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Cyclooxygenase-2 increases hypoxia-inducible factor-1 and vascular endothelial growth factor to promote angiogenesis in gastric carcinoma

Shih-Pei Huang[1], Ming-Shiang Wu[1], Chia-Tung Shun[2], Hsiu-Po Wang[3], Chang-Yao Hsieh[4], Min-Liang Kuo[5] & Jaw-Town Lin[1,*]

[1]*Department of Internal Medicine,* [2]*Department of Forensic Medicine and Pathology,* [3]*Department of Emergency Medicine,* [4]*Department of Oncology, National Taiwan University Hospital, National Taiwan University College of Medicine, and* [5]*Laboratory of Molecular and Cellular Toxicology, Institute of Toxicology, National Taiwan University College of Medicine, No. 7, Chung-Shan S. Road, Taipei 100, Taiwan*

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Summary

Cyclooxygenase-2 (COX-2) is an inducible enzyme important in inflammation and which is overexpressed in a variety of cancers. This study investigated its role in angiogenesis of gastric carcinoma (GC). Immunohistochemical examination of surgical specimens showed a positive correlation among COX-2, vascular endothelial growth factor (VEGF), and vasculature in GC. After transfection with a COX-2-expressing vector, the AGS GC cell line showed increases in both proliferation and tube formation of human umbilical vein endothelial cells (HUVECs). These *in vitro* angiogenic effects on HUVECs were reduced either by blocking VEGF or NS-398, a COX-2 inhibitor. To elucidate the mechanism by which COX-2 increases angiogenesis, we established a COX-2-expressing clone, AGS/COX-2, and its vector control clone, AGS/pcDNA3, and verified their functions by determining prostaglandin E2 (PGE2). Among 6 angiogenesis-associated factors, VEGF was considerably expressed in AGS/COX-2. After reducing hypoxia-inducible factor-1α (HIF-1α) protein by antisense HIF-1α transfection, VEGF production was reduced in AGS/COX-2 cells in a dose-dependent manner. We found that HIF-1α increased concomitantly with VEGF after exogenous PGE2 stimulation to wild-type AGS cells, but this effect was blocked by SC19220, a PGE2 receptor antagonist. In addition, pretreatment with NS-398 to reduce PGE2 also effectively suppressed HIF-1α protein accumulation and achieved a similar inhibitory effect on VEGF production as did antisense HIF-1α transfection. Our work supports the COX-2/PGE2/HIF-1α/VEGF pathway possibly contributing to tumor angiogenesis in GC.

Introduction

Cancers develop through multiple mechanistic strategies, including signaling growth, continuing replication, evading apoptosis, and promoting angiogenesis [1]. Evidence is rapidly accumulating that chronic inflammation may contribute to carcinogenesis through upregulation of growth, angiogenesis, and metastasis in a number of neoplasms, including gastric carcinoma (GC) [2]. Clinical studies in patients with chronic inflammatory disease demonstrated an effect on reducing colorectal cancer risk and mortality after long-term use of aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) [3]. Cyclooxygenase-2 (COX-2), an inducible enzyme pivotal in the inflammatory response, converts arachidonic acid to the

*To whom correspondence should be addressed. Fax: +886-2-23947899; E-mail: jawtown@ha.mc.ntu.edu.tw

prostaglandins required to initiate and maintain reactions during the inflammatory process [4]. COX-2 is blocked by NSAIDs and is now the most studied therapeutic target of NSAIDs [4].

Angiogenesis, the process by which new vascular networks develop from preexisting vessels, plays a crucial role in tumor initiation, progression, invasion, and metastasis and is generally accepted as an indicator of the prognosis [5, 6]. The angiogenic process is finely controlled by balancing a vast array of pro- and anti-angiogenic factors. Among them, vascular endothelial growth factor (VEGF) is one of the most potent tumor angiogenic factors, capable of promoting proliferation and migration of endothelial cells and increasing vascular permeability [7]. The main stimulus for VEGF expression comes from hypoxia, which activates the VEGF gene through increasing hypoxia-inducible factor-1 (HIF-1) [8]. HIF-1 is a heterodimeric transcriptional factor consisting of HIF-1α, a regulated subunit, and HIF-1β, a constitutive subunit [9], and is important in activating multiple genes involved in angiogenesis, cell survival, and invasion [10]. HIF-1α is primarily regulated by an oxygen concentration change [9] via degradation through the ubiquitin-proteasome pathway [11]. Interestingly, recent studies discovered that HIF-1α is also regulated by oxygen-independent mechanisms, mainly activation of oncogenes and mutation of tumor suppressor genes [10].

