Differential Suppression of Vascular Permeability and Corneal Angiogenesis by Nonsteroidal Anti-inflammatory Drugs

Pouya Pakneshan,1 Amy E. Birsner,2 Irit Adini,1 Christian M. Becker,1,3 and Robert J. D’Amato1,4

PURPOSE. Angiogenesis, the formation of new capillary blood vessels, is an essential biological process under physiological conditions, including embryonic development, reproduction, and wound repair. Under pathologic conditions, this process plays a critical role in a variety of diseases such as cancer, rheumatoid arthritis, atherosclerosis, endometriosis, diabetic retinopathy, and age-related macular degeneration. The purpose of this study was to examine the effects of cyclooxygenase inhibitors on basic fibroblast growth factor (bFGF)- and vascular endothelial growth factor (VEGF)-mediated ocular neovascularization and permeability.

METHODS. A modified Miles vascular permeability assay was used to examine VEGF-induced vascular hyperpermeability, and the mouse corneal model of angiogenesis was used to compare the efficacy of systemic treatment with different nonsteroidal anti-inflammatory drugs (NSAIDs) on bFGF- and VEGF-induced angiogenesis.

RESULTS. The authors demonstrated that systemic application of most NSAIDs, but not acetaminophen, blocked VEGF-induced permeability in mice. However, systemic treatment of mice with NSAIDs resulted in the differential inhibition of bFGF-induced (5%–50%) and VEGF-induced (3%–60%) corneal angiogenesis. The selective COX-2 inhibitors were more effective at suppressing bFGF-induced angiogenesis than VEGF-induced angiogenesis.

CONCLUSIONS. Though most NSAIDs are effective at suppressing vascular leak, there exists a differential efficacy at suppressing the angiogenic response of specific cytokines such as bFGF and VEGF. (Invest Ophthalmol Vis Sci. 2008;49:3909–3913) DOI: 10.1167/iovs.07-1527

Angiogenesis is the sprouting of new capillary blood vessels from existing vessels. Under physiological conditions, angiogenesis is an essential biological process that satisfies the increasing metabolic demand for nutrients and oxygen in rapidly expanding tissues that depend on adequate blood supply.1,2 Physiological angiogenesis in adults occurs in reproduction and wound repair, and the process is local and transient.3 Under pathologic conditions, angiogenesis plays a central role in cancer and many non-neoplastic diseases, such as rheumatoid arthritis, atherosclerosis, infantile hemangiomas, endometriosis, diabetic retinopathy, and age-related macular degeneration.4 In tumors, proliferating and migrating endothelial cells form tubelike structures that lead to the development of extensive sprouts and eventually to mature blood vessels that are highly tortuous, lack sufficient mural cells such as pericytes, and are hyperpermeable. The increased permeability of these vessels results in the leakage of plasma proteins, which in turn participate in extracellular matrix (ECM) remodeling and tissue destruction.5

The process of angiogenesis is regulated by a delicate balance between a range of angiogenic factors and inhibitors.6 Various stimuli have been reported to shift this balance toward the angiogenic phenotype. Spontaneous mutations can cause the upregulation of oncogenes or the downregulation of tumor-suppressor genes, which are known to trigger the growth of new blood vessels.7 These responses are amplified by accompanying inflammatory responses and environmental factors such as mechanical and metabolic stress. The changes lead to excessive production of pro-angiogenic cytokines, such as vascular endothelial growth factor (VEGF), and to reduced expression of endogenous angiogenesis inhibitors, such as endostatin or thrombospondin-1.8 Inhibiting angiogenesis is, therefore, considered a promising therapeutic approach to fight cancer, age-related macular degeneration, and other angiogenesis-dependent diseases.

