Cyclooxygenase-2-selective Nonsteroidal Anti-Inflammatory Drugs Inhibit Hepatocyte Growth Factor/Scatter Factor-induced Angiogenesis

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ABSTRACT

Epidemiological studies have indicated a reduced risk of malignancies with the use of nonsteroidal anti-inflammatory drugs (NSAIDs), although the exact mechanisms are debated. NSAIDs inhibit angiogenesis, which is a key step for tumor growth. Hepatocyte growth factor/scatter factor (HGF/SF), a potent and independent angiogenic factor, has been implicated in tumorigenesis, but limited knowledge exists on the potential targets for inhibiting HGF/SF-induced pathological angiogenesis. The current study was designed to elucidate the possible role of cyclooxygenase (COX) downstream of HGF/SF during angiogenesis and to evaluate the potential for harnessing NSAIDs as a therapeutic strategy. Known NSAIDs were classified as COX-1 or COX-2 selective based on their activity in a platelet aggregation experiment. The inhibitors were administered into a polyethylene polyurethane scaffold implant in mice at the selected doses, and the total neovascularization after the administration of HGF/SF was quantified using a 133Xenon clearance technique, vessel counts, and immunohistochemistry. Angiogenesis was also quantized into chemoinvasion, migration, proliferation, and tube formation events in vitro, and the effects of the NSAIDs were evaluated on HGF/SF-induced activity of human umbilical vein endothelial cells (HUVECs). HGF/SF accelerated the angiogenic process in the murine implant, and this activity was inhibited by COX-2-selective meloxicam and NS398. The COX-1 inhibitors ketoprofen and SC560 failed to inhibit the HGF/SF-induced angiogenic events in vitro and in vivo. A COX-2 blockade inhibited the HGF/SF-induced chemoinvasion and migration of human umbilical vein endothelial cells, without affecting the proliferative or tubulogenic responses. Western blots revealed the induction COX-2 expression after HGF/SF treatment, and the pharmacological inhibition of COX-2 executed a temporal inhibition of phosphorylation of the mitogen-activated protein kinase. The current study, for the first time, implicates COX-2 as a downstream signal during HGF/SF-induced angiogenesis, temporally impinging on the mitogen-activated protein kinase signaling. However, the mediation is restricted to only the early events of the angiogenic process, emphasizing the chemopreventive role for NSAIDs. Few therapeutic options currently exist for HGF/SF-induced pathological angiogenesis, and the vast knowledge on COX-2 inhibitors can be harnessed to design a newer therapeutic approach.

INTRODUCTION

Epidemiological studies have indicated that the use of NSAIDs is associated with a reduced risk of malignancies of the gastrointestinal tract (1) and the pancreas (2). Furthermore, a recent meta-analysis has suggested a reduced risk of breast cancer with the use of NSAIDs (3). NSAIDs act primarily through the inhibition of the COX enzyme, which is involved in arachidonic acid metabolism, and which exists as a constitutive COX-1 and an inducible COX-2 isoform (4). Indeed, numerous studies have demonstrated the overexpression of COX-2, but not COX-1, in premalignant lesions and human malignancies with poor prognosis (5, 6), providing a rationale for the chemopreventive activity of NSAIDs. In a murine model of human familial adenomatous polyposis, the genetic inactivation of COX-2 was shown to reduce substantially the frequency and size of intestinal polyps (7), whereas the selective inhibition of COX-2 was shown to reduce the polyp burden in patients (8). Interestingly, a recent study reported the separation of COX-2 inhibition from the apoptosis-inducing activity of NSAIDs (9), spurring further an intense debate focusing on many mechanisms for the anticancer activity of NSAIDs.