Earlier clinical studies confirmed an association between COX-2 overexpression and GC occurrence [13]. An animal model of GC also showed that celecoxib, a selective COX-2 inhibitor, is effective for the chemoprevention of tumor development [14]. The known mechanisms by which COX-2 promotes carcinogenesis include evasion from apoptosis, suppression of immunity, and promotion of invasiveness [15]. Recently, COX-2 was also found to play a role in tumor angiogenesis [16]. However, the mechanism by which COX-2 promotes angiogenesis in GC remains obscure, especially its effects on various angiogenic factors. Therefore, we conducted the present study to investigate the clinical relationships among COX-2, VEGF, and tumor vascularization on surgical specimens of GC, to clarify the effects of COX-2 on growth and the function of vascular endothelial cells, and to assess the role of COX-2 in the regulation of angiogenic factors in GC cells.

Materials and methods

Surgical specimens

Surgical specimens of GCs from National Taiwan University Hospital were collected for histological examinations. The Institutional Review Board of National Taiwan University Hospital approved the clinical study protocol. Early GC is defined as cancer limited to the mucosa or submucosa, regardless of lymph node metastasis, and advanced GC as cancer invasion beyond the submucosa [17]. Tumors were subclassified into cardiac cancers and non-cardiac cancers for those at the antrum, body, or both. Laurén's histological type [18] and the status of *Helicobacter pylori* (*H. pylori*) infection were also determined.

Immunohistochemical staining

Five-micrometer-thick sections serially cut from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated, and antigen-retrieved. After quenching the endogenous peroxidase activity, sections were blocked for nonspecific binding, incubated with antibodies against COX-2 (1:100 dilution, Cayman Chemical, Ann Arbor, MI, USA) and VEGF (1:50 dilution, R&D Systems, Minneapolis, MN, USA), respectively, and then incubated with a biotinylated secondary antibody. Immunoreactivity was visualized with diaminobenzidine as the chromogen. COX-2 and VEGF immunoreactivities were scored as previously described [9, 20]. A high-COX-2 tumor was defined as a tumor possessing more than 10% positively stained GC cells and a low COX-2 tumor as one with less than 10% positively stained cells (Figure 1a, b). A high-VEGF tumor was defined as a tumor with more than 50% positively stained GC cells and a low-VEGF tumor as one with less than 50% positively stained cells (Figure 1c, d).

Microvessel density (MVD) assessment

Tumor vascularization was assessed by the MVD as previously described [21]. An anti-CD34 monoclonal