Recently, several angiogenesis inhibitors have been approved by the US Food and Drug Administration (FDA). The first class of drugs developed specifically as angiogenesis inhibitors was the VEGF inhibitors. Bevacizumab (Avastin; Genentech, South San Francisco, CA) was approved for colorectal cancer and non-small-cell lung cancer treatment.9 Ranibizumab (Lucentis; Genentech) was approved for the treatment of age-related macular degeneration. Pegaptanib (Macugen; OSI Pharmaceuticals, Farmingdale, NY) also received FDA approval for the treatment of patients with age-related macular degeneration.10 Additional drugs targeting VEGF and other angiogenic factors are in late-stage clinical testing.10

Many older, established drugs, such as thalidomide, indomethacin, steroids, and spironolactone, have anti-angiogenic properties in addition to their known activities.11–14 The discovery of dual roles and new mechanisms for a well-established drug with known toxicity profiles provides an attractive approach for the treatment of angiogenic diseases.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed in the clinic for the treatment of pain and inflammation because of their anti-inflammatory, analgesic, and anti-pyretic properties. NSAIDs inhibit the catalytic activity of the cyclooxygenase isoenzymes COX-1 and COX-2. These enzymes

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3909
are responsible for the conversion of arachidonic acid to prostaglandin H$_2$ (PGH$_2$). Thus, NSAIDs block the production of prostaglandins (PGs) and thromboxanes (TXs), which are the two main classes of lipid-derived pro-inflammatory molecules. PGs are known to play a role in physiological and pathologic angiogenesis.\textsuperscript{15,17} It has been demonstrated that PG$_E_2$ can induce VEGF production and increase basic fibroblast growth factor (bFGF) mRNA levels.\textsuperscript{18,19} It has also been shown that bFGF can promote the expression of PGs such as PG$_E_2$, partly through the induction of COX-2.\textsuperscript{20,21} Most NSAIDs act as non-selective inhibitors of COX-1 and COX-2 and are almost equally effective as anti-inflammatory agents in the clinic when used at equivalent doses. Differences are mainly found in their toxicity profile, route of administration, and elimination half-life. Common adverse effects include upper intestinal bleeding and renal failure. Increasing evidence suggests that COX-2, the inducible isoform of COX, plays a role in promoting angiogenesis primarily by inducing the proliferation and migration of endothelial cells and by inhibiting apoptosis. Specific inhibitors of COX-2 have, in fact, been shown to inhibit angiogenesis in vitro and in vivo.\textsuperscript{22–24}

Several laboratories, including ours, have shown that NSAIDs can inhibit angiogenesis in the cornea, in tumors, and in endometriosis.\textsuperscript{12,15,25–26} In this study, we used the corneal model of angiogenesis to compare the efficacy of systemic treatment with a spectrum of clinically important NSAIDs on bFGF- and VEGF-induced angiogenesis in the mouse cornea. It should be noted that bFGF leads to local production of VEGF by stromal cells and macrophages in the cornea, contributing to the total neovascular response.\textsuperscript{29} Thus, specific inhibitors of VEGF will diminish a component of bFGF-induced angiogenesis. We also examined the inhibitory effect of NSAIDs on VEGF-induced vascular hyperpermeability in the skin of mice using a modified Miles assay.

**Materials and Methods**

**Mouse Handling**

All animal experiments were performed on 8- to 10-week-old male C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were kept at least 1 week before the experiments. They were housed in groups of five in microisolator cages in the animal facility at Children’s Hospital Boston in a 12-hour on/12-hour off light cycle and were fed autoclaved water and chow ad libitum. Experiments were performed in accordance with federal and institutional guidelines approved by the Institutional Animal Care and Use Committee and with adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were performed under general anesthesia with 2.5% tribromoethanol (Avertin; Sigma Aldrich, St. Louis, MO).

