Role of Cyclooxygenases in Angiogenesis

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Abstract: Angiogenesis is the process by which new blood vessels are formed. This process supports normal physiology as well as contributes to progression of disease. Progressive rheumatoid arthritis and growth of tumors are two pathologies to which angiogenesis contributes. In arthritis, we know that prostaglandins (PGs) and the enzyme cyclooxygenase-2, which catalyses prostaglandin production, are inflammatory mediators. These mediators are involved in rheumatoid arthritis and cancer-induced angiogenic processes. We discuss, herein, recent findings on the expression of cyclooxygenases in both rheumatoid arthritis and human cancer, and the links between COX-2, PGs, and angiogenesis. We also propose a model for the possible mechanistic interaction of the various cell types involved in angiogenesis.

Introduction

Angiogenesis is the process by which new blood vessels are formed and grow from existing, quiescent vascular endothelium. Angiogenesis is known to occur in human physiology during reproduction and wound healing, and, pathologically, in malignant tumor growth, metastatic cancer, rheumatoid arthritis (RA), diabetic retinopathy, and age related macular degeneration[1]. Inflammatory mediators are thought to contribute to these processes. In fact, some of the inflammatory prostaglandins, products of arachidonic acid metabolism, are pro-angiogenic[2]. This review explores the recent literature and some of our own studies on the role of cyclooxygenase in both tumor and arthritis induced angiogenesis, and suggests a model for the interaction of the different cell types involved.

Prostaglandins are derived from arachidonic acid by either of two enzymes, cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed in most tissues, and COX-1 derived prostaglandins perform many functions. In the stomach and upper gastrointestinal tract, for instance, COX-1 derived PGs are protective of the mucosal lining and help prevent ulcerations. In the platelet, COX-1 derived thromboxane A2 is important for platelet function, particularly aggregation and clotting [3]. Constitutive COX-2 expression was detected in the brain, and in some cells in other organs, the function of which in these locations is not well understood. COX-2 is induced in reproductive tissues during ovulation, implantation and labor. No COX-2 was detected in quiescent vasculature in normal tissues. COX-2 production elsewhere is highly regulated, and is induced in inflammatory cells including those associated with arthritis, and in tumor cells by cytokines and tumor promoters [3-7]. Initially, anti-inflammatory drugs were identified based on their capacity to inhibit the cyclooxygenase obtained from a tissue that contains mainly COX-1, and their ability to relieve pain and inflammation in animal models, now known to be COX-2 mediated. Thus, traditional non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, diclofenac, and indomethacin are inhibitors of both cyclooxygenases. With the discovery of two separate isoforms, small molecule enzyme inhibitors were designed which can inhibit either COX-1 or COX-2 at doses which do not inhibit the other isoform [8,9]. The specificity of some of these compounds has been demonstrated in vitro and in vivo (Table (1)). The pharmacological analyses obtained by use of these tools has greatly expanded our understanding of the roles of cyclooxygenase in arthritis and pain [3,4,8-11]. In this review we will discuss the role of cyclooxygenases in angiogenesis and possible mechanisms by which this process may occur.

COX-2 Expression and Angiogenesis

Human rheumatoid arthritis is a disease state characterized by chronic inflammation. The major prostanoid produced by the arthritic synovium is PGE2,
which in RA can induce bone resorption and angiogenesis [12]. Immunolocalization of the COX enzymes in human RA reveals that COX-2 is expressed in the invading neovascular endothelium as well as the tissue macrophages and synovial fibroblasts in the inflamed joint [13]. Furthermore, COX-2 inhibitors were both anti-inflammatory in animal models of arthritis, including rat adjuvant-induced arthritis[14], and anti-angiogenic in a bFGF-induced sponge implant model [15]. Since COX-2 is expressed in cell types which either constitute or may contribute to angiogenesis, it is possible that some of the therapeutic effects of inhibiting COX-2 in RA may be due to inhibition of angiogenesis.

The rheumatoid pannus has similar properties to a malignant tumor [16]. The cells involved in both diseases include fibroblasts, vascular endothelial cells, immune cells, and platelets. The in vivo processes including hypoxia, growth factor production, cytokine activated COX-2 induction, prostaglandin production, vascular endothelial growth factor (VEGF)-mediated vascular permeability, and neovascularization, are strikingly similar[13,14]. With the findings that COX-2 mRNA is induced both in chemically-induced colon tumors [17] and in adjuvant-induced arthritis [14] in rodents, we compared the localized expression of COX-2 in tumors with that found in arthritis.