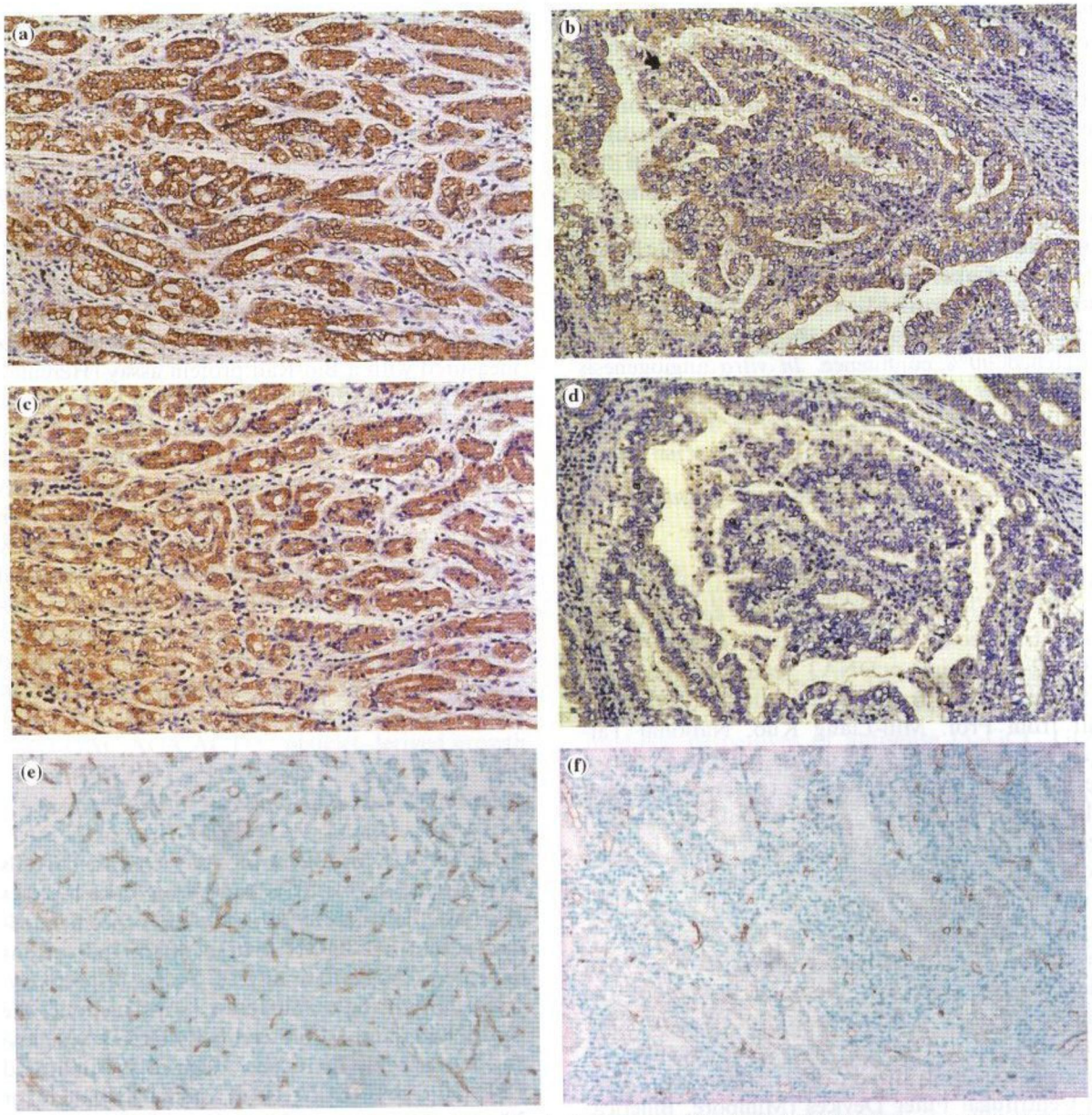

Figure 1. Photomicrographs representative of immunohistochemical staining in GC. (a) High-COX-2 immunoreactivity (more than 10% positive); (b) low-COX-2 immunoreactivity (less than 10% positive); (c) high-VEGF immunoreactivity (more than 50% positive); (d) low-VEGF immunoreactivity (less than 50% positive); (e) high-MVD; and (f) low-MVD. Tumor microvessels were stained with an anti-CD34 antibody which recognized endothelial cells (original magnification: a–d, ×100; e and f, ×40).

antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to recognize CD34 on the endothelium (Figure 1e, f) [22]. After screening at low-power field (×40), microvessels of the five most intensely vascularized areas (i.e., hot spots) were counted at high-power field (×200). The average count of these five hot spots was taken as the MVD to represent the extent of tumor vascularization.

GC cell line and culture

The GC cell line, AGS (CRL-1739), obtained from American Type Culture Collection (Manassas, VA, USA) was grown in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and antibiotics, at 37 °C in a humidified 95% air/5% CO_2 atmosphere.

Preparation of human umbilical vein endothelial cells (HUVECs)

HUVECs were harvested from fresh human umbilical cord veins by collagenase treatment according to a previously described protocol [23]. HUVECs were cultured in M199 complete medium (Gibco BRL) containing 20% fetal bovine serum, 50 IU/ml heparin, 20 μg/ml ECGS (Upstate, Lake Placid, NY, USA), in a humidified atmosphere of 5% CO_2 at 37 °C. HUVECs were subcultured when the cell density approached 90% confluence. *In vitro* angiogenesis assays were performed at passage 2–3.

Transient transfection of COX-2 and preparation of conditioned medium

AGS cells were seeded at a density of 1×10^5 cells/well in a 6-well plate for 24 h and then transfected with indicated doses of the COX-2-expressing pcDNA3 vector containing a 1.9-kbp cDNA fragment of human COX-2 (a gift from Prof. Min-Liang Kuo, National Taiwan University) or 5 μg of the pcDNA3 control vector using the TransFast™ reagent (Promega, Madison, WI, USA), a liposomal transfection reagent, according to the manufacturer's instructions. After 4 h of transfection, cells were recovered in complete medium for 20 h and then replaced in serum-free medium for another 24 h to collect the conditioned medium (CM). When the CM was collected, the total protein of the transfected cells was harvested for Western blot analysis. CM in HUVEC assays was prepared by supernatants centrifuged using Amicron® Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA, USA) at $4000 \times g$ for 15 min for a 10-fold concentrate.

Establishment of the COX-2-expressing GC clone

AGS cells were transfected with the COX-2-expressing pcDNA3 or control vector only using the TransFast™ reagent. After 24 h of transfection, cells were replaced in complete medium with 800 μg/ml G418. Stable G418-resistant transfectants (the COX-2-expressing clone AGS/COX-2 and the control clone AGS/pcDNA3) were selected and then expanded.