**Drug Treatments**

Mice were treated with various NSAIDs purchased from Sigma Aldrich. Treatments consisted of commonly used NSAIDs, at their maximally tolerated dose, that produced no overt toxicity and no weight loss in excess of 5% per mouse. The doses were consistent with those found in the literature for each NSAID. Mice were treated once daily (unless otherwise noted) by oral gavage or subcutaneous injections with indomethacin (5 mg/kg, subcutaneously), rofecoxib (40 mg/kg orally), celecoxib (68 mg/kg orally twice a day), naproxen (10 and 20 mg/kg orally), ketoprofen (80 mg/kg orally), ibuprofen (20 and 25 mg/kg subcutaneously), aspirin (160 mg/kg orally), and acetaminophen (100 mg/kg orally).

**Miles Vascular Permeability Assay**

Before the experiment, mice were treated with NSAIDs or placebo for 5 days. Three mice were used for each group, and each drug was tested in at least two independent experiments. A modified vascular permeability assay was then performed as previously described.\textsuperscript{30–33} In summary, anesthetized mice kept on warming blankets were injected with 100 μL Evans Blue intravenously into the orbital plexus (1% solution in 0.9% sterile saline). Ten minutes later, two 50 μL boluses of both recombinant human VEGF165 (1 ng/μL) and phosphate-buffered saline (PBS) were injected intradermally into previously shaved skin on the back of each mouse. Approximately 20 minutes after the injections, the mice were humanely killed, the dorsal skin was removed, and the four lesions on the back of each mouse were excised with an 8-mm biopsy punch (Miltex, Bethpage, NY). Evans Blue was then extracted over 5 days at room temperature in formamide (Sigma Aldrich). The absorption of these lesions was measured at 620 nm and then normalized using absorbance of the PBS lesions. The inhibitory effect was also expressed as a percentage representing the difference in VEGF-induced permeability in mice treated with the drug compared with control mice treated with vehicle alone.

**Corneal Micropocket Assay**

Corneal micropocket assay was performed as previously described by Rogers et al.\textsuperscript{34} and is reviewed in detail by Kenyon et al.\textsuperscript{35} Pellets containing 80 ng carrier-free recombinant human bFGF or 160 ng VEGF165 (R&D Systems, Minneapolis, MN) were implanted into micropockets created in the corneas of five anesthetized mice per group. Mice were treated daily during the length of the experiment, 5 (bFGF) or 6 (VEGF) days, with the drugs or placebo. Vascular response was then determined after 5 days for the bFGF pellets and 6 days for the VEGF pellets using a slit lamp. Because the activity of VEGF was lower than that of bFGF, a higher concentration of VEGF was used in the assay over the course of 6 days, as previously optimized.\textsuperscript{34,35} The area of neovascularization was calculated as a vessel area by measuring vessel length from the limbus and clock hours around the cornea, as previously described and illustrated,\textsuperscript{34,35} and by using the following equation: Vessel area (mm$^2$) = [Vessel length × clock hours × 0.02π]. The inhibitory effect is expressed as a percentage representing the difference in vessel area induced by bFGF or VEGF in mice treated with the drug compared with control mice treated with vehicle alone.

**Statistical Analysis**

Statistical significance was determined with the unpaired Student’s $t$-test. All statistical analyses were two sided. $P < 0.05$ was considered statistically significant.

**Results**

**Effect of NSAIDs and Acetaminophen on VEGF-Induced Hyperpermeability**

To examine the effect of systemic NSAIDs on VEGF-induced hyperpermeability, we treated mice with various NSAIDs or acetaminophen for 5 days and carried out a modified Miles vascular permeability assay. Although acetaminophen had no effect on VEGF-induced hyperpermeability, the NSAIDs significantly decreased vascular permeability in mice (Table 1). Treatment with indomethacin caused an average of 89% suppression in VEGF-induced hyperpermeability. Naproxen, celecoxib, and rofecoxib also had strong inhibitory effects, showing an average of 86%, 81%, and 74% inhibition, respectively. Systemic treatment with ibuprofen, aspirin, and ketoprofen resulted in 71%, 71%, and 69% inhibition, respectively (Table 1).