In a classical study, Tsuji et al. (10) demonstrated that COXs are implicated in tumor angiogenesis, suggesting a novel mechanism for the anticancer activity of NSAIDs. Angiogenesis, the formation of new blood vessels from an existing vascular bed, is a key step in tumor growth and metastasis (11). Many molecules that induce tumor angiogenesis have been characterized, including VEGF, FGF, angiopeptins, HGF/SF, and others, acting through mediators such as VEGF, tyrosine kinases, nitric oxide, prostaglandins, and so forth (12). The past decade has seen a concentration of efforts to develop inhibitors and antagonists to block pathological angiogenesis, primarily targeting VEGF (13). However, in a recent study, we have demonstrated that HGF/SF, which is associated with an aggressive phenotype in multiple cancer pathologies and has been implicated in angiogenesis (14), can independently and sufficiently induce angiogenesis even in the presence of a VEGF blockade (15). This finding emphasized the need for newer strategies that target a broad spectrum, but site-specific, blockade.

HGF/SF was discovered independently both as a mitogen for hepatocytes and as a fibroblast-derived factor that induced scattering in polarized epithelial cells (16, 17). An elevated level of HGF/SF has been implicated in malignancies of the gastrointestinal tract (18). Lamzus et al. (19) demonstrated that HGF/SF conferred a growth advantage to human breast cancer xenotransplants, linked with a higher microvessel density. Strategies to block HGF/SF-induced angiogenesis and tumor growth have included using a mixture of three antibodies or ribozymes (20, 21); however, these technologies need to be evaluated further before clinical applications. Interestingly, in a recent study, HGF/SF was shown to trigger the activation of the COX-2 gene in gastric epithelial cells (22). The same group also demonstrated that angiogenesis was susceptible to the inhibition of COX (23). Therefore, the current study was designed to elucidate the interaction between HGF/SF and COX and evaluate the possibility of harnessing NSAIDs in the modulation of HGF/SF-induced angiogenesis.

MATERIALS AND METHODS

Preparation of HGF/SF. Murine recombinant HGF/SF was used for the mouse experiments and was generated using the NSO mouse melanoma cell line transfected by electroporation with a mouse HGF/SF cDNA. For experi-
ments involving human endothelial cells, the NSO cells were transfected with a human HGF/SF cDNA to generate recombinant human HGF/SF. The proteins were purified by elution through a heparin-Sepharose column and a Mono Q column.

Chemicals. The 5-LOX inhibitor NDGA and the preferential COX-1 inhibitor ketoprofen were from Sigma Chemical Co. The selective COX-2 inhibitor NS398 [N-2(Cyclohexyloxy)-4-nitrophenyl)methanesulfonamide] was procured from RBI, whereas the selective COX-1 inhibitor SC560 [5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoroethyl]pyrazole] was obtained from Calbiochem. The 5-LOX-activating protein inhibitor L-655-238 was obtained from Alexis Corporation, whereas LY294002, a PI3K inhibitor, was purchased from Tocris. Meloxicam was a gift from Prof. S. K. Gupta (All India Institute of Medical Sciences, New Delhi, India). Hynorm and Hypnovel were obtained from Janssen Pharmaceutica and Roche, respectively. Radioactive 133Xe was purchased from DuPont Pharma. Biomatix was obtained from TCS Biologicals.

Platelet Aggregation Experiments. Platelet aggregation was performed according to the protocol of Born (24), using a Payton Dual Channel Aggregation Module linked to a Kipp and Zonen chart recorder. Light transmission through platelet-poor plasma was fixed as 100%, whereas light transmission through platelet-rich plasma was set as 0%. Exclusion criteria included intake involving human endothelial cells, the NSO cells were transfected with a human HGF/SF cDNA to generate recombinant human HGF/SF. The proteins were purified by elution through a heparin-Sepharose column and a Mono Q column.

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belling the vessels using an endothelial-specific marker and stereol-
as a function of the total vessels entering the implant, by immunola-
plant murine model. The neovascularization response was quantified
of HGF/SF was studied using a polyether-polyurethane scaffold im-
level of significance set at
ANOVA, followed by Newman-Keuls or Bonferroni’s post hoc test, with the
level of significance set at P < 0.05.

