of the host and secure a vascular supply in order to grow [34,35]. To induce new vessel formation, the cancer must induce the expression of angiogenic growth factors and their receptors and regulate the expression of proteolytic enzymes and adhesion molecules [36]. In a manner similar to the blastocyst, the malignant tumor forces the host tissue to grant it a supply of oxygen and nutrients that enables the tumor expansion, which otherwise is confined to 1-2 mm by the maximum oxygen diffusion limit. Progression from limited tumor growth to invasion and metastases requires degradation of the extracellular matrix by proteolysis by metalloproteases (MMP, matrixins), induction of angiogenesis, tumor cell migration, and modification of cell adhesion [37,38].

COX-2 is important because it induces metalloproteinase and strongly induces VEGF, the common angiogenic factor, which thereupon directs tumor-induced angiogenesis [39]. Together, these factors contribute to tumor growth, invasion, and metastasis. Magnetic resonance imaging (MRI) shows that tumor-induced angiogenesis follows a common pattern, leading to a well-perfused tumor periphery but a residual necrotic core. Host vessels enter the periphery of the tumor, and the perfusion front moves toward the center of the tumor [40].

B. COX-2 expression in cancer tissues

Enhanced COX-2 expression has been demonstrated in numerous types of cancer cells and tissues, such as colorectal cancer [5]. For example, COX-2 expression was increased in all but 2 of 20 lung cancers, 14 of 20 colon adenocarcinomas, and 11 of 20 breast tumors, but not in epithelial cells of the corresponding non-tumor tissue. Expression of COX-2 was minimal in the stromal cells of the tumors and in normal tissues [41]. Furthermore, immunohistochemical expression of COX-2 was significantly increased in malignant cells of all but 1 of 13 cervical carcinomas, compared to non-tumor cervical tissue [42].

High expression levels of COX-2 may foretell a poor prognosis; for instance, in 24 patients with cervical carcinoma who had been treated with radiation, increased expression of COX-2 was associated with shorter survival [43]. In patients with gastric cancer, the tissue COX-2 levels were significantly higher in invasive versus non-invasive tumors [44]. A study of 63 patients with colorectal cancer showed that high COX-2 expression correlated with tumor recurrence, and particularly with hematogenous spread of the tumors [45]. The follow-up period in this study (6 to 98 mo, average
60 mo) was significantly longer than in earlier studies that did not show correlation between COX-2 expression and survival. The Kaplan-Meier curves clearly showed longer survival for patients with low expression of COX-2 in their tumor tissues [45]. Prostatic tissue cancer cells showed significantly elevated, mainly intracytoplasmic, expression of COX-2 compared to the COX-2 expressed along the cell membrane in benign prostatic hyperplasia. The intensity of staining of COX-2 was significantly higher in poorly-differentiated versus well-differentiated prostate cancers and correlated with the Gleason grade [46]. Other studies corroborate these findings. For instance, significant elevations of COX-2 protein and mRNA were demonstrated in prostate cancer cells by immunostaining and RT-PCR in a series of 28 carcinomas, compared to controls (8 cases of benign prostatic hyperplasia and 8 prostate samples that were free of tumor) [47]. In another series of prostatic carcinoma, all but 4 of 31 cancers showed intense and uniform immunostaining for COX-2 [48]. COX-2 was expressed in all but 2 of 28 primary skin melanomas (16 showing moderate to strong expression), but not in 4 benign nevi [49]. All except 1 of 29 retinoblastomas over-expressed COX-2 [50].

In a study of 100 patients with colon cancer, those with COX-2-expressing tumors had significantly shorter survival time than those with COX-2-negative tumors; moreover, correlation was noted between COX-2 expression and microvascular density [7]. Immunostaining for CD-34 to show the microvascular density of the tumors revealed that a large number of small vessels had formed around areas that expressed COX-2 [7]. In addition, a study of paraffin-embedded tumor tissue from 120 patients with breast cancer showed that a high tumor MVD correlated with the histological grade and with poor survival [51]. In another study of patients with breast cancer, detecting increased MVD of axillary lymph nodes by iv digital subtraction angiography predicted lymph node metastases [52], suggesting that MVD may be reliably determined using non-invasive techniques.

These findings in malignant tumors suggest that high COX-2 expression is associated with tumor-induced angiogenesis, invasion, and metastases.

C. VEGF in cancer tissues

Enhanced expression of VEGF has been demonstrated in, for instance, ovarian [53], breast [54], renal [55,56], and esophageal [57] cancers and in malignant melanomas [58]. The malignant cells in breast cancers showed variable expression of VEGF, but no VEGF expression was found in epithelial cells in areas of breasts that were free of tumor [54]. VEGF expression was increased in 55 prostate cancers compared to 5 cases of prostatic adenomas and 20 normal prostate samples; elevation of VEGF correlated with tumor MVD, tumor grade, and stage [59]. In 85 patients that underwent surgical resection of stage I non-small cell lung cancers, high tumor MVD and poor prognosis correlated with high levels of VEGF expression [60].