**Effect of NSAIDs on Corneal Angiogenesis**

To examine the effect of NSAIDs on angiogenesis, we used a previously optimized corneal micropocket assay.\textsuperscript{34} Systemic treatment of mice with NSAIDs and acetaminophen resulted in differential inhibition of bFGF- and VEGF-induced angiogenesis from the limbal corneal vessels. Inhibition of corneal vascular-
### Differential Suppression of Angiogenesis by NSAIDs

Inflammatory processes such as infections and wound healing are also associated with increased vessel permeability and angiogenesis. COX-1 and COX-2 are enzymes responsible for the production of a number of different prostaglandins, including PGE₂, which mediate the inflammatory response. PGE₂ has been shown to be associated with angiogenesis and is known to stimulate VEGF expression in rat gastric microvascular endothelial cells. Furthermore, VEGF has been demonstrated to increase COX-2 expression at the transcriptional and post-transcriptional levels in human umbilical vascular endothelial cells, creating a positive feedback mechanism. Studies have shown that COX-2–derived prostaglandins are important for uterine vascular permeability and angiogenesis. In the uterus of COX2⁻/⁻ mice, the expression of VEGF and its receptor are reduced with an accompanying decrease in vascular permeability. Further, several reports have shown that inducible COX-2 modulates the expression of VEGF and its receptors. Inducible COX-2 has also been implicated as an important pro-angiogenic protein in human cancer models. Additionally, the COX product PGE₂ has been found to enhance VEGF-induced extravasation in vivo, supporting the synergistic interaction between these two signaling pathways.

NSAIDs are drugs with anti-inflammatory, antipyretic, and analgesic effects classified by their selectivity in inhibiting COX-1 and COX-2, with varying specificity for one or the other. They have been shown to inhibit tumor growth, though different mechanisms have been proposed. In this study, we examined the effects of several COX inhibitors on bFGF- and VEGF-mediated angiogenesis in a model of growth factor–dependent corneal neovascularization and in an animal model of VEGF-induced permeability. We found that the extent of this suppressive effect varies between different drugs. One of the NSAIDs tested was indomethacin, which is commonly used to reduce fever, pain, stiffness, and swelling. It works by inhibiting the production of prostaglandins known to cause these symptoms. Indomethacin can inhibit oxygen-induced retinopathy of prematurity in animals. Findings in newborns, however, have not always been consistent. Although indomethacin treatment reduced the incidence of patent ductus arteriosus in premature infants, it did not have a significant effect on the outcome of retinopathy of prematurity. Indomethacin is a methyalted indole derivative and a member of the arylandolic acid family. It has two additional modes of action with clinical importance: it inhibits the motility of polymorphonuclear leukocytes, similar to colchicine, and it uncouples oxidative phosphorylation in cartilaginous or hepatic mitochondria similar to salicylates. Our data showed that indomethacin po-

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### Table 1. Effect of NSAIDs on VEGF-Induced Hyperpermeability

<table>
<thead>
<tr>
<th>NSAID</th>
<th>Dose (mg/kg)</th>
<th>Percentage Inhibition of VEGF-Induced Hyperpermeability</th>
<th>Percentage Inhibition of bFGF-Induced Hyperpermeability</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>5</td>
<td>89</td>
<td>66</td>
<td>0.05</td>
</tr>
<tr>
<td>Naproxen</td>
<td>10</td>
<td>86</td>
<td>3</td>
<td>0.05</td>
</tr>
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<td>Celecoxib</td>
<td>68</td>
<td>81</td>
<td>13</td>
<td>0.05</td>
</tr>
<tr>
<td>Rofecoxib</td>
<td>40</td>
<td>74</td>
<td>9</td>
<td>0.05</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>25</td>
<td>71</td>
<td>9</td>
<td>0.05</td>
</tr>
<tr>
<td>Aspirin</td>
<td>160</td>
<td>71</td>
<td>18</td>
<td>0.001</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>80</td>
<td>69</td>
<td>41</td>
<td>0.001</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>100</td>
<td>0</td>
<td>13</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The inhibitory effect is expressed in percentages representing the differences in VEGF-induced permeability in mice treated with drug compared with control mice treated with vehicle alone.