RESULTS

Selectivity of NSAIDs for COX-1 and COX-2 Isoenzymes. To test for the concentration range at which the NSAIDs exhibit selectivity for COX isoforms, we studied their activity on the aggregation of human platelets. As shown in Fig. 1A, collagen induced complete aggregation of the platelets, which was completely inhibited by ketoprofen at a concentra-
tion of $10^{-6}$ m, the concentration at which neither NS398 nor meloxic-
am had any inhibitory effects. Significant inhibition of aggregation was observed at $10^{-5}$ m for meloxicam and $10^{-4}$ m for NS398 (Fig. 1A and B), suggesting that the selectivity ratio for COX1:COX-2 decreases in the order of ketoprofen $\rightarrow$ meloxicam $\rightarrow$ NS398 (Fig. 1C). Indeed, in an extensive study using Chinese hamster ovary cells transfected with COX-1 or COX-2, Riendeau et al. (26) demonstrated that meloxicam and NS398 were selective inhibitors of COX-2 with an IC$_{50}$ in the nanomolar range and a COX-2/COX-1 selectivity ratio of 300, although we found NS398 to be less potent than meloxicam in inhibiting COX-1. The same study also reported that ketoprofen failed to completely inhibit COX-2.

Induction of Angiogenesis by HGF/SF in Vivo. The in vivo effect of HGF/SF was studied using a polyether-polyurethane scaffold implant murine model. The neovascularization response was quantified as a function of the total vessels entering the implant, by immunola-
belling the vessels using an endothelial-specific marker and stereol-
ology and by measuring the clearance of a radioactive tracer after it is injected into the implant. As shown in Fig. 2, A–D, the administration of HGF/SF (30 ng/scaffold) resulted in a temporal acceleration of the angiogenic process, with a peak vascularization evident at day 15. Peak vascularization was reached on day 28 in the control groups. Fig. 2 (inset, Table 1) shows the half-life of clearance of the radioactive $^{133}$Xe from the implants as measured on days 7, 15, and 28. During this period, the total clearance from the skin remained constant, excluding any effect of environmental parameters (Fig. 2E). Interestingly, although the total clearance of $^{133}$Xe was unaltered between days 15 and 28 in the HGF/SF-treated group, more vessels were evident on day 15. Furthermore, there was no difference between HGF/SF and vehicle-treated groups by day 28, and the window of discrimination offered on day 15 was harnessed to evaluate the effects of the selective inhibitors of the COX enzymes.

Effect of NSAIDs on HGF/SF-induced Angiogenesis in Vivo. To study the involvement of COX-1 and COX-2 in HGF/SF-induced angiogenesis, the selective pharmacological inhibitors were adminis-
tered into the implants before the administration of the growth factor. The vehicle used was 0.05% ethanol in PBS, and the angiogenic outcome in this group was not different from an untreated implant. As shown in Fig. 3, A–C, meloxicam (1 nmol; $\sim 5 \times 10^{-6}$ m) blocked the HGF/SF-induced neovascularization as evident from vessel counts, $^{133}$Xe clearance, and immunolabeling studies. The dose of meloxicam was selected such that it would inhibit COX-2 in addition to partially inhibiting COX-1, therefore, acting as a nonspecific in-
hibitor of COX.

The COX-2 selective NSAID NS398 also exhibited a dose-depen-
dent inhibition of HGF/SF-induced angiogenesis (Fig. 3, D and E), without altering the basal neovascularization. Treatment with neither meloxicam nor NS398 resulted in any abnormalities or loss of body weight at the doses used (Fig. 3F).

Ketoprofen, at a dose range where it selectively inhibited COX-1, did not inhibit the HGF/SF-induced neovascularization (Fig. 3, G and H). Fig. 3 (inset, Table 2) shows the half-life ($T_{1/2}$) of clearance of $^{133}$Xe from the implants, in which a shorter $T_{1/2}$ indicates a faster clearance resulting from enhanced blood flow. Ketoprofen failed to
block the HGF/SF-induced increase in functional vasculature, which was inhibited by both meloxicam and NS398.