Immunostaining of VEGF might be an indicator of aggressiveness in serous ovarian tumors. Firstly, VEGF immunostaining of tumor tissue was significantly greater in 32 invasive tumors, compared to 16 borderline and 10 benign ovarian tumors. Secondly, there was significant correlation between VEGF elevation and metalloproteinase-2 (MMP-2) elevation [53]. Moreover, in 44 cases of hepatocellular carcinoma, the microvascular density correlated significantly with the expression levels of VEGF mRNA. Poorly encapsulated tumors showed higher levels than well-encapsulated tumors. Interestingly, serum VEGF concentration was significantly higher in patients with tumors, compared to patients with benign liver disease [61]. However, high MVD does not always indicate a poor prognosis, particularly in cases with extensive tumor necrosis. For instance, in a series of 49 localized renal cell carcinomas, MVD was inversely correlated with the magnitude of tumor necrosis; low MVD and extensive necrosis predicted a poor outcome [62]. And, in a study of 69 patients with stage I-II non-small-cell lung carcinomas, the cancer cells, stromal fibroblasts, and endothelial cells in the tumors all exhibited strong VEGF staining. However, the level of tumor microvascular density by CD34 immunostaining in these cases correlated neither with the tumor levels of VEGF and its receptors, nor with the patients' survival [63]. Determining the levels of VEGF and its receptors flt-1 and KDR/flk-1 by immunostaining in 35
adenocarcinomas, 6 papillary serous carcinomas, and 6 carcinosarcomas was ineffective in predicting metastases, recurrence, or survival [64]. On the other hand, immunostaining for VEGF may be of use to differentiate malignant melanomas from benign nevi. Cytoplasmic VEGF was found in almost half of 45 malignant melanomas and the intensity of immunostaining was related to the Clark level. In contrast, VEGF was not detected in benign nevi [58].

**D. Haptoglobin**

It has been reported that the multifunctional haptoglobin molecule is angiogenic (for a review see [5]). Haptoglobin expression is regulated by dexamethasone and by IL-6, which is induced by PGE2 [65]. Whether haptoglobin induces VEGF is unknown. Haptoglobin expression has been detected in human breast carcinoma tissue extracts and cell lines [66,67]. Furthermore, in vitro cell lines established from squamous cell and ovarian cancers and from embryonic lung secrete a multi-protein complex that has subunits with sequence homology to the β chain of haptoglobin [68].

**E. Vascular patterns in malignant tumors**

Although tumor-induced angiogenesis resembles the angiogenesis observed at sites of inflammation, there are important differences between physiological angiogenesis and cancer-induced angiogenesis. In addition to increased microvascular density in and around many tumors, the pattern of neoplasia-induced vascularization differs from normal vascular patterns. Measuring and evaluating this difference might have significant diagnostic and prognostic value. To illustrate, in uveal melanomas, the presence of parallel vessels with cross-linking indicates a poor prognosis [69]. Moreover, the fractal character of tumor-induced new vessel proliferation reflects a higher degree of irregular branching and vessel distribution, compared to physiological angiogenesis. This difference is probably caused by more variable local distribution of vascular growth factors within the tumor, owing to the inherent genetic instability and progressive molecular diversity of malignant cells [70]. Caution is advised when extensive necrosis is present. In the renal carcinoma cases mentioned above, the microvascular complexity as evidenced by the fractal dimension of vessels visualized with CD34 staining in the tumors correlated inversely with the extent of tumor necrosis, which predicted a poor outcome [62].

**F. Cancer cell supernatants**

Supernatants of cultured cells from several types of cancer contain angiogenic factors, such as fibroblast growth factor-2 (FGF-2) in prostate cancer [71] and TGF-β, PGE2, and VEGF in squamous cell cancer [72]. Supernatants of cultured cells from head and neck squamous cell cancer, breast cancer, and liver cancer secrete PGE2, VEGF, and TGF-β and induce in vitro angiogenesis [72-74]. VEGF expression is elevated in the cancer tissue and cell lines derived from breast tumors [54,75]. Prostaglandin E2 induces VEGF in a variety of cell lines and VEGF expression strongly stimulates angiogenesis in vitro. As examples, PGE2 induces VEGF in cell lines derived from the rat osteoblasts [76] and Müller cells [3], as well as human rheumatoid synovial fibroblasts [4] and monocytes [77].

Metalloproteases play an important role in tumor cell invasion by proteolysis of the extracellular matrix. As a case in point, cultured prostate cancer cells release proMMP-2, proMMP-9, and MMP-9 into the medium and invade through Matrigel. The factor release and tumor cell invasion can be inhibited by a PLA2 inhibitor, 4-bromophenacyl bromide, and by a selective COX-2 inhibitor, NS-398. Inhibition of lipoxygenase with esculetin does not affect MMP secretion. On the other hand, the tumor cell invasion can be restored by PGE2 supplementation. These experiments indicate that cyclooxygenase-2 induces metalloproteinases (MMP) via PGE2 [78].