### Table 2. Inhibitory Effects of NSAIDs on bFGF- and VEGF-Induced Angiogenesis in the Corneal Neovascularization Assay

<table>
<thead>
<tr>
<th>NSAID</th>
<th>Dose (mg/kg)</th>
<th>Percentage Inhibition of bFGF-Induced Angiogenesis</th>
<th>Percentage Inhibition of VEGF-Induced Angiogenesis</th>
<th>No. of Eyes per Group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>5</td>
<td>57</td>
<td>66</td>
<td>13</td>
<td>0.001</td>
</tr>
<tr>
<td>Naproxen</td>
<td>10</td>
<td>44</td>
<td>41</td>
<td>8</td>
<td>0.001</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>68</td>
<td>43</td>
<td>13</td>
<td>18</td>
<td>0.001</td>
</tr>
<tr>
<td>Rofecoxib</td>
<td>40</td>
<td>44</td>
<td>3</td>
<td>6</td>
<td>0.005</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>20</td>
<td>30</td>
<td>41</td>
<td>8</td>
<td>0.001</td>
</tr>
<tr>
<td>Aspirin</td>
<td>160</td>
<td>14</td>
<td>4</td>
<td>8</td>
<td>0.02</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>80</td>
<td>14</td>
<td>41</td>
<td>8</td>
<td>0.05</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>100</td>
<td>0.7</td>
<td>13.3</td>
<td>8</td>
<td>0.95</td>
</tr>
</tbody>
</table>

The inhibitory effect is expressed in percentages representing the differences in vessel areas induced by bFGF or VEGF in mice treated with drug compared with control mice treated with vehicle alone.

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**Differential Suppression of Angiogenesis by NSAIDs**

Angiogenic cytokines such as bFGF and VEGF are strong chemottractants for endothelial cells and also induce endothelial mitosis. It has been known that VEGF induction leads to the recruitment of endothelial cells, followed by the formation of tube-like structures. VEGF also acts as a survival factor for the newly formed vessels. Initially identified as vascular permeability factor, VEGF exerts permeabilizing effects more potent than those of histamine. Indeed, hyperpermeability of blood vessels is a central characteristic in the pathogenesis of many diseases. In age-related macular degeneration, for example, leaky choroidal neovascularization causes exacerbations of blood cells, fluid lipids, and proteins, which lead to the degeneration of retinal photoreceptors.

Inhibitory effects on bFGF-induced angiogenesis and from 3% to 66% for VEGF-induced angiogenesis (Table 2). Daily treatment with indomethacin (5 mg/kg) had the most potent inhibitory effect on bFGF-stimulated (57%) and VEGF-stimulated (66%) angiogenesis in the mouse cornea. Treatment with celecoxib (68 mg/kg twice daily) resulted in strong inhibition of bFGF-induced angiogenesis (45%) but weak inhibition of VEGF-induced angiogenesis (13%). Daily treatment with rofecoxib (40 mg/kg) also resulted in strong inhibition of bFGF (44%) but no significant inhibition of VEGF-induced angiogenesis. Orally administered ketoprofen (80 mg/kg) and naproxen (10 mg/kg) daily in mice suppressed bFGF-induced vessel growth by 30% and 32% and VEGF-induced vessel growth by 41% and 21%, respectively. Systemic therapy with ibuprofen, aspirin, and acetaminophen did not have a significant inhibitory effect in either assay (Table 2).