Effect of NSAIDs on HGF/SF-induced wound Regeneration and Cell Proliferation. To elucidate the interaction between COX isoenzymes and HGF/SF, we used a multiple scratch model to mimic the pathophysiological situation and amplify biochemical signals.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>7th Day</th>
<th>15th Day</th>
<th>28th Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>304±10 s</td>
<td>171±13 s</td>
<td>101±12 s</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>144±12 s</td>
<td>113±2 s</td>
<td>164±2 s</td>
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Fig. 2. Temporal effect of HGF/SF on angiogenesis into a scaffold implanted in vivo. A sterile polyester-polyurethane scaffold was implanted s.c. on the dorsum of a mouse. Treatment with HGF/SF (30 ng/scaffold) or vehicle was started 24 h after implantation and continued for 10 days. Angiogenesis into the scaffold was quantified using immuno- staining, and vessel count. A, the confocal immunofluorescence micrographs of scaffold sections labeled with an antibody against von Willebrand factor to delineate all endothelial cells. The blood vessels are seen as white overlaid on the phase contrast image of the scaffold section. The images were at an original magnification of ×400; the final image size is 141 × 141 μm in area. Images were captured with a resolution of 512 × 512 pixels using a Leica TCS-NT confocal microscope. Antibody binding sites were visualized using a species-specific secondary antibody conjugated to FITC. The fluorochrome was excited using a 488-nm laser line, and the emitted light was captured using 530/30 nm bandpass filters. Controls undertaken by omitting the primary antibody were imaged using the same settings for laser power and gain and showed no specific fluorescence. Angiogenesis was quantified using 133Xe clearance from the scaffolds (B), vessel counts (C), and vessel density (D). E, graph shows the clearance of 133Xe from the skin on the experimental days, included as a control for environmental effects. Table 1 shows the T1/2 of clearance of the radioactive Xe from the scaffold. A shorter T1/2 denotes a more functional vasculature. *, P < 0.05; ***, P < 0.001 versus day 7 vehicle-treated control values; +, P < 0.01 versus concurrent vehicle-treated group. Data shown are mean ± SE (n = 6–10).

Effect of the Inhibition of the Lipoxygenase Pathway on HGF/SF-induced Angiogenesis. The arachidonate metabolism can traverse down the LOX pathway and result in the synthesis of leukotrienes that have been implicated in angiogenesis (29). To dissect out any role of LOXs in HGF/SF-induced angiogenesis, we used NDGA and L655238, which at the concentrations used inhibited LOX (30) and 5-LOX-activating protein (31), respectively. As shown in supplementary Fig. 1, neither NDGA nor L655238 exerted any effect on HGF/SF-induced angiogenesis or cell proliferation, although loss of cells was evident at high concentrations.

Fig. 5 shows the effects of COX-1-selective inhibitors, ketoprofen and SC560, on the HGF/SF-induced regeneration and cell proliferation. Unlike COX-2 inhibitors, ketoprofen failed to inhibit the HGF/SF-induced regeneration or cell proliferation at concentrations selective for COX-1. SC560 is a selective COX-1 inhibitor (IC50 for COX-1, 9 nM; IC50 for COX2, 50 μM; Ref. 28) and was used to confirm the observation with ketoprofen. As shown in Fig. 5, SC560 failed to alter HGF/SF-induced regeneration at concentrations at which it selectively blocks COX-1.

Effect of COX-1 and COX-2 Inhibitors on HGF/SF-induced Chemoinvasion of Endothelial Cells. One of the earliest steps during angiogenesis is the migration of endothelial cells after invading the basement membrane. To dissect out the possible roles for COX-1 and COX-2 in this phenomenon, we used a chemoinvasion assay in which the endothelial cells were stimulated to invade into a matrix and migrate through pores between two chambers of a transwell. As shown in Fig. 6, the HUVECs demonstrated a strong chemoinvasive behavior toward HGF/SF. In the presence of constant blockade of COX-2 by NS398, the chemoinvasion of cells toward HGF/SF reverted to basal levels in a concentration-dependent manner. In contrast, the highly selective COX-1 inhibitor SC560 failed to alter the HGF/SF-induced chemoinvasion at a COX-1-selective concentration.