We examined the expression of COX-1 and 2 in several common human cancers. The angiogenic vasculature within and adjacent to tumors expresses COX-2 in addition to the neoplastic epithelial cells of human cancers including colon[18], lung, breast, prostate, pancreatic, and head and neck squamous cell carcinoma [19,20]. In normal tissue epithelium COX-2 expression was only detected focally and at low levels in some colonic crypt epithelial cells, pulmonary peribronchial glands, pancreatic ductal epithelium, and brain. Thus COX-2 expression is induced in cancerous epithelium, and, importantly, in tumor invading neovasculature. Immunohistochemical staining showed COX-1 to be broadly distributed in normal epithelium where COX-2 is not expressed, and also broadly distributed in cancerous epithelium where COX-2 is expressed. In vascular endothelium, COX-1 immunostaining appeared only in normal, established vessels not associated with cancerous lesions. Taken together, these data show that COX-2, but not COX-1, is induced in malignant epithelium, and is also expressed in cells that comprise the angiogenic response to cancer.

We also examined COX-2 staining of metastatic nodules. COX-2 positive angiogenic blood vessels were clearly detected in livers containing metastatic colon carcinoma, and in lymph nodes invaded by pancreatic metastatic lesions. COX-2 was also strongly expressed in the neoplastic cells comprising the metastatic nodes. Thus, COX-2 is present in the angiogenic vasculature that occurs in rheumatoid arthritis, primary tumors, and in metastatic disease. Reducing the levels of COX-2 derived prostaglandins in arthritis relieves the symptoms of this disease, symptoms which include angiogenesis. We wondered whether inhibition of COX-2 derived prostaglandins would have a direct inhibitory effect on in vivo angiogenesis.

### Inhibition of Prostaglandin Synthesis Suppresses Angiogenesis

Conventional non-steroidal anti-inflammatory drugs limit PG synthesis by inhibiting both COX-1 and COX-2, although they derive their anti-inflammatory properties from their ability to inhibit COX-2. As early as 1991, epidemiological studies showed that regular use of aspirin or other traditional NSAIDs may reduce the risk of death from colon cancer [21]. Numerous studies demonstrate that traditional NSAIDs can reduce tumor growth and angiogenesis in experimental models. For instance, chronic oral indomethacin dosing, starting at 6 months of age, delays by 11-12 weeks the initial development of spontaneous mammary tumors in retired breeder C3H/HEJ mice, increases by 2-fold the spontaneous regression of these tumors over controls, and significantly prolongs the lifespan of mice bearing multiple tumors[22]. These tumors from animals treated with indomethacin exhibit a reduction in vascularity among other anti-tumor effects. This study suggests that endogenous prostaglandins promote the development, growth, neovascularization and metastasis of spontaneous mammary tumors and that all these effects are reduced by indomethacin. Topical administration of the traditional NSAID diclofenac inhibits angiogenesis in mice bearing syngeneic, subcutaneous Colon-26 tumors, with an increase in apoptosis and tumor necrosis [23,24]. These studies show that inhibition of PG synthesis limits angiogenesis, indicating that PGs are important mediators of this process.

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<th>in vitro IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>in vivo ED&lt;sub&gt;50&lt;/sub&gt; (mg/kg)</th>
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<tr>
<td>COX-1</td>
<td>17.6</td>
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<tr>
<td>COX-2</td>
<td>0.009</td>
<td>0.3</td>
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<tr>
<td>Indomethacin</td>
<td>0.1</td>
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1 Inhibition of human recombinant enzyme

2 COX-1 inhibition assessed as inhibition of gastric prostaglandins; COX-2 inhibition assessed as inhibition of air pouch prostaglandins.
In an in vivo model of angiogenesis, the chorioallantoic membrane (CAM) technique, a small hole is made in the shell of a fertilized chicken egg, and growth factor is injected into the membrane before sealing with a glass cover slip. Angiogenesis can be observed through the cover slip, and use of labeled sulphate allows quantitation of the angiogenesis following harvest of the membrane two days later. This study shows that bFGF-generated prostanoids correlate to angiogenesis, and both are inhibited by indomethacin [25]. The use of conventional NSAIDs in all these in vivo and in vitro studies demonstrates the anti-angiogenic effect of limiting PGs, however, whether the efficacy of these inhibitors is due to their capacity to inhibit COX-1, COX-2, or both, cannot be determined.