**G. Tumor xenograft-induced angiogenesis**

Experiments have shown that COX-2 and VEGF are essential angiogenic proteins that determine the growth of various xenografted human cancer cells. In addition, such experiments have corroborated the sequential propagation of the angiogenic signal from COX-2 via PGE2 to VEGF. The tumor-induced COX-2 expression in the host tissue located adjacent to the malignant tumor adds to the angiogenesis
induced by the COX-2 produced by the cancer cells, and both enhance tumor growth.

The significance of host COX-2 expression in cancer growth is illustrated by the proliferation of Lewis lung cancer cells xenografted into the COX-2(-/-) mouse. The xenograft growth is significantly reduced compared to that in the wild-type mouse. Expression of VEGF by host COX-2 null fibroblasts is reduced >90% and is comparable to the reduced VEGF expression by wild-type murine fibroblasts following treatment with a selective COX-2 inhibitor [79].

The importance of VEGF in tumor-induced angiogenesis is evidenced by the effect of neutralizing anti-VEGF antibody in nude mice xenografted with tumor. The antibody treatment completely inhibits the angiogenesis by xenografted cell lines derived from human rhabdomyosarcoma and prostate cancer. The tumors become dormant after the initial angiogenesis-independent growth phase [80], thus indicating the critical importance of tumor-induced new vessel formation in the tumor growth.

Molecular Pathology

A. Angiogenic factors

1. Cyclooxygenase and carcinogenesis
Transgenic overexpression of either COX-1 or COX-2 can transform cells [81,82]. No sequence aberration of the COX-2 gene has been reported in malignant cells. A great variety of cancer cells and tissues overexpress COX-2 and prostaglandins that reduce cancer cell apoptosis [6] and induce angiogenesis via VEGF. NSAIDs inhibit the growth of such cancers and the anti-neoplastic effects of the NSAIDs are partly due to COX-2 inhibition [5].

On the other hand, neoplastic transformation appears to be independent of cyclooxygenase. Thus, Ha-ras or SV40 cause in vitro transformation of fibroblasts that lack COX-1 or COX-2, or both. By comparison, when the genes for either or both enzyme isoforms are present, the corresponding protein is highly expressed in the transformed cells. These results suggest that cyclooxygenases play a role in tumorigenesis at a later step [83].

Several oncogenes and growth factors can induce COX-2 expression. In cultured CaCo-2 colon carcinoma cells, COX-2 is upregulated by insulin-like growth factor (IGF)-II via the IGF-I receptor [84]. Besides, TGF-α, epidermal growth factor (EGF), TGF-β, and hepatocyte growth factor (HGF) can all induce COX-2 [5,6].

2. Prostaglandin and carcinogenesis
Prostaglandin E2 induces VEGF and basic fibroblast growth factor (bFGF) [3]. There is evidence that bFGF might contribute to cancer-induced new vessel formation. For example, when co-cultured with bovine aortic endothelial cells, 4 human glioma cell lines that expressed high levels of bFGF mRNA induced the endothelial cells to form tubes. The tube formation was blocked by supplementation with anti-bFGF antibody. Moreover, 3 human glioma cell lines with low expression of bFGF failed to induce tube formation [85].

PGE2 induces the expression of metalloproteinases by a multistep process involving NF-kappaB. It was recently suggested that PGE2 might enhance the invasive potential of colorectal carcinoma through activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt/PKB) pathway. PGE2 treatment of LS-174 human colorectal carcinoma cells increased their motility and altered their shape [86]. Prostaglandin E2 suppresses both cellular and humoral immunity [2,87] (reviewed in [6]). Inhibition of cyclooxygenase with NSAIDs restores the immune reactivity [5,88].

3. VEGF and carcinogenesis
Vascular endothelial cell growth factor (VEGF) is the primary member of a family of growth factors. VEGF and its homologues can convey regulatory signals via receptors KDR/Flk-1, Flt-1, and Flt-4 [89] (Fig.2). However, VEGF and its homologues have different effects on different cells. For instance, the orf paracoxvirus encodes the VEGF-E homologue [90] that causes orf disease in sheep and is associated with vascular proliferation at the site of infection in human erythema multiforme [91,92].

The VEGF gene is located at chromosome region 6p12 [93]. A 600 bp genomic fragment was mapped to chromosome region 6p12 and was highly amplified in almost half of a series of human non-small cell lung cancer; however, it lacked sequence homology to any known protein [94].
VEGF stimulates the activation of cPLA2 and the release of arachidonic acid by human umbilical vein endothelial cells in vitro [95]. VEGF induces COX-1 but not COX-2 in cultured endothelial cells; it has been suggested that COX-1 has a physiological role in maintenance of vascular structures [96]. Such a feedback loop might help to maintain a basal level of VEGF expression.