Effect of COX-1 and COX-2 Inhibitors on HGF/SF-induced Tube Formation. The administration of HGF/SF (10⁻9 M) induced significant endothelial tubulogenesis compared with vehicle treatment. None of the COX-1 and COX-2 inhibitors were found to alter the basal or HGF/SF-induced tubulogenesis (Fig. 7).

Effect of HGF/SF on COX-2 Protein Expression. As shown in Fig. 8A, the treatment of an injured monolayer of HUVECs with HGF/SF induced a strong expression of COX-2 protein in the cells. This expression was susceptible to the inhibition by a PI3K inhibitor, LY294002. We could not detect any COX-1 in the experiment.

Effect of COX-2 Inhibitors on HGF/SF-induced Phosphorylation of MAPK. We have demonstrated previously that HGF/SF can induce a rapid and prolonged temporal phosphorylation of MAPKs (ERK1/2; Ref. 15). Because the HGF/SF-induced MAPK phosphorylation returns to basal levels before 24 h, the time point at which cellular phenotypes were quantified, we evaluated the effects of the COX-1- and COX-2-selective NSAIDs on the early (30 min) and delayed (12 h) phases of HGF/SF-induced phosphorylation of ERK1 and ERK2. As shown in Fig. 8, neither NS398 nor meloxicam inhibited the early phase. In contrast, the late phase of MAPK phosphorylation was susceptible to the inhibition of COX-2.

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NSAIDS INHIBIT HGF/SF-INDUCED ANGIOGENESIS
Fig. 3. Effect of NSAIDs on HGF/SF-induced angiogenesis in a murine scaffold-granuloma model. Sterile polyether polyurethane scaffolds were implanted s.c. on the dorsum of mice. Treatment was started 24 h after implantation and continued for 10 days. Angiogenesis into the scaffold was quantified using immunohistochemistry, 133Xe clearance, and vessel count. A, confocal immunofluorescence micrographs of HGF/SF- and HGF/SF + meloxicam-treated scaffold sections labeled with antibody von Willebrand factor to delineate all endothelial cells. The images were at an original magnification of ×400; the final image size is 141 × 141 μm in area. Images were captured with a resolution of 1024 × 1024 pixels using a Leica TCS-NT confocal microscope. The fluorochrome was excited using a 488-nm laser line, and the emitted light was captured using 530/30-nm bandpass filters. Controls undertaken by omitting the primary antibody were imaged using the same settings for laser power and gain and showed no specific fluorescence. Nuclear staining was done with DAPI. The effect of meloxicam on HGF/SF-induced angiogenesis was quantified using the total 133Xe cleared from the implant at the end of 6 min (B), and as the total vessel count (C), quantified as the number of vessels entering an implant. The graph shows the effect of NS398 on HGF/SF-induced neovascularization quantified as 133Xe clearance (D) and vessel counts (E). F, the effect of treatment with the NSAIDs on the body weight of the animals. Treatment with ketoprofen, a COX-1 inhibitor, failed to block HGF/SF-induced increase in the 133Xe clearance (G) and vessel counts (H). *, P < 0.05; ***, P < 0.001 versus vehicle-treated controls; #, P < 0.05 versus HGF/SF-treated group. Table 2 shows the T1/2 of clearance of 133Xe from the scaffolds in the different treatment groups. Data shown are mean ± SE (n = 3–8).