Western blot analysis

Cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM NaF, 1 mM Na_3VO_4, 2 mM phenylmethyl-sulfonylfluoride, 1 μg/ml aprotinin, and leupeptin in PBS). Lysates were centrifuged at 12,000 rpm for 25 min at 4 °C. The protein concentration was measured with a Bio-Rad protein assay (Hercules, CA, USA). Each 50 μg of protein sample was separated by 12.5% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and respectively probed with antibodies against COX-1, COX-2, HIF-1α, and HIF-1β purchased from Santa Cruz Biotechnology. Anti-β-actin antibody (Sigma Chemical, St. Louis, MO, USA) was used as a loading control. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies, followed by an enhanced chemiluminescent detection system (ECL, Boehringer Mannheim, Germany).

HUVEC proliferation and tube formation assays

In the HUVEC proliferation assay, endothelial cells were seeded at 5×10^3 cells/well on 96-well plates and cultured with 100 μl of HUVEC-specific medium, serum-free RPMI 1640 medium, and various indicated CM containing 2% serum, respectively. According to the manufacturer's instructions, the neutralizing anti-VEGF antibody (R&D Systems) and non-specific IgG were added to the indicated CM, adjusted to 10 μg/ml, and incubated for 30 min prior to experiments to effectively block VEGF and as the antibody control, respectively. The same medium was changed on the 4th day. Viable endothelial cells were assessed by the MTT assay after culturing for 7 d. The HUVEC tube formation assay was used to evaluate the effect of COX-2 on the functional activity of angiogenesis [24]. Briefly, 300 μl of Matrigel was pipetted into a 96-well plate and allowed to polymerize for 1 h at 37 °C. HUVECs were seeded at 5×10^3 cells/well with the indicated CM and incubated for 6 h. The formation of tube networks was observed using an inverted microscope.

ELISA determination of PGE2 and VEGF

Levels of PGE2 and VEGF in the supernatants were determined by commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. Each measurement was repeated in triplicate, and the average value was recorded as pg/ml.

RNA isolation and reverse transcriptase-PCR of angiogenic factors

Total RNA was extracted and purified from GC cells using the RNAzol B reagent (Biotecx Laboratories, South Loop East Houston, TX, USA). The RT-PCR was performed using an RT-PCR kit (Life Technologies, Gaithersburg, MD, USA). The sequences of the primers were as follows: VEGF (58 °C): sense 5′-CCT GGT GGA CAT CTT CCA GGA GTA CC-3′ and antisense 5′-CTC ACC GCC TCG GCT TGT CA-3′; basic fibroblast growth factor (bFGF) (55 °C): sense 5′-AGT CTC CTG GAG AAA GCT-3′ and antisense 5′-CCC TTG ATG GAC ACA ACT-3′; platelet-derived growth factor (PDGF) (58 °C): sense 5′-AAG CAT GTG CCG GAG AAG CG-3′ and antisense 5′-TCC TCT AAC CTC ACC TGG AC-3′; interleukin-6 (IL-6) (56 °C): sense 5′-ATG TAG CCG CCC CAC ACA GA-3′ and antisense 5′-CAT CCA TCT TTT TCA GCC AT-3′; interleukin-8 (IL-8) (56 °C): sense 5′-CTC TCT TGG CAG CCT TCC TGA TT-3′ and antisense 5′-AAC TTC TCC ACA ACC CTC TGC AC-3′; p53 (55 °C): sense 5′-TAC TCC CCT GCC CTC AAC AAG A-3′ and antisense 5′-GAA TAT TTC ACC CTT CAG GTG CTA AG-3′; and β-actin (56 °C): sense 5′-CGT CTG GAC CTG GCT GGC CGG GAC-3′ and antisense 5′-CTA GAA GCA TTT GCG GTG GAC GAT G-3′. In total, 30 cycles were performed with each cycle consisting of 1.5 min at 72 °C, 1 min at 94 °C, and 1 min at 55–58 °C depending on each gene, with a final extension of 8 min at 72 °C. All amplifications were carried out within the linear range of the assay. β-Actin reactions were amplified for 25 cycles. The products were separated on a 2% agarose gel, stained with 1 μg/ml ethidium bromide, and quantified using an IS-1000 digital imaging system. Results were confirmed by running at least three replicate experiments.

Exogenous PGE2 stimulation and the blocking effect of the PGE2 EP1 receptor antagonist, SC19220, in AGS cells

Wild-type AGS cells were seeded at a density of 1×10^5 cells/well in a 6-well plate for 24 h, serum-starved for 24 h, pretreated with the indicated doses of the PGE2 EP1 receptor antagonist, SC19220 (Cayman Chemical, Ann Arbor, MI, USA), and then stimulated with PGE2 (2.5 μM) (Cayman Chemical) for the indicated duration. Total proteins of AGS cells stimulated by PGE2 for 6 h were collected for Western blot analysis of HIF-1α, and the supernatants in those stimulated by PGE2 for 24 h were harvested for ELISA determination of VEGF.