4. Aging and carcinogenesis
With advancing age, both angiogenic and anti-angiogenic changes have been observed. In vitro studies suggest that as the generation of reactive oxygen species (ROS) increases with advancing age, there is increased expression of COX-2. The ROS reduces IkappaB expression and enhances the expression of NF-kappaB, a strong COX-2 inducer [97]. On the other hand, as the oxidative stress increases with age, the mitochondrial function may decline [98,99], and there is some evidence that decline of endothelial energy metabolism may inhibit angiogenesis [100].

B. Anti-angiogenic proteins
Physiological angiogenesis is the result of a dynamic balance between angiogenic and anti-angiogenic factors [101]. Orderly angiogenesis is essential for the endometrial cycle, for inflammation, and for the granulation tissue of wound repair [29]. Deranged expression of anti-angiogenic factors caused by viruses or by loss of function of tumor suppressor genes has been linked to various human malignancies. Because they can counterbalance the angiogenic effects of COX-2, a few anti-angiogenic factors are discussed below.

1. Thrombospondin-1 (TSP-1)
Reports on the role of the glycoprotein TSP-1 in carcinogenesis are controversial. Some reports
indicate that thrombospondin inhibits angiogenesis in vitro, while other reports state the opposite. Some studies find elevated levels of TSP-1 in cancer tissues, while other studies report decreased levels. However, as explained below, the strongest evidence suggests that TSP-1 is a major anti-angiogenic protein. Most importantly, transgenic expression of TSP-1 can limit tumor angiogenesis and growth. For instance, injection of an adenovirus containing TSP-1 plasmid into pre-established tumors reduces tumor-induced angiogenesis, tumor growth, and metastatic spread. The overexpression of TSP-1 in squamous carcinoma cell lines inhibits their in vivo growth. Depending upon the cell-line used, overexpression of TSP-1 either prevents xenograft growth or, when a tumor develops, it exhibits marked central necrosis [102].

Cholangiocarcinoma is a tumor with low vascularity compared to hepatocellular carcinoma. When tumor tissues were compared to the matching non-tumor tissue, there were significant differences in the ratio of TSP-1 to VEGF expression in these two types of tumor. Cholangiocarcinomas showed much higher TSP-1 and lower VEGF expression than hepatocellular carcinomas [103]. These findings seem to corroborate that TSP-1 can inhibit VEGF expression and reduce tumor-induced angiogenesis. On the other hand, TSP-1 was strongly expressed and the expression levels were associated with high microvascular density in 87 of 98 pancreatic carcinomas. It was proposed that TSP-1 expression played a principal role in the new vessel formation and the spread of these tumors [104]. However, in vitro, thrombospondin-1 inhibits endothelial cell migration and tube formation. The inhibitory signal is transmitted through CD36, an endothelial cell transmembrane glycoprotein and is completely dependent on its expression [105]. As discussed below, both TSP-1 and CD36 are downregulated by the platelet endothelial cell adhesion molecule (PECAM) [106].

In vitro assays and tissue culture experiments indicate that TSP-1 may also inhibit angiogenesis by inhibiting MMP activity [107]. Almost half of 99 malignant melanomas expressed mutant p53 and significantly lower TSP-1 levels and higher microvessel counts than the tumors with the wild-type p53. Besides, the presence of mutated p53 was significantly more frequent and the TSP-1 levels significantly lower in the tumor metastases than in the primary tumors [108]. In contrast, immunostaining for p53 and TSP-1 in a series of 65 colon cancer tissues showed accumulation of p53 in 42 cases with reciprocal expression of TSP-1 in the areas that expressed p53, suggesting that p53 can suppress TSP-1 in vivo [109].

Surprisingly, a TSP-1 plasmid expression vector inserted into a human prostate-carcinoma cell-line failed to reduce the growth of the cancer cells in vitro. However, when the cells were transfected along with a liposomal agent and then xenografted into nude mice, there was reduced microvessel density and extensive necrosis in the tumors compared to xenografted but non-transfected tumor cells [101].

Mevalonate induces the expression of TSP-1 (Fig. 3). As a case in point, incubation of cultured human vascular smooth muscle cells for 24 hr with lovastatin at concentrations as low as 1 µM/L significantly inhibited TSP-1 expression. The expression of TSP-1 was restored by co-incubation with mevalonate [110].

2. Metalloproteinase inhibitors
Introducing a recombinant adenovirus containing the matrix metalloproteinase inhibitor TIMP-2 into tumor cells xenografted into mice inhibits tumor development, but the degree of inhibition is tumor cell dependent. In the case of MDA-MB231 human breast cancer cells, transfection completely inhibits tumor development from the xenografted cells. In contrast, treatment of murine LLC lung cancer and C51 colon cancer cells with the recombinant virus reduces the tumor establishment by about one half. Furthermore, injection of the adenovirus into pre-established tumors reduces tumor-induced angiogenesis, tumor growth, and metastatic spread [111].