Inhibition of COX-2 Suppresses Angiogenesis

In an initial effort to address this issue, we used inhibitors of COX-1 and inhibitors of COX-2 to characterize each isoenzyme in an in vivo rat matrigel assay (Fig. (1)). We subcutaneously implanted matrigel plugs containing the inflammatory reagent carrageenan, and observed after 4 days, some development of new blood vessels together with a robust cellular influx including endothelial cells and macrophages. Oral dosing of indomethacin, or the anti-inflammatory drug dexamethasone, dramatically inhibited the cellular influx and neovascularization, whereas treatment with a COX-1 inhibitor SC-560 did not. Interestingly, we used, as a experimental control, a matrigel plug containing basic fibroblast growth factor (bFGF). Four days later, we observed the plug full of new blood vessels; an effect that was dramatically augmented compared to the carrageenan plug. This angiogenesis was blocked by the COX-2 inhibitor SC-236, and also by indomethacin, suggesting that inhibition of COX-2 impairs bFGF-induced angiogenesis in vivo (Fig. (1)). Similar to our finding, another COX-2 inhibitor significantly reduces the hemoglobin content of bFGF injected rat sponge implants [26] indicating that angiogenesis is reduced by COX-2 inhibition. Taken together, these results suggest that bFGF may require the induction of COX-2, not COX-1, to generate neovascularization in vivo, and led us to further evaluate the role of COX-2 in angiogenesis.

A clear, quantitative model of angiogenesis in vivo is the corneal micropocket assay. Since the cornea is normally avascular except for the limbic vessels which

![Fig. (1)](image_url)

Fig. (1). Inhibition of carrageenan and bFGF induced angiogenesis.

Matrigel plugs containing either saline, carrageenan, or bFGF were implanted subcutaneously in mice. Dosing by gavage BID with indomethacin, dexamethasone, or SC-236 was performed for the duration of the experiment. After 4 days the plugs were resected and photographed. Both carrageenan and bFGF induced neovascularization, however bFGF was more effective. Inhibitors of COX-2 reduced neovascularization in this model.
circle the outer perimeter, it is possible to visualize and measure any new blood vessels produced within it. We induced angiogenesis in the mouse cornea by surgically implanting a hydron/sucralfate pellet containing 50 ng bFGF, approximately 1mm from the temporal limbus [27]. Cyclooxygenase inhibitors were administered by gavage beginning the day before surgery and continuing the length of the study. Four days after surgery, the corneas were examined under a slit lamp microscope and the neovascular response was calculated by measuring the area of the cornea covered by new blood vessels. Using this model we were able to quantitatively determine the degree of neovascularization of bFGF-induced angiogenesis and analyze the pharmacological effects of cyclooxygenase inhibitors.

We also used immunohistochemistry to localize COX expression in the cells of the angiogenic corneas. COX-2 was clearly detected in the angiogenic blood vessels and vascular associated cells, but was not expressed in the pre-existing limbic vessels within the cornea. In contrast, COX-1 was observed in the existing limbic vasculature, but not in the new blood vessel cells invading the corneal stroma. Taken together, these observations suggest that COX-1 is the enzyme present in mature endothelial cells while COX-2 expression is associated with the generation of new blood vessels.

In a study comparing the effects of traditional NSAIDs on bFGF-induced angiogenesis in this model, the potent COX inhibitors ketoprofen, indomethacin, ibuprofen, and sulindac inhibit angiogenesis by 30%, 59%, 6%, and 50% respectively [28]. We also evaluated COX-1 and COX-2 inhibitors in this model for their ability to inhibit bFGF-induced neovascularization. In addition, the ability of the compounds to inhibit COX-1 was tested in the same animals by measuring serum thromboxane-B2 (TXB2), a prostanoid derived from platelet COX-1 [9,10]. The COX-2 inhibitor, SC-236, inhibited the angiogenic response (Table 2) [29] at doses which did not significantly inhibit COX-1 activity in platelets. Celecoxib, a COX-2 inhibitor which at therapeutic doses in humans does not inhibit COX-1, and is commercially available to relieve the signs and symptoms of osteo- and rheumatoid arthritis in adults [30-32], demonstrated similar anti-angiogenic activity [33]. COX-1 inhibition with SC-560 did not affect the angiogenic process at doses that clearly inhibited platelet COX-1 activity. The traditional NSAID indomethacin inhibited both angiogenesis and COX-1 activity. The immunolocalization of COX-2 to the neovascular cells in the cornea, and the potent suppression of angiogenesis with COX-2 inhibitors at doses which do not inhibit COX-1 suggest the active participation of COX-2 in bFGF-induced angiogenesis.