Anti-sense HIF-1α oligonucleotide and COX-2 inhibitor, NS-398, treatment of COX-2-expressing GC cells [25]

HIF-1α was depleted in GC cells as described previously [26] by loading phosphorothioate-modified derivatives of antisense (5′-GCC GGC GCC CTC CAT-3′) or control sense (5′-ATG GAG GGC GCC GGC-3′) oligonucleotides. AGS/COX-2 cells were washed in serum-free medium and then transfected with the indicated doses of HIF-1α antisense or control sense oligonucleotides. Cells were incubated for 4 h at 37 °C and then replaced with complete medium for 20 h. To block COX-2 function, AGS/COX-2 was pretreated with the indicated doses of NS-398, a COX-2 inhibitor, for 1 h, which was then washed out before serum starvation. After a total period of 24 h of serum starvation, the total protein was harvested for Western blot analysis of HIF-1α and HIF-1β expressions and the supernatant for ELISA determination of VEGF.

Statistical analysis

Continuous data are expressed as the mean ± SD and were compared by Student's *t*-test. Categorical data were analyzed by Fisher's exact test. A *p*-value of <0.05 was considered a statistically significant difference.

Table 1. Demographic features, COX-2[a] and VEGF[b] immunoreactivities, and tumor vasculature of 54 GC patients.

	Total	High-COX-2[c]	Low-COX-2[c]	*p*-Value
No. of cases	54	42	12	
Age (yr)	59.9 ± 13.5	60.6 ± 11.8	57.7 ± 18.7	0.616
Gender (M:F)[d]	28:26	23:19	5:7	0.520
VEGF (H:L)[e]	27:27	25:17	2:10	0.019
MVD[f]	37.8 ± 31.4	43.6 ± 32.2	17.8 ± 17.7	0.001

[a]COX-2, cyclooxygenase-2.
[b]VEGF, vascular endothelial growth factor.
[c]High COX-2, COX-2 immunoreactivity positive in more than 10% of cancer cells; low COX-2, COX-2 immunoreactivity positive in less than 10% of cancer cells.
[d]M, male; F, female.
[e]H, high immunoreactivity of VEGF, defined as more than 50% of cancer cells positive; L, low immunoreactivity of VEGF, defined as less than 50% of cancer cells positive.
[f]MVD, microvessel density, a measure of tumor vascularization expressed as microvessel count (mean ± SD) per high-power field (×200).

Results

COX-2 positively correlated with VEGF and tumor vasculature in GC

This study enrolled a total of 54 GC patients (28 males and 26 females, aged 59.9 ± 13.5 yr). Forty-two patients had high-COX-2 tumors and 12 had low-COX-2 tumors. Both age and gender distributions were similar between these two groups (Table 1). In general, high-COX-2 tumors developed significant vascularization compared with low-COX-2 tumors (43.6 ± 32.2 vs. 17.8 ± 17.1/HPF, $p = 0.001$). The trend of VEGF expression was the same with tumor angiogenesis, i.e., high-COX-2 tumors exhibited significantly high VEGF activity (59%), compared with low-COX-2 tumors (17%, $p = 0.019$). After confirming that both COX-2 and VEGF were positively correlated with the MVD ($p = 0.001$ and 0.011, respectively), we performed a stepwise linear regression and found that the significance of COX-2 activity on determining the MVD was considerably influenced by VEGF expression (COX-2, $p = 0.100$; VEGF, $p = 0.012$). Next, we investigated the association of COX-2 with the vasculature in different clinicopathological subsets of GC (Table 2). Lauren's diffuse type, non-cardiac, and *H. pylori* infection GCs exhibited statistically positive correlations between COX-2 activity and the level of tumor vascularization. In this series, all cardiac GCs had both high COX-2 activity and high MVDs, but all early GCs had low MVDs. Taken as a whole, the investigation on surgical specimens supported the association of COX-2 with VEGF and angiogenesis in GC.

Overexpression of COX-2 in GC cells promotes in vitro angiogenesis

To understand the effects of COX-2 on angiogenesis in GC, we transiently transfected the GC cell line,

Table 2. Tumor vascularization in different subsets of GC stratified by COX-2[a] immunoreactivity.