**DISCUSSION**

Angiogenic cytokines such as bFGF and VEGF are strong chemotactants for endothelial cells and also induce endothelial mitosis. It has been known that VEGF induction leads to the recruitment of endothelial cells, followed by the formation of tube-like structures. VEGF also acts as a survival factor for the newly formed vessels. Initially identified as vascular permeability factor, VEGF exerts permeabilizing effects more potent than those of histamine. Indeed, hyperpermeability of blood vessels is a central characteristic in the pathogenesis of many diseases. In age-related macular degeneration, for example, leaky choroidal neovascularization causes exacerbations of blood cells, fluid lipids, and proteins, which lead to the degeneration of retinal photoreceptors.

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tently inhibits bFGF- and VEGF-induced angiogenesis in the cornea micropocket assay and strongly suppresses VEGF-induced hyperpermeability in the Miles assay.

The selective NSAIDs examined in this study were celecoxib (Celebrex; Pfizer, New York, NY) and rofecoxib (Vioxx; Merck, Whitehouse Station, NJ). Celecoxib is used in the clinic for patients with osteoarthritis, rheumatoid arthritis, acute pain, and menstrual pain and symptoms. It also reduces the number of colon and rectum polyps in patients with familial adenomatous polyposis. Unlike the traditional NSAIDs that inhibit COX-1 and COX-2, celecoxib and rofecoxib are inhibitors of COX-2 alone. Although they had less inhibitory effects on angiogenesis than indomethacin, celecoxib and rofecoxib blocked bFGF-induced angiogenesis significantly by an average of 43% and 44%. However, these treatments had little or no effect on VEGF-induced corneal angiogenesis (Table 2). These data show that the selective inhibition of COX-2 suppresses bFGF-induced neovascularization more effectively than VEGF-induced neovascularization. In contrast, indomethacin and ketoprofen, which are nonselective COX-1 and COX-2 inhibitors, suppressed bFGF- and VEGF-induced angiogenesis significantly. Naproxen suppressed bFGF-induced angiogenesis slightly more effectively than VEGF-induced angiogenesis, but the difference was not significant. Ibuprofen and aspirin, which are also nonselective COX-1 and COX-2 inhibitors, effectively suppressed permeability in the Miles assay but did not have a strong effect on corneal angiogenesis in our model.

The permeability-inducing effect of VEGF is easily suppressed by NSAIDs. Additionally, most NSAIDs suppressed the angiogenesis-stimulating effect of bFGF. However, the selective inhibitors of COX-2 were only effective at suppressing bFGF-induced, not VEGF-induced, angiogenesis. We have previously shown that after implantation of a bFGF pellet in the cornea, some VEGF is produced locally by stromal cells and macrophages, contributing to the total neovascular response. In fact, the inhibition of VEGF with a soluble VEGF receptor can block up to 50% of the neovascularization induced by a bFGF pellet implanted in the cornea by neutralizing this locally produced VEGF. We have found that COX-2-selective inhibitors demonstrate a similar pattern, wherein these agents inhibited approximately 50% of the bFGF-induced angiogenesis but did not directly inhibit VEGF-induced angiogenesis when a VEGF pellet was implanted. Recent reports have shown that celecoxib can directly inhibit VEGF mRNA and protein expression in models of diabetic retinopathy and cancer. Thus, it is probable that the effects of celecoxib and rofecoxib on bFGF-induced corneal neovascularization result from inhibition of the local upregulation and production of VEGF after bFGF pellet implantation. Therefore, selective inhibitors of COX-2 would be best used clinically to treat angiogenic disorders early in the course of disease, before the upregulation and secretion of large amounts of VEGF. Once VEGF is present, selective COX-2 inhibitors would be less active in directly inhibiting VEGF-stimulated angiogenesis, though they would still block VEGF-induced permeability.

In conclusion, NSAIDs have differential effects on growth factor–induced angiogenesis and leakage. Knowledge of these differential effects will enable the selection of the most effective therapy with the least toxicity for the treatment of a particular pathologic process dependent on bFGF or VEGF.

References