COX-2 is apparently involved in the pathway to neovascularization, however, other studies suggest a possible role for COX-1 in this process. For instance, tube formation by human umbilical vein endothelial cells (HUVECs) in co-culture with HCT-116 colon cancer cells may be COX-1 dependent [34]. HCT-116 cells lack COX-1 or -2 activity, but provide a source of growth factors which will stimulate tube formation in HUVECs. Tube formation is blocked by aspirin, and by a COX-1 antisense oligonucleotide, but not by an experimental COX-2 inhibitor, or by a COX-2 antisense oligonucleotide [35]. These results suggest that given a steady supply of growth factors, HUVECs depend on COX-1 to form tubes in vitro. COX-2 inhibition, however, potently reduced proliferation and the production of angiogenic factors by a cancer cell line which does express COX-2. The authors conclude that COX-2 is the isoenzyme which contributes to tumor growth and growth factor production in some cancer

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<th>Table 2. Inhibition of COX-2 Suppresses bFGF-Induced Angiogenesisa</th>
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<td><strong>Dose in mg/kg/day</strong></td>
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<td>COX-2 inhibitor, SC-236</td>
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<td>COX-1 &amp; COX-2 inhibitor, indomethacin</td>
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a Expressed as % inhibition of vehicle-treated control values. Data from average of 6 mice per group.

SC-236, a COX-2 inhibitor, dose dependently inhibited the corneal area of neovascularization induced by bFGF in mice. Indomethacin, a COX-1 and COX-2 inhibitor, also inhibited the angiogenic response, but the COX-1 inhibitor SC-560 had no effect. SC-236 did not significantly inhibit mouse stomach COX-1 activity as measured by PGE2 production, or platelet COX-1 activity as measured by serum TXB2 production. Indomethacin, a COX-1 and COX-2 inhibitor, and the COX-1 inhibitor SC-560, potently inhibited mouse stomach COX-1 and platelet COX-1.
cells, and that COX-1 activity may be necessary for endothelial cells to form new vessels [34].

Other reports have looked at the role of platelets in angiogenesis, a cell type that is a rich source of COX-1 enzyme. During activation, platelets release platelet derived growth factor (PDGF) which has angiogenic effects on endothelial cells [35]. Activated platelets also cause the release of arachidonic acid from cell membranes, the substrate for COX enzymes. The main prostanoid that platelets produce is thromboxane-A$_2$ (TXA$_2$) via thromboxane synthase activity. TXA$_2$ is a potent vasoconstrictor and is important for platelet aggregation. Thrombocytopenia reduces tumor vascularization similar to what is observed after administration of diclofenac-sodium [36]. Whether the reduced vascularization is due to the lack of TXA$_2$ or PDGF is unclear, however, this study indicates that platelet activity may be important in angiogenesis. Platelets may also contribute to metastasis, since thrombocytopenic mice show decreased metastatic spread of tumor cells compared to platelet replete controls [37,38]. It is thought that platelets aggregate around tumor cells in transport to distal capillary beds and aid in implantation. If inhibition of COX-1 derived thromboxane synthase decreases the platelet/Es ability to aggregate around tumor cells, then there may be a role for COX-1 in metastasis.

Prostaglandin-Dependent Mechanisms of Angiogenesis

It is clear that angiogenesis is not dependent on a single mediator or cell type. In fact, different cell types, enzymes, integrins, growth factors, and small molecules interact in a complex manner and temporal sequence. What is the underlying mechanism that links growth factor induced angiogenesis to COX-2 expression? Based on our data and that of others, we propose a simple model to explain the interaction between neoplastic cells, fibroblasts, and endothelial cells in COX-2 mediated angiogenesis (Fig. (2)). Basic FGF, a growth factor produced by many tumors, can induce COX-2 messenger RNA (mRNA) expression and protein in several cell lines including bone derived endothelial cells [39], rat gastric mucosal epithelial [40], and RA synovial cells [41]. There have been few studies to date, however, that show a direct connection between bFGF and COX-2 activity or expression in vascular endothelial cells. For instance, in an in vitro study of HUVECs, transforming growth factor beta

Fig. (2). Model of AngiogenesisûInteraction of cell types.