3. Angiostatin
Angiostatin can diminish vascular density and branching. For instance, in the quail chorioallantoic membrane assay, it reduced angiogenesis by almost 70% compared to the normal rate of developmental angiogenesis [112]. There is evidence that angiostatin affects angiogenesis by inhibiting the surface F0F1-ATPase activity of endothelial cells, thereby reducing endothelial cell metabolism [100].
C. Biphasic growth factors

Biphasic growth factors are characterized by an ability to induce tubulogenesis (i.e., the formation of tubular structures) in vitro. Several are morphogens that are intimately involved in vasculogenesis and angiogenesis. The present author speculates that a biphasic growth factor is involved in establishing the curvature of a blood vessel wall and thereby determines the vessel diameter [113]. Several biphasic morphogens that are expressed during embryogenesis are re-expressed during inflammation and repair, and cancer growth, invasion, and metastasis. The important biphasic morphogens in connection with COX-2 and cancer-induced angiogenesis are TGF-β, TGF-α, and PECAM.

1. TGF-β

Transforming growth factor β (TGF-β) induces COX-2 via the TGF-β type 2 receptor (TGFBR2) [5,6]. It is secreted as a latent protein and converted to a 25 kDa active form. There is some evidence that TSP-1 can bring about this activation in vitro [114]. Other investigators found no evidence of such activation in vitro [115]. However, supernatants from glioma cell lines contain high levels of TSP-1 that can activate TGF-β [116].

Because TGF-β is a bimodal growth factor, a protein that activates it would appear to have bimodal properties as well. Assuming that TSP-1 can activate latent TGF-β in certain cells, this might explain the observation that TSP-1 at low concentrations stimulated the growth of cultured bovine endothelial cells while it inhibited their growth at higher concentrations [117]. In addition, activation of TGF-β might explain how TSP-1 enhances tumor invasion by breast cancer cells in vitro [118]. This impression is supported by in vitro experiments using pancreatic tumor cells; supplementation by either TSP-1 or TGF-β produced upregulation of tumor cell invasion that was completely reversed by...
antibodies against either urokinase plasminogen activator or its receptor [119].

TGF-β is of interest because it is abundantly expressed in the same areas as COX-2 in colon carcinoma tissues. This observation suggests a close collaboration in the process of carcinogenesis [120] especially because TGF-β1 has been described as a powerful regulator of COX-2 expression [121]. Most important, from an etiologic point of view, deregulation of TGF-β1 expression may be an early event in colon carcinogenesis [122]. Aberrations in TGF-receptors, such as repression of the expression of the TGF-type II receptors, have been shown to contribute to carcinogenesis [123].

There is evidence that the different isoforms of TGF-β may have varying roles in carcinogenesis, and that their effects may differ in different types of malignancies. To cite an instance, in skin cancers, TGF-β1 was found to be associated with highly differentiated tumors, TGF-β3 was associated with tumor stroma growth and angiogenesis, and TGF-β2 with invading, very malignant cells [124]. Yet, in prior studies in xenografted nude mice, expression of TGF-β2 was higher than that of the β1 isoform in the poorly invasive DU145 prostate carcinoma cell line compared to the highly metastatic PC-3M prostate carcinoma cell line [125].

TGF-β modulates angiogenesis by regulating vascular endothelial cell proliferation and migration. Besides, it affects the extracellular matrix and the synthesis of adhesion molecules [36]. TGF-β stimulates smooth muscle cells at low concentration and inhibits them at high concentration [126,127].

It has similar effects on capillary lumen formation induced in vitro by bFGF or VEGF [128]. The TGF-β signals via type I (ALK-1), type II, and type III (endoglin) receptors and Smad proteins, which regulate genes involved in angiogenesis [129,130].

Participation of TGF-β in angiogenesis is corroborated by results from the mouse corneal model of inflammation induced by silver nitrate. When mice were injected into the femoral muscle with an adenovirus that expressed a soluble ligand binding part of the TGF-β type II receptor, the corneal angiogenesis was significantly reduced when compared to control mice [131].

The TGFBR2(-/-) mouse dies about 10 days post-coitus due to defects in yolk sac vasculogenesis [129] that closely resemble those that occur in the TGF-β1 null mouse [17]. In like manner, mice lacking the TGF-β Type I receptor (TGFBR1) die of defective yolk sac and placental vascular development. Cultured vascular endothelial cells from such mice show defective fibronectin production and abnormal migration, but enhanced proliferation, compared to control cells from wild-type mice [132].

Another example of the importance of TGF-β and its receptors in proper angiogenesis is illustrated by mutations of the TGF-β1 and TGF-β3 binding protein endoglin (ENG, CD105) and ALK-1 genes. Such mutations are responsible for the vascular malformations seen in hereditary hemorrhagic teleangiectasia (HHT) types 1 and 2 respectively (Fig. 1) [130,133].

Endoglin is an essential factor for angiogenesis during embryogenesis. It is expressed on the surface of endothelial cells. Lack of endoglin does not affect vasculogenesis but it is essential for normal vascular maturation as part of angiogenesis. This is evident in endoglin null mice, which die by gestational day 11.5 from defective development of vascular smooth cells and aborted endothelial remodeling [133]. These experiments confirm that TGF-β signaling through its receptors plays an important role in the regulation of angiogenesis. The importance of TGF-β signaling in cancer-induced angiogenesis is further validated by a report that elevated levels of endoglin in breast cancer tissues predict a high risk of developing metastatic disease. Strong upregulation was detected by immunostaining in the tissues of 92 breast cancer patients [134].