DISCUSSION

In the present study, we report that HGF/SF induces angiogenesis by signaling through COX-2, and targeting this isoenzyme using selective COX-2-selective NSAIDs could be harnessed as a therapeutic approach for the inhibition of HGF/SF-induced pathological angiogenesis. Although the NSAIDs were administered locally to precisely dissect the role of COX isoforms in HGF/SF-induced angiogenesis, preliminary results suggest that orally administered NS398, but not ketoprofen, retained the inhibitory effect (see supplementary Fig. 2). Furthermore, we demonstrated that there is a temporal relationship between COX-2 and HGF/SF-induced phosphorylation of MAPK. Using a deconstructural approach in which we replicated the key steps of angiogenesis in vitro, we demonstrated that HGF/SF could promote the tubulogenesis, chemoinvasion, proliferation, and chemokinesis of endothelial cells but that COX-2 is implicated only during the chemoinvasive and the chemokinesis steps.

HGF/SF has been implicated in tumorigenesis and associated angiogenesis (32). Furthermore, it also promotes angiogenesis underlying diabetic retinopathy (33), psoriasis (34), and arthritis (35), both in cohesion and independently of other angiogenic factors. Interestingly, however, research into the mechanisms of angiogenic action of HGF/SF and the development of effective therapeutics against it has been overshadowed by the importance being placed on VEGF.

An interesting approach for inhibiting the HGF/SF-induced angiogenesis would be to block the signal transduction cascade. A few studies had reported a link between HGF/SF and COX-2. For example, it was demonstrated that prostaglandins strongly induce HGF/SF expression in skin fibroblasts (36). Jones et al. (22) demonstrated that HGF/SF could trigger the activation of the COX-2 gene, and HGF/SF was shown to enhance the activity of cytosolic phospholipase A2 and COX and increase the levels of prostaglandin E2 in human gastric carcinoma (37). Furthermore, COX-2 products were shown to accelerate the healing of ulcers by HGF/SF (38). Indeed, the products of COX-mediated metabolism have been implicated in inducing angiogenesis (39). However, despite the circumstantial evidence supporting the involvement of COX in HGF/SF signaling, there has been no study to elucidate the exact implications. In the current study, we evaluated the neovascular response induced by a daily dosing of HGF/SF into a polymer scaffold implant (25). Compared with vehicle-treated controls, by day 15, the administration of HGF/SF induced a significant increase in the total 133Xe cleared, vessel counts, and vessel density, with a decrease in the T1/2 of 133Xe clearance, allowing the pharmacological modulation with NSAIDs to dissect out the involvement of COX in HGF/SF-induced angiogenesis. Interestingly, this discrimination was lost by day 28, suggesting that HGF/SF did not alter the maximal angiogenesis but accelerated the process. Furthermore, at day 28, although the total 133Xe clearance was similar to day 15 in the HGF/SF-treated group, the vessel counts were lower and the half-life was higher. This was consistent with the observations of Reed et al. (40) and could possibly be attributable to active vascular remodeling.

Although the involvement of COX in the angiogenic process is well documented, there exists some disagreement on the extent to which each isofrom is involved. We used NS398 as a highly selective COX-2 inhibitor, meloxicam as a nonselective inhibitor with a preference for COX-2, and ketoprofen as a selective COX-1 inhibitor (41, 42). However, before using the NSAIDs, the dosage range at which each selectively blocks the COX-1 isofrom was established using a platelet aggregation experiment, which involves only the COX-1 isofrom (43). At a concentration range at which NS398 and meloxicam...
cam had no inhibitory activity on platelet aggregation, both the drugs blocked HGF/SF-induced angiogenesis. In contrast, ketoprofen failed to block HGF/SF-induced angiogenesis, although it inhibited platelet aggregation, suggesting that COX-2 is the key isoform involved in mediating HGF/SF-induced angiogenesis. This is supported further by the overexpression of COX-2 after treatment with HGF/SF, and, indeed, a recent study reported that HGF/SF and prostacyclin, a COX-mediated metabolite, synergize for enhanced angiogenesis (44). Other eicosanoids, such as thromboxane-A2, were shown to modulate COX-2-dependent endothelial cell migration (45), and COX-2 inhibitors were demonstrated to block angiogenesis induced via a prostaglandin E2-EP3 signaling (46). An interesting point of note is that the pharmacological inhibition of COX-2 did not completely inhibit HGF/SF-induced angiogenesis, suggesting the involvement of additional pathways.