	Microvessel density[b]		
	High-COX-2[c]	Low-COX-2[c]	*p*-Value
Tumor stage			
Advanced	44.5 ±32.0	28.3 ± 16.2	0.058
Early	5	3.2 ± 2.5	–
Lauren's type			
Intestinal type	40.2 ± 29.9	34.3 ± 22.5	0.746
Diffuse type[d]	51.1 ± 37.0	12.3 ± 12.9	0.003
Location			
Non-cardiac[d]	40.7 ± 30.0	17.8 ± 17.7	0.017
Cardiac	55.6 ± 40.3	–	–
Helicobacter pylori			
Present[d]	42.6 ± 35.1	14.3 ± 15.4	0.007
Absent	45.1 ± 27.9	21.3 ± 20.5	0.073

[a]COX-2, cyclooxygenase-2.
[b]MVD, microvessel density, a measure of tumor vascularization expressed as microvessel count (mean ± SD) per high-power field (×200).
[c]High COX-2, COX-2 immunoreactivity in more than 10% of cancer cells; low COX-2, COX-2 immunoreactivity in less than 10% of cancer cells.
[d]$p < 0.05$ by Fisher's exact test.

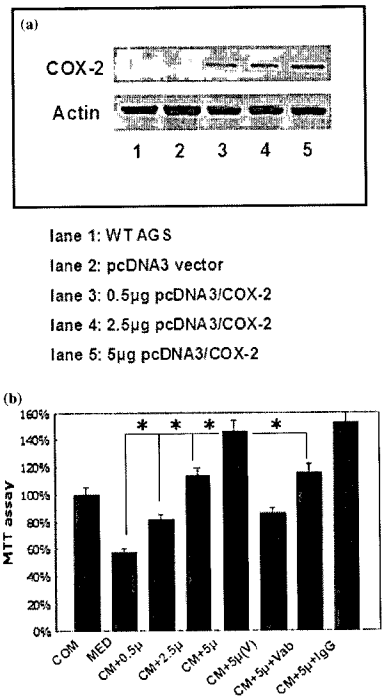

Figure 2. Effect of COX-2 expression in GC cells on HUVEC growth. AGS cells were transfected with the indicated dose of the vector for 4 h and then allowed to recover in complete medium for another 20 h. Cell lysates were subjected to Western blot analysis of COX-2 after transient transfection, and supernatants were harvested for the HUVEC growth assay. (a) Western blot analysis: lane 1, wild-type AGS; lane 2, AGS transfected with 5 μg of the pcDNA3 control vector; lanes 3, 4, and 5, AGS transfected with 0.5, 2.5, and 5 μg of the COX-2-expressing pcDNA3 vector, respectively. COX-2 protein increased as the dose of COX-2 transfected increased. (b) MTT assay of HUVECs as an assessment of growth: column 1, HUVEC-specific culture medium (COM, positive control); column 2, serum-free RPMI 1640 CM (MED, negative control); columns 3, 4, and 5, CM of transfection with 0.5, 2.5, and 5 μg of a COX-2-expressing pcDNA3 vector, respectively; column 6, CM of transfection with 5 μg of the pcDNA3 control vector; column 7, CM of transfection with 5 μg of the COX-2-expressing pcDNA3 vector and blockage by an anti-VEGF antibody; column 8, CM of transfection with 5 μg of the COX-2-expressing pcDNA3 vector and blockage by a nonspecific IgG. Each study was done with three independent experiments, and all results were used for calculating the mean and standard deviation. As the dose of COX-2 transfected increased, HUVECs grew significantly in comparison with the positive and negative controls (*$p < 0.05$ by t-test). Moreover, the effect of COX-2 was effectively prohibited by a neutralizing antibody against VEGF (*$p < 0.05$ by t-test) but not by the nonspecific IgG.

AGS, with a COX-2-expressing vector and then harvested the culture medium supernatants for HUVEC growth and tube formation assays. Western blotting analysis confirmed that COX-2 expression increased in a dose-dependent manner after transient transfection (Figure 2a). Using the obtained CM to culture HUVECs, we found that endothelial cells considerably proliferated as the dose of COX-2 transfection increased from 0.5 to 5 μg ($p < 0.05$), which was even greater than those cultured in HUVEC-specific culture medium (Figure 2b). Although HUVEC growth slightly increased in the vector control group, the extent was considerably less than that of the positive control. Notably, neutralizing antibody against VEGF significantly blocked ($p < 0.05$) although it did not completely abolish the COX-2 effect, as compared with non-specific blocking IgG. Subsequently, we selected and obtained a stable COX-2-overexpressing clone, AGS/COX-2, and a vector control clone, AGS/pcDNA3. Both clones were verified by Western blotting analysis of COX-2 protein expression and ELISA determination of PGE2 levels as an enzymatic functional assessment (Figure 3). In the HUVEC tube formation assay, AGS/