Endoglin is highly expressed in some malignant tumors. It might find use as a tumor marker because the protein was detected in the plasma of patients who had early breast cancer. Analysis of plasmas obtained before any treatment was given revealed that patients who had high plasma levels of endoglin had a high risk of developing metastatic disease [134]. Also, the endothelial-specific expression of endoglin favors its use as an endothelial cell specific marker. As an example, in paraffin-embedded cervical cancer tissues immunostaining for endoglin was more sensitive than immunostaining factor VIII for
visualizing capillaries and was a better predictor than factor VIII for lymph node metastases [135].

2. TGF-α
TGF-α induces COX-2 by binding to the EGF receptor (EGFR) [136,137]. Supplementation with TGF-α of cultured cervical cancer cells corroborates these findings. TGF-α supplementation markedly increased COX-2 expression [42]. Presence of EGFR, but not of TGF-α, in 86 breast cancers was associated with poor prognosis. However, intensity of TGF-α immunostaining reflected the extent of angiogenesis in the tumor [137].

3. PECAM
Platelet endothelial cell adhesion molecule (PECAM-1) has a bimodal effect, stimulating endothelial cell morphogenesis at low concentrations and inhibiting it at high concentrations. PECAM is found at sites of cell-to-cell contact in polyoma middle T-transformed mouse brain endothelial cells. These cells express high levels of PECAM-1 that inhibit the expression of TSP-1 and TSP-1 receptor CD36 and form hemangiomas in the mouse [138]. The bimodal character of PECAM may be related to the activation of latent TGF-β by TSP-1, which regulates the formation of active, bimodal TGF-β.
inhibition of COX-2 expression and enhanced angiogenesis. Furthermore, CMV increases tumor-induced angiogenesis independently of p53 expression by depressing TSP-1 expression. For instance, infecting p53-defective astrocytoma cells with CMV showed that the virus significantly reduced the TSP-1 mRNA and protein levels. Supplementation with CMV anti-sense oligonucleotides fully blocked the abolition of TSP-1 expression [144].

Human papilloma virus (HPV) is associated with angiogenesis in cervical cancer. One study examined 230 cervical tissues from 6 groups of 31 to 43 subjects, including a control group and 5 groups with tumors that ranged from low grade, intraepithelial lesions to poorly differentiated squamous cell carcinomas. There was good correlation between the extent of new vessel formation and the degree of histological abnormality. Importantly, the amount of angiogenesis determined by CD34 immunostaining was associated with the presence of HPV types 16 and 18 [148].

The E6 oncoprotein of the human papilloma virus (HPV)-16 upregulates the VEGF gene via a promoter region that contains 4 Sp-1 sites. VEGF is highly expressed in carcinomas of the cervix, with high levels in cells that are HPV-16 positive [149].

The latent membrane protein (LMP-1) of the Epstein-Barr virus induces COX-2 via NF-kappB. COX-2 is often expressed in preinvasive nasopharyngeal lesions that harbor the virus and in nasopharyngeal carcinomas. In vitro studies suggest that EBV, present in over two-thirds of such tumors, contributes to VEGF expression and tumor-induced angiogenesis [150].

### D. Regulation of COX-2 expression

Several oncogenes and growth factors can induce COX-2 (Fig. 2) (for review, see [6]). For example, EGF induces COX-2. TGF-β enhances COX-2 expression induced by EGF both in mink lung and rat intestinal epithelial cells but not in a mink lung cell line that lacks the TGF-β type I receptor. Inhibition of the EGF receptor tyrosine kinase activity with AG1478 entirely abolished the expression of COX-2 induced either by EGF alone or by a combination of the two growth factors [151].

A prior report from the same laboratory documented that TGF-β strongly induces COX-2 and that COX-2 can participate in the inhibitory signaling pathway of TGF-β. In other studies the in vitro inhibitory part of the biphasic effects of TGF-β via COX-2 and PGE2 was blocked by indomethacin and restored by supplementation with PGE2 [152].

Oncostatin, a member of the IL-6 family of cytokines, is a potent growth factor for Kaposi’s sarcoma. It is a strong inducer of COX-2 expression in cultured human aortic smooth muscle cells [153]. The angiogenic effect of oncostatin depends upon the cell type. It was angiogenic in vitro for dermal microvascular endothelial cells but not for macrovascular, human umbilical vein endothelial cells. It induced COX-2 in the former, but not in the latter, and the angiogenic effect was nullified by selective inhibition of COX-2 [154]. Oncostatin was angiogenic in vivo in the rabbit corneal model, but it slightly inhibited calf pulmonary artery endothelial cells proliferation in vitro [155], suggesting that it might be a bimodal angiogenic factor.