The angiogenic phenotype is a culmination of sequential temporal events. We, therefore, recruited in vitro assays using endothelial cells for a quantized study of the angiogenic process. Blockade of the monolayer regeneration after a pharmacological inhibition of COX-2 using NS398 or meloxicam and the inability of ketoprofen or SC560, a highly selective COX-1 inhibitor, supported the in vitro findings that COX-2 was required for HGF/SF-induced angiogenesis. Interestingly, even though the LOX products have been implicated in promoting angiogenesis (47), this alternative pathway of arachidonic acid metabolism seems not to be involved with HGF/SF-induced angiogenesis in the current study. In a previous study, we had dissected out a chemokinetic and a proliferation component in this assay, using paclitaxel and actinomycin D (48). This same study also revealed that endothelial cells start replicating after 18 h, and, based on the finding, any experiment in which cell proliferation could skew results was terminated at 16 h. Interestingly, in the present study, although treatment with the COX-2 inhibitors blocked the total regeneration, HGF/SF-induced proliferation was not altered, suggesting that it was the migratory component that was being inhibited. The inhibition of HGF/SF-induced chemo-invasion by NS398 and the failure of SC560 to do so, further supported the implication of COX-2 in mediating the migratory phenotype in endothelial cells. This was in contrast with the reported implication of COX-2 in the VEGF pathway, in which COX-2 selective NSAIDs were found to inhibit cell proliferation (49).

Fig. 4. Effect of COX-2-selective NSAIDs on the HGF/SF-induced repair process. A, photomicrographs depict the regeneration in different treatment groups 24 h after injury. B and C, the concentration-effect curves of NS398 on the basal and HGF/SF-induced wound regeneration and cell count, respectively. D and E, the concentration-effect curves of meloxicam on the basal and HGF/SF-induced wound regeneration and cell count, respectively. Where the NSAIDs were used, the cells were preincubated with the inhibitors for 1 h before the lesioning and addition of HGF/SF. COX blockade was maintained for the entire duration of the study. Data expressed are mean ± SE of at least six separate experiments with duplicate/quadruplicate wells in each. In the wound recovery experiment, data are shown as percentages of 0 h values: *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus HGF/SF-treated controls; +, P < 0.001 versus vehicle-treated controls.

Fig. 5. Effect of COX-1-selective NSAIDs on the HGF/SF-induced repair process. A, photomicrographs depict the recovery in different treatment groups 24 h after injury. B and C, the concentration-effect curves of ketoprofen on the basal and HGF/SF-induced wound regeneration and cell count, respectively. D and E, the concentration-effect curves of SC560 on the basal and HGF/SF-induced wound regeneration and cell count, respectively. Where the NSAIDs were used, the cells were preincubated with the inhibitors for 1 h before the lesioning and addition of HGF/SF. COX blockade was maintained for the entire duration of the study. Data expressed are mean ± SE of at least three separate experiments with duplicate/quadruplicate wells in each. In the wound recovery experiment, data are shown as percentages of 0 h values: *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus HGF/SF-treated controls; +, P < 0.001 versus vehicle-treated controls.
Furthermore, VEGF-induced tube formation was also reportedly inhibited by NS398 (23, 50), whereas in the present study HGF/SF-induced tubulogenesis remained unaltered by either the COX-1- or COX-2-selective NSAIDs. This suggests that the temporal implication of COX-2 during angiogenesis is dependent on the cytokine and that HGF/SF-induced tubulogenesis proceeds via a COX-2-independent mechanism. Interestingly, Tsujii et al. (10) demonstrated that COX-2 could up-regulate the expression of COX-1, and this could be suppressed by a combination of neutralizing antibodies (anti-VEGF, anti-FGF-2, and anti-platelet-derived growth factor or anti-transforming growth factor α/β2) that could not inhibit COX-2 expression. This observation, in light of the current findings, suggests that COX-2 induction could proceed through a VEGF- or FGF-independent pathway and that HGF/SF could be a likely candidate. This is supported further by our previous observation that HGF/SF could induce angiogenesis in the presence of a VEGF receptor blockade (15).