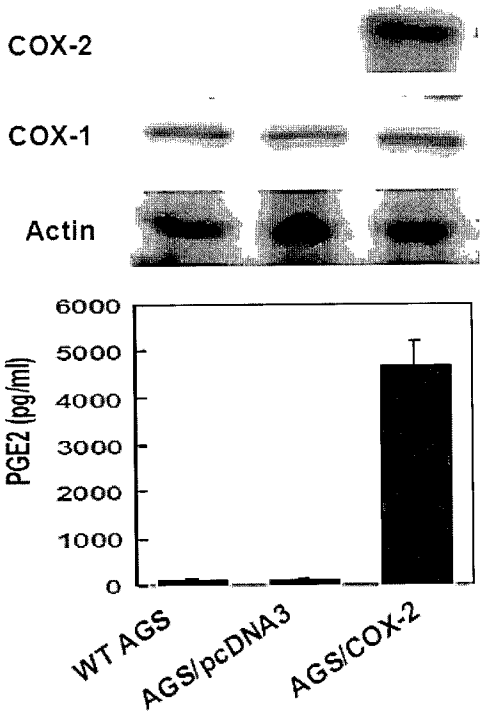

Figure 3. Western blot analysis of COX-2 protein and ELISA determination of PGE2 as the functional assay for COX-2 in AGS cells. Lane 1, wild-type AGS; lane 2, AGS/pcDNA3, the stable transfectant with the pcDNA3 control vector; lane 3, AGS/COX-2, the stable transfectant with the COX-2-expressing pcDNA3 vector. The results confirmed that COX-2 was overexpressed in AGS/COX-2 and functioned to convert arachidonic acid to PGE2.

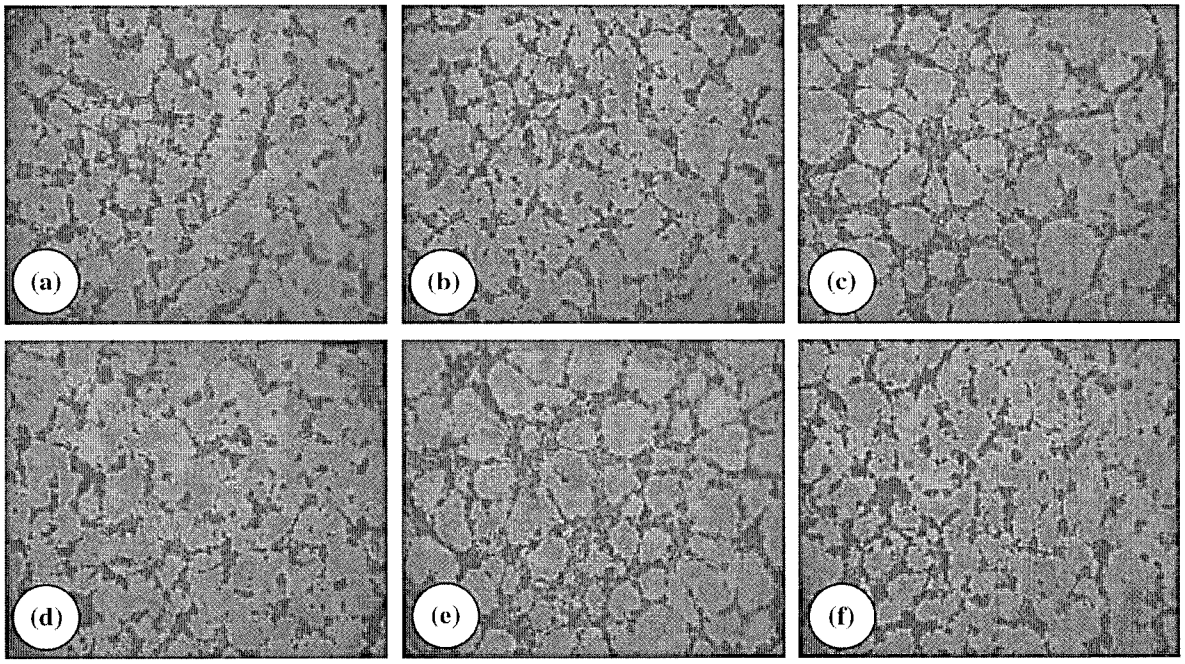

Figure 4. Effect of COX-2 expression in GC cells on HUVEC tube formation. HUVECs were cultured with each indicated CM for 6 h to allow network formation arising from endothelial cell migration. (a) Wild-type AGS; (b) AGS/pcDNA3 as a vector control; (c) AGS/COX-2 overexpressing COX-2; (d) AGS/COX-2, neutralized by the anti-VEGF antibody; (e) AGS/COX-2, blocked by non-specific IgG; (f) AGS/COX-2, pretreated with NS-398, a COX-2 inhibitor, for 1 h. COX-2 expression induced prominent HUVEC tube formation, but wild-type AGS, the vector control, those blocked by the anti-VEGF antibody, and those suppressed by the COX-2 inhibitor cause HUVECs to have a dispersed distribution.

