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

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ABSTRACT

Epidepidemiological 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 polyether polyurethane scaffold implant in mice at the selected doses, and the total neovascularization after the administration of HGF/SF was quantified using a 133Xe 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 blocker 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 of 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 kinases. 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 NSAIDs6 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, Tsujii 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, angiopoietins, 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 electropropagation 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-trifluoromethylpyrazole] 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). Hynpom and Hynpovel were obtained from Janssen Pharmaceutica and Roche, respectively. Radioactive 113mXe 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 of NSAIDs or eicosapentaenoic acid-rich diet within 7 days before donating blood. Informed consent and approval from the donors was requisitioned before collection of 3 ml of venous blood. Platelet aggregation was induced using collagen (40 µg/ml) after pretreatment with the various inhibitors of COXs. Data were represented as light transmission as percentages of untreated controls.

Murine Angiogenesis Model. Male BALB/c mice (20 g; Tucks, United Kingdom) were anesthetized with 4% isoflurane and maintained on 2% isoflurane, using a mixture of oxygen (0.8 liters/min) and nitrous oxide (0.6 liters/min). Two bilateral s.c. air pockets were created in the dorsal subscapular region using a pair of curved blunt forceps. Two sterile polyether polyurethane sponges (160 mm3; Acerc Associates, Bucks, United Kingdom) were inserted into the pockets, and the incision was closed with silk sutures (Mersilk, United Kingdom).

The treatment with vehicle or peptides/drugs was started 24 h (considered as day 0) after the implantation of the scaffolds and continued for 10 days. A Precision Glide 30-gauge needle (Sigma Chemical Co.) was used to deliver the injection, and the total volume administered into the scaffold was kept constant at 40 µl/scaffold. All of the in vivo procedures were approved and confirmed to the United Kingdom Home Office guidelines.

Assay for Functional Status of Neovasculature. On day 15, the animals were anesthetized using a combination of fentanyl citrate/fluanisone and midazolam (diluted 1:1:20 in saline). Vascularization was assessed as a function of the blood flow through the implants by direct injection of 113mXe-containing saline into the scaffold and by monitoring its clearance over a 6-min period. Radioactivity was measured using a microprocessor scalar ratemeter (Nuclear Enterprise), linked to a collimated low energy X-ray/gamma ray NaI-crystal with an AI-entrance window, on an HG-type mount coupled to an NE 5289C premultiplier. The rate meter was connected to an Epson LX850 printer. The data were expressed as the percentage of 113mXe cleared at every 40 s, calculated according to the formula:

\[
\frac{\text{Initial count} - \text{count at } t \text{ (s)}}{\text{Initial count}} \times 100\%.
\]

Although the background radioactivity was negligible, it was subtracted from the counts in the calculations. The neovascularization in the scaffold was correlated with the total 113mXe cleared in a 6-min period and also as a function of the T½ clearance of 113mXe from the scaffold. The T½ clearance was calculated by nonlinear regression curve fitting using a one-phase exponential decay equation.

Macroscopic Vessel Count. After the 113mXe clearance measurements, the animals were sacrificed by cervical dislocation, and the dorsal skin flap was everted. The gross angiogenic response was photographed using a macro lens connected to a Nikon Single Lens Reflex (SLR) camera. Prints were developed on Kodak plates. Angiogenesis was quantified as the in-growth of vessels in the scaffold-granuloma tissue.

Immunostaining for Endothelial Cells. Thin sections (12 µm) were probed overnight with the primary antibody against human von Willebrand factor (1:2000 dilution; DAKO). Antibody-binding sites were visualized using a biotin/avidin antibody-conjugated to FITC (1:150 dilution; Vector Laboratories, United Kingdom). The fluorochrome was excited using a 488-nm laser line, and the emitted light was captured using 530/30-nm bandpass filter. The images were captured at a resolution of 512 × 512 pixels using a Leica TCS-NT confocal microscope. Controls were run alongside, by omitting the primary antibody.

Morphometry. An axial strip sampling technique (25) was used to calculate the numerical density of blood vessels. Using a 96-point square lattice, the volume fraction (Vv) occupied by the fibrovascular growth and the number of vascular profiles within the field area were recorded. Length density per mm3 of sponge was then calculated as:

\[
\text{Length density per mm}^3 = \frac{L_v}{V_v}.
\]

In Vitro Tube Formation Assay. HUVECs, between passages 2 and 6, were plated on 6-well plates and grown to confluence. The cells were synchronized in 1% serum-containing media for 24 h, after which they were plated in 24-well plates (Costar), each well coated with 200 µl of Matrigel (extracellular matrix extracted from Engelbreth-Holm-Swarm murine sarcoma, diluted 1:3 in PBS). The drugs and growth factors were added in the appropriate dilutions in the media, and the cells were allowed to incubate for 16 h. Wherever appropriate, cells were pretreated with the inhibitors, which were maintained for the entire duration of the experiments. At the end of 16 h, the cells were fixed in 10% formalin and visualized with a Nikon Diaphot inverted microscope using a ×20 objective. This time point was selected to avoid any contribution of the cell proliferation component to tubulogenesis.