Inhibition of integrins can block the migration of endothelial cells, and a recent observation by Dormond et al. (51) suggests that COX-2-selective NSAIDs can block V3 integrin-mediated angiogenesis (51). Furthermore, in different cell types, HGF/SF has been reported to exert antiapoptotic effects through Bcl-2, the expression of which...
can be modulated by prostaglandins (52, 53). Similar mechanisms could be active here and need additional studies, but we explored the involvement of MAPK. We have demonstrated recently that HGF/SF induced "a rapid and a delayed" biphasic phosphorylation of the MAPK module in endothelial cells and the pharmacological inhibition of MEK could block HGF/SF-induced migration and proliferation. Furthermore, Jones et al. (23) demonstrated that NSAIIDs could block a VEGF-induced rapid phosphorylation of MAPK and inhibit angiogenesis (23). In the current study, both meloxicam and NS398 inhibited the delayed phase of MAPK phosphorylation without altering the rapid phase. This temporal distinction in activation may explain the differences in the phenotypic outcomes when endothelial cells are treated with different cytokines in the presence of NSAIIDs. Furthermore, the escape of the early MAPK phosphorylation from NSAIID inhibition could possibly account for pathways that contribute to residual angiogenesis in the presence of a COX-2 blockade. Intriguingly, we also demonstrated that the inhibition of PI3K could block the HGF/SF-induced delayed phase phosphorylation of MEK and block angiogenesis (54). Furthermore, in a recent study, LY294002, a PI3K inhibitor, was shown to decrease the expression of COX-2 in keratinocytes (55). This was consistent with the findings of this study in which treatment of an injured monolayer of HUVECs with HGF/SF resulted in the overexpression of COX-2, which was susceptible to the inhibition by the PI3K inhibitor LY294002. This raises the possibility of the existence of a HGF/SF→PI3K→COX-2→MAPK link during angiogenesis, but additional studies are warranted. Although epidemiological data strongly support the chemopreventive effects of NSAIIDs for gastrointestinal malignancies and benefits in other solid tumors, the precise mechanism is not yet clear. The current study for the first time demonstrates that COX-2 plays a significant role in the early stages of HGF/SF-induced angiogenesis, and stresses further the position of NSAIIDs in chemopreventive therapeutics. This enthusiasm should be tempered by caution arising from suggestions that COX-2 inhibitors may be associated with adverse cardiovascular events as reported in the Vioxx Gastrointestinal Outcome Research trial (56). Contrasting reports to this trial has evolved from the Celecoxib Long-Term Arthritis Safety Study (57). Nonetheless, the selective expression of COX-2 in pathological vasculature and the normal vasculature expressing COX-1 makes the former isoform an attractive therapeutic target (58). Furthermore, because COX-2 and HGF/SF are coexpressed at high levels in multiple malignancies, it may be possible to generalize the paradigm developed for the use of NSAIIDs in HGF/SF-induced angiogenesis to these conditions, especially given the extensive clinical data that exist on the COX-2-selective NSAIIDs, which can be harnessed to design clinical trials.

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Also, note that this text appears to be a mixture of scientific literature citations and some terms that may be related to the effects or consequences of NSAID inhibition of HGF/SF-induced angiogenesis.
Cyclooxygenase-2-selective Nonsteroidal Anti-Inflammatory Drugs Inhibit Hepatocyte Growth Factor/Scatter Factor-induced Angiogenesis

Shiladitya Sengupta, Lynda A. Sellers, Tereza Cindrova, et al.


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