In tumor cells, COX-2 derived prostaglandins upregulate the production of growth factors including VEGF which can act directly on endothelial cells, and bFGF which stimulates COX-2 upregulation in fibroblasts. COX-2 derived prostaglandins in fibroblasts stimulate VEGF production which acts on endothelial cells in a paracrine fashion to again upregulate COX-2 and facilitate vascular permeability and angiogenesis. COX-2 inhibitors block the production of prostaglandins, and thus prevent growth factor induced angiogenesis.
TGFbeta. They remark that regulation of PGI\(_2\) capillaries, but these cells failed to respond to bovine aortic endothelial cells, and human retinal did report bFGF stimulated PGI\(_2\) release from fetal fibroblasts obtained from human RA tissues, PGE\(_2\) vascular smooth muscle cells, and synovial lining cells, sections showed that VEGF mRNA and protein are necessarily be the target endothelial cell. The source of VEGF in cancer and arthritis may not be the fibroblast appears to be an important source of VEGF, and prostaglandins could be a mediator of VEGF production in these cells. Fibroblasts help support the endothelial cells invading both the tumor and arthritic environment. Basic FGF induces angiogenesis and acts upon fibroblasts as a cytokine. In fibroblasts, PGE\(_2\) produced from cytokine-induced COX-2 upregulates VEGF production [50]. In a paracrine manner, then, VEGF may stimulate angiogenesis in vascular endothelial cells (Fig. (2)) [2,57].

Both VEGF and prostaglandins can be produced by tumor cells themselves, and this VEGF production may be regulated by tumor derived prostaglandins. For example, tumor cells transfected to over-express COX-2 in vitro produced 4-8 times as much of a variety of growth factors, including VEGF, as non-transfected cells. This over-production is reduced to basal levels with an experimental COX-2 inhibitor [34]. Taken together, these data suggest that fibroblasts which are activated by cytokines, such as interleukin-1 or bFGF, induce COX-2 expression resulting in the synthesis of large amounts of PGE\(_2\), which in turn will upregulate VEGF production (Fig. (2)). Whether the source of VEGF is the fibroblast, the tumor cell, or both, VEGF acting on endothelial cells will stimulate the growth of new blood vessels. The same process appears to occur in inflammatory diseases, such as RA, as well as in cancer.

VEGF increases permeability of vascular endothelial cells, a necessary part of the angiogenic process. After intravenous administration of contrast dye to guinea pigs and mice, vascular leakiness was observed at sites of intradermal injection of VEGF, indicating increased vascular permeability. Blockade of PGs by indomethacin attenuates the vascular permeability [58,59]. Thus, PGs contribute to the pronounced permeability produced by VEGF on vascular endothelial cells [58,59]. Possible sources of VEGF may include tumor cells, synovial fibroblasts, macrophages, tumor-invading fibroblasts, or endothelial cells themselves. Prostaglandins derived from the inducible COX-2 enzyme seem likely to be involved in the regulation of the synthesis and activity of this highly pro-angiogenic molecule.

**Conclusions**

In summary, we have established the presence of COX-2 in angiogenic endothelial cells in two disease states and demonstrated the potent inhibition of angiogenesis in vivo with COX-2 inhibitors at doses which do not inhibit COX-1. But there is much about COX-2 in angiogenesis that remains to be explained. Much of the scientific discussion in cancer and angiogenesis has been focused on either tumor cells or...
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endothelial cells, but we suggest that the interplay of these and other cell types involved in angiogenesis will prove to be critically important. Fibroblasts may play a major role in the neovascularization of both cancer and arthritis, as well as platelets, pericytes, and immune cells such as macrophages and leukocytes. Further elucidation of the roles of COX-1 and COX-2 in these numerous cell types will provide the basis for greater understanding of the mechanism(s) by which prostaglandins mediate angiogenesis.

Abbreviations

PGs = Prostaglandins
RA = Rheumatoid arthritis
COX-1 = Cyclooxygenase-1
COX-2 = Cyclooxygenase-2
NSAIDs = Non-steroidal anti-inflammatory drugs
VEGF = Vascular endothelial growth factor
CAM = Chorioallanthoic membrane
bFGF = Basic fibroblast growth factor
TXB₂ = Thromboxane-B₂
HUVECs = Human umbilical vein endothelial cells
PDGF = Platelet derived growth factor
TXA₂ = Thromboxane-A₂
mRNA = Messenger RNA
TGFβ = Transforming growth factor beta
PGI₂ = Prostaglandin I₂

References