Endothelial cells form connected tube-like structures with branches when plated on appropriate matrices. The number of branches per view field was counted, and an average of 18 fields were recorded across the two perpendicular axes per well.

Mechanical Injury Regeneration Model. A confluent monolayer of synchronized endothelial cells was scraped with a multichannel wounder, thereby causing 11 parallel lesions, 400 µm wide. Some of the coverslips were fixed immediately after wounding and served as time zero (T0) controls. At 24 h after lesion, the experiments were stopped by washing the coverslips with ice-cold PBS and fixing in 4% formaldehyde. Recovery of the denuded area was quantified by use of a Leica Q500, semiautomated, computerized image analysis system. The image analysis system integrates the discrete shifts in the boundaries of the injury with time and ignores the stray migration of individual cells to avoid any bias. For each coverslip, four fields of view were selected at random. The area of lesion in each field of view was measured, and by use of the formula for T = t0, the lesion area was then converted to give percentage of regeneration relative to T0 values.

Proliferation Assay. The monolayer of endothelial cells were “wounded” as above and cultured in 1% FCS with vehicle or peptides/drugs for a period of 24 h. At the end of this period, they were washed in ice-cold PBS, trypsinized, and counted with a hemocytometer using the trypan blue exclusion method.

Chemoinvasion Assay. Synchronized HUVECs were trypsinized and plated on the top chamber of a biomatrix-coated Transwell (pore size, 8 µm; Costar) at a density of 10⁶ cells/well. The drugs and growth factors were added in the appropriate chambers, and the cells were allowed to incubate for 16 h. The cells that had migrated to the lower side of the membrane were fixed in 10% formalin and stained with H&E. The cells on the top side of the membrane were wiped off using a cotton bud, and those on the lower surface were visualized at ×20 objective using a Nikon Diaphot inverted microscope. The number of cells in a view field was counted, and an average of five random fields were recorded across the two perpendicular axes per well.

Western Blot Detection of the Expression of COX-2 and Phosphorylation of ERKs. A confluent monolayer of cells was wounded as described previously, and the cellular proteins were solubilized by rapid mixing with sample buffer (three times) under reducing conditions. Equivalent amounts of protein per sample were resolved electrophoretically on 10% polyacrylamide gels and transferred onto a nitrocellulose (0.22 µm) membrane. Anti-phospho-ERK1/2 antibody (1:800 dilution; New England Biolabs and Cell Signaling Technologies, respectively), and anti-ERK1/ERK2 total protein antibodies were probed overnight with the primary antibody against human von Willebrand factor (1:2000 dilution; DAKO). Antibody-binding sites were visualized using a biotin/avidin antibody-conjugated to FITC (1:150 dilution; Vector Laboratories, United Kingdom). The fluorochrome was excited using a 488-nm laser line, and the emitted light was captured using 530/30-nm bandpass filter. The images were captured at a resolution of 512 × 512 pixels using a Leica TCS-NT confocal microscope. Controls were run alongside, by omitting the primary antibody.

Morphometry. An axial strip sampling technique (25) was used to calculate the numerical density of blood vessels. Using a 96-point square lattice, the volume fraction (Vv) occupied by the fibrovascular growth and the number of vascular profiles within the field area were recorded. Length density per mm³ of sponge was then calculated as:

\[
\text{Length density per mm}^3 = \frac{L_v}{V_v}.
\]
(1:500 dilution; Santa Cruz Biotechnology) were used to probe the membrane. For the detection of COX-2, the membranes were probed with a 1:200 dilution of a monoclonal antibody against COX-2 (Santa Cruz Biotechnology). Protein level normalization was achieved by probing the blots using a 1:1000 dilution of a polyclonal antibody against β-actin. Where LY294002, the PI3K inhibitor was used, the cells were incubated with the drug for 1 h before wounding. The signal was amplified using a 1:2000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad), and the immunocomplexes were visualized using enhanced chemiluminescence detection (Amer- sham Life Science).

**Statistics.** All experiments were repeated at least three times with replicates, and the data were expressed as mean SE. Data were tested using 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 isosforms, 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 concentration of \( 10^{-6} \) m, the concentration at which neither NS398 nor meloxicam 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 > meloxicam > 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 immunolabelling the vessels using an endothelial-specific marker and stereology 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 administered 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 nmole; \( ~\sim 5 \times 10^{-6} \) m) blocked the HGF/SF-induced neovascularization as evident from vessel counts, \(^{133}\)Xe clearance, and immunolabelling 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 inhibitor of COX.