### Discussion and Hypothesis

The experimental findings that have been discussed show that caution is needed in interpreting the sometimes contradictory data. There are clearly differences in the ways different cell types respond to various cytokines and growth factors. The following hypothesis attempts to explain some of these differences. The present hypothesis differs from the angiogenic balance theory that considers that a balance between TSP-1 and VEGF expression determines angiogenesis. Rather, the present hypothesis proposes that cancer-induced angiogenesis is determined mainly by the differential signal input that controls VEGF expression (Figs. 4 and 5), which in turn governs angiogenesis.

In this view, the VEGF system is comparable to an operational amplifier, with negative feedback via VEGF induction of TSP-1, which inhibits VEGF, (Fig. 5). Evidence that such a negative feedback loop exists was found in the murine model of retinal new vessel formation [156]. The importance of the negative feedback is that it may ensure a linear response of the VEGF-mediated angiogenesis. The amount of new vessel formation responds to the
magnitude of the differential input to COX-2 and TSP-1. All angiogenic and anti-angiogenic signals that regulate the expression of vascular endothelial growth factor (VEGF). COX-2 increases VEGF and metalloproteinases (MMP) and thrombospondin (TSP-1) inhibits VEGF and MMP expression. Overexpression of COX-2 by cancer cells increases VEGF and MMP expression, inhibits apoptosis, and promotes angiogenesis, invasion, and metastatic spread. Loss of function of the tumor suppressor protein p53 increases tumor-induced angiogenesis by reducing TSP-1 and increasing COX-2 expression, resulting in reduced inhibition and increased stimulation of VEGF by TSP-1 and COX-2 respectively. Cytomegalovirus (CMV) inhibits TSP-1 expression, which decreases the inhibition of VEGF expression. Human papilloma virus (HVP) contains a VEGF-like segment. Both viruses induce angiogenesis in the cervical cancer.

TSP-1 may paradoxically behave as an angiogenic agonist. Located predominantly at the tumor invasion front, TSP-1 mRNA was strongly expressed in all but 11 of a series of 98 pancreatic carcinomas. TSP-1 levels correlated with the tumor microvascular density. It was concluded that TSP-1 induces angiogenesis [104]. However, the malignant tumor might over-express TSP-1 and provide a strongly inhibitory signal to VEGF, but might be overruled by a stronger angiogenic signal to VEGF – for example, caused by powerful overexpression of COX-2. In the cited experiments, COX-2 expression was not analyzed, so this suggestion appears reasonable, but speculative.

Furthermore, this hypothesis may explain why loss of the tumor-suppressor protein p53 function is so significant to tumor growth. Such loss not only enhances COX-2 but in addition reduces TSP-1 expression, which together greatly increase VEGF protein expression, angiogenesis, tumor invasion, and metastasis.

In most cases of cervical cancer, p53 is not mutated, but rather is elevated [148]. One might speculate that p53 expression could be induced in vivo in response to a malignant tumor, to defend against tumor-induced angiogenesis in an attempt...
to combat tumor growth, invasion, and metastasis. A better understanding of the pathophysiology involved in the growth of a specific tumor might be obtained by determining the levels of both angiogenic and anti-angiogenic factors that affect VEGF expression, and not assaying only VEGF. Finally, the role of interference of energy metabolism in the inhibition of angiogenesis is most interesting. Angiostatin inhibits the surface F0F1-ATPase activity of endothelial cells [100]. In comparison, the cholesterol-lowering statin drugs inhibit coenzyme Q10 synthesis and thereby mitochondrial oxidative phosphorylation and ATP generation [99]. Such statins might thereby inhibit angiogenesis, and at the same time enhance angiogenesis by reducing TSP-1 expression.

**Diagnosis and Therapy**

**A. Tumor markers**

Several proteins that are angiogenesis regulators have been detected in blood and some are elevated in patients with cancer. However, their potential use to detect or monitor post-operative patients for tumor recurrence needs clarification [157,158].

Tumor vascular density is a prognostic factor for a variety of cancers. It reflects the importance of the vascular supply to the tumor growth. Furthermore, the fractal character of tumor-induced vessel formation exhibits differences compared to the fractal character of physiological new vessel formation. Tumor-induced vessels follow a more irregular course, probably due to inherent regional genetic differences in the tumor cell population. Magnetic resonance imaging can detect alterations in vessel permeability and permits in vivo evaluation of experimental tumor-induced angiogenesis. In the future it might be used in clinical studies to follow the reduction in cancer-induced angiogenesis that is achieved by COX-2 inhibition [159,160].

**B. Anticancer therapy with NSAIDs**

1. **Non-selective NSAIDs**

Many epidemiological studies, a large number of in vitro and animal experiments, and several clinical studies have demonstrated that non-selective NSAIDs can restrain the development and growth of different types of cancer [5]. Non-selective NSAIDs (nsNSAIDs) differ in their ability to inhibit the two known isoforms of cyclooxygenase (COX)
[5], but most of them significantly inhibit COX-1 in platelets. Especially after prolonged use, they can cause bleeding and gastrointestinal ulceration and uncouple mitochondria [161].