The COX-2 selective NSAID NS398 also exhibited a dose-dependent 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
Figure 2. Temporal effect of HGF/SF on angiogenesis into a scaffold implanted in vivo. A sterile polyether-polyurethane scaffold was implanted under the dorsal skinfold chamber of athymic mice. 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 immunofluorescence microscopy 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 TMR. The fluorescein isothiocyanate (FITC) 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 (D), vessel counts (C), and vessel density (D). E, graph shows the clearance of 133Xe from the skin on the experimental days, including 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.

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±1 s</td>
<td>101±2 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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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. Contact-inhibited endothelial cells were injured using a multichannel wounder (27), switching them from a physiological “off” state to a pathophysiological “on” state. As shown in Fig. 4, the addition of HGF/SF (10^-9 M) induced a significant regeneration into the denuded area. Furthermore, HGF/SF induced a significant proliferation of endothelial cells (Fig. 4). Both NS398 and meloxicam blocked the HGF/SF-induced regeneration in a concentration-dependent manner without affecting the basal regeneration or the HGF/SF-induced cell proliferation (Fig. 4, A–E).

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.

Effect of the Inhibition of the Lipoxygenase Pathway on HGF/SF-induced Angiogenesis. The arachidonic 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 regeneration or cell proliferation, although loss of cells was evident at high concentrations.

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Fig. 3. Effect of NSAIDs on HGF/SF-induced angiogenesis in a murine scaffold-granuloma model. Sterile polyetherurethane 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, 133 Xe 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 fluorescein 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 133 Xe 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 133 Xe 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 133 Xe 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 T 1/2 of clearance of 133 Xe 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 deconstructional 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 underlyng 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 133 Xe cleared, vessel counts, and vessel density, with a decrease in the T 1/2 of 133 Xe 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 133 Xe 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 meloxi-
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 this 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 chemoinvasion 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).
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) 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

Fig. 6. Effect of COX-1 and COX-2 inhibition on HGF/SF-induced chemoinvasion. A, photomicrographs of H&E images of cells migrated under defined conditions. Data shown are mean ± SE where n = 3, ***, P < 0.001 versus vehicle-treated control; #, P < 0.001 versus HGF/SF (lower chamber)-induced chemoinvasion.

Fig. 7. Effect of COX-inhibitors on HGF/SF-induced cord-like structure/tube formation by HUVECs. A, photomicrographs depict a random field of view and show the alignment of HUVECs under different conditions. Where the selective COX inhibitors were used, they were added to the cell media 1 h before plating. B, the effect of the treatments as quantified by number of branch-points per field of view. The data represents mean ± SE from n = 3, with 18 fields of view captured per well, across the two axes. ***, P < 0.001 versus vehicle-treated group. V, vehicle; H, HGF/SF (1 nM); NS, NS398 (1 μM), a COX-2-selective inhibitor; M, meloxicam (10 μM), a preferential inhibitor of COX-2; K, ketoprofen (1 μM), a selective COX-1 inhibitor.

Fig. 8. Western blot to detect changes in the levels of COX-2 expression after treatment with HGF/SF and the effect of COX-inhibitors on the HGF/SF-induced changes in phosphorylation of ERK1 and ERK2 as a function of time. A confluent monolayer of HUVECs was injured using a multichannel wounding and incubated for fixed lengths of time in the presence of HGF/SF (1 nM). A, probing with an antibody against COX-2 demonstrates the HGF/SF-induced expression of COX-2, which is susceptible to the PI3K inhibitor LY294002. The blot was normalized to β-actin levels. B, meloxicam and NS398, both selective for COX-2, were added 1 h before injury. At fixed time points, the cells were lysed with 3x sample buffer, and equal amounts of proteins were resolved using a 10% SDS-PAGE gel. The phosphorylation changes of ERK1 and ERK2 were probed using a 1:800 diluted monoclonal phospho-specific antibody against ERK raised in rabbit. Equal loading of ERK1 and ERK2 protein was confirmed by reprobing the blots using a 1:500 dilution of a polyclonal antibody raised in goat, against ERK1 and ERK2. V, vehicle; H, HGF/SF (1 nM); NS, NS398 (10 μM); M, meloxicam (10 μM); LY, LY294002.
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 NSAIDs 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 NSAIDs. Furthermore, the escape of the early MAPK phosphorylation from NSAID inhibition could possibly account for pathways that contribute to residual angiogenesis in the presence of a COX-2 blocker. 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 NSAIDs 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 NSAIDs 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 NSAIDs in HGF/SF-induced angiogenesis to these conditions, especially given the extensive clinical data that exist on the COX-2-selective NSAIDs, which can be harnessed to design clinical trials.

REFERENCES

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

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