Some anti-neoplastic effects of NSAIDs might be independent of cyclooxygenase inhibition. For instance, NSAIDs suppress colony formation on soft agar by transformed fibroblasts that either possess or lack COX-1 or COX-2 [83]. These anti-neoplastic effects might be due, in part, to the mitochondrial uncoupling that is common to many non-selective NSAIDs.

2. Selective COX-2 inhibitors
NSAIDs that inhibit COX-2 restore tumor cell apoptosis in vitro and reduce in vivo COX-2-tumor-induced angiogenesis. Furthermore, COX-2 inhibition lowers the synthesis of metalloproteinases and reduces matrix proteolysis. Also, as noted above, by inhibiting the COX-2 synthesis induced by the tumor in the adjacent host tissue, such agents obstruct the growth of xenografted tumors in which COX-2 has been silenced by methylation [79,162]. These selective COX-2 inhibitors might have great potential for treatment of human cancer. In comparison to nonselective NSAIDs, selective COX-2 inhibitors have fewer gastrointestinal side effects, do not interfere with platelet function, and do not uncouple mitochondria. Selective COX-2 inhibitors have shown significant anti-cancer effects in a variety of animal tumor xenograft models. They inhibit the growth and spread of the malignant cells [5,6].

The US Food and Drug Administration (FDA) has approved celecoxib and rofecoxib for treatment of rheumatoid arthritis and osteoarthritis respectively. A review of >50 clinical studies involving 13,000 patients and lasting 12 wk to 2 yr concluded that these drugs are well tolerated [163]. Twice daily doses of celecoxib at 200 mg [164] and 400 mg [165] have been well tolerated by arthritis patients. It is encouraging that selective COX-2 inhibitors might prevent the development of cancer in patients with premalignant conditions such as adenomatous polyposis of the colon. A 6-mo clinical trial demonstrated that celecoxib, 400 mg twice daily (30 patients), but not 100 mg twice a day (32 patients) significantly reduced the number of polyps, compared to 15 patients given placebo [166].

The patient’s physician should carefully supervise any selective COX-2 therapy. For example, it has been advised that while gastrointestinal bleeding is not a problem in the use of the selective COX-2 inhibitors, the possible development of dyspepsia and renal problems should be carefully monitored [167]. Renal side effects might appear due to inhibition of COX-2 in the macula densa and medullary interstitial cells [168]. Second, because of the role COX-2 plays in implantation, COX-2 inhibition is not advisable in women who desire to conceive. Pregnant women should not use NSAIDs that inhibit COX-2 because the enzyme prevents the premature closure of the fetal ductus arteriosus [169]. Third, because the statin type of cholesterol-lowering drugs also lowers the synthesis of mevalonate and thereby the synthesis of the anti-angiogenic protein TSP-1, it might be reasonable to re-evaluate the need for statin therapy in patients with cancer. Fourth, analysis of 3 studies involving >17,000 rheumatoid arthritis patients showed an increase of thromboembolic events (eg, myocardial infarction, stroke) in patients who took rofecoxib 50 mg daily, compared to those who took celecoxib 400 mg twice daily plus low dose aspirin [170].

Conclusions
High expression levels of cyclooxygenase (COX-2), vascular endothelial growth factor (VEGF), and metalloproteinase (MMP), and low thrombospondin (TSP-1) expression in cancer tissues is associated with high tumor vascular density (MVD), a prognostic factor in human malignancies that predicts aggressive growth, invasion, and metastases. The human cancer derails the normal balance between angiogenic (COX-2) and anti-angiogenic factors (TSP-1) that control the expression of VEGF, the pivotal mediator of new vessel formation. By expressing COX-2, which via PGE2 induces VEGF, the solid cancer forces its host to provide a vascular supply, which enables the tumor to grow beyond 1-2 mm.

The importance of COX-2 in angiogenesis is evidenced by its role in implantation-induced new vessel formation. The trophoblast of the implanting blastocyst induces maternal COX-2 expression, which is essential for the placental vasculogenesis and angiogenesis that are required for successful
implantation. In granulation tissue at the sites of inflammation, and in a variety of solid tumors, COX-2 is re-expressed.

Chronic inflammation is a risk factor for cancer. The exact reason is not known, but in vitro, transgenic overexpression of COX-2 is capable of transforming cells. On the other hand, COX-2 negative cells can also be transformed. No gene abnormality of COX-2 in human cancer has been reported, but COX-2 can be induced by a variety of oncogenes, cytokines, growth factors, and carcinogens – for example, benzo[a]pyrene in tobacco smoke. In a large variety of human cancers, COX-2 is upregulated, producing eicosanoids such as PGE2, which induces VEGF and thereby angiogenesis. By inhibiting COX-2 in experimental cancers that overexpress this enzyme, the balance between angiogenic and anti-angiogenic signals, which control VEGF expression and angiogenesis, can be restored, causing tumor necrosis and tumor regression to a small, dormant state.

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