COX-2 effectively promoted network-like formation of HUVECs (Figure 4c), whereas HUVECs were distributed in a dispersed manner with wild-type AGS and AGS/pcDNA3 (Figure 4a, b). Meanwhile, both neutralizing anti-VEGF antibody and the selective COX-2 inhibitor, NS-398, also inhibited HUVEC tube formation (Figure 4d, f), as compared with non-specific blocking IgG (Figure 4e). Collectively, the results showed that HUVEC growth and function were both enhanced by COX-2 overexpression in GC cells, and this provides experimental evidence supporting the findings observed in our clinical study.

Angiogenic factor, VEGF, and HIF-1α were upregulated by COX-2 overexpression through a PGE2-dependent pathway

We wondered which pathway and factors are involved in GC angiogenesis influenced by COX-2. Six candidate angiogenic factors were screened, including VEGF, bFGF, PDGF, IL-6, IL-8, and p53, by RT-PCR of mRNAs from wild-type AGS, AGS/pcDNA3, and AGS/COX-2 cells (Figure 5). We found that VEGF was markedly upregulated after GC cells overexpressed COX-2. IL-8 and bFGF transcripts were modestly increased, whereas the other three genes were not changed. Regarding subtle changes in PDGF, p53, and bFGF in AGS/pcDNA3 in comparison to wild-type AGS cells, a clonal effect was considered. The result further supported the role of VEGF in angiogenesis caused by COX-2. We next attempted to clarify whether the blockage of HIF-1α accumulation in COX-2-overexpressing GC cells can prohibit the effect of COX-2 on VEGF induction, since VEGF is primarily regulated by HIF-1α, and whether HIF-1α is capable of being regulated independently of hypoxic stimulation. Our experiments confirmed that HIF-1α accumulation and VEGF induction caused by COX-2 were concomitantly reduced in a dose-dependent response to transfection of antisense HIF-1α (Figure 6a). PGE2 is a prostaglandin produced by COX-2 catalysis and is important in mediating COX-2's effects. We found that exogenous PGE2 stimulation effectively induced HIF-1α accumulation and simultaneously increased VEGF production in AGS cells; these effects could be reversed by SC19220,

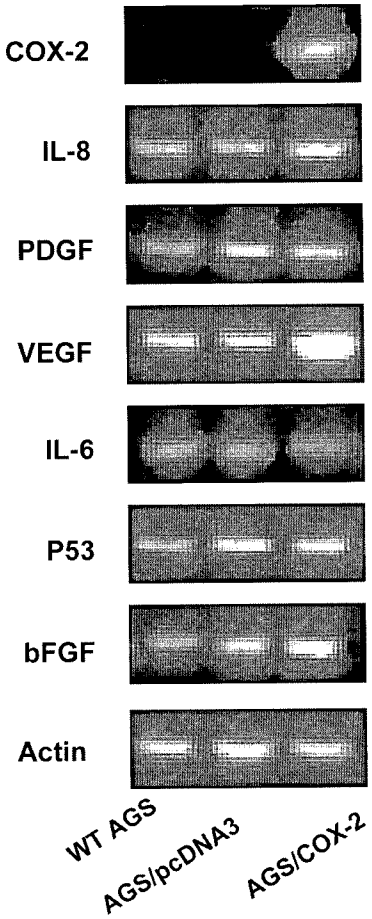

Figure 5. Effect of COX-2 overexpression on induction of angiogenic factors. RT-PCR from total RNA was used to evaluate 6 angiogenic factors, including IL-8, PDGF, VEGF, IL-6, p53, and bFGF. Lane 1, wild-type AGS; lane 2, AGS/pcDNA3; lane 3, AGS/COX-2. VEGF gene expression was markedly increased by COX-2 overexpression; bFGF and IL-8 were modestly increased; but the other three factors did not substantially change.

an antagonist of the PGE2 EP1 receptor, in a dose-dependent manner (Figure 6b). NS-398 is an inhibitor which blocks PGE2 conversion from arachidonic acid by COX-2. Finally, we investigated whether the blockage of PGE2 production by NS-398 can suppress VEGF production. Results were compatible with the above experiments, showing that VEGF expression and HIF-1α accumulation concomitantly decreased in a dose-dependent response to the pretreatment of NS-398 (Figure 6c). Accordingly, COX-2 may increase VEGF by sequential signaling via PGE2 and then HIF-1α in GC cells.

Discussion

An emerging issue in cancer research is the focus on the mechanistic link between chronic inflammation and carcinogenesis, including tumor angiogenesis [2]. COX-2 is an important inducible enzyme mediating inflammatory processes and is highly expressed in a diversity of cancers [4]. Recently, accumulating evidence has indicated that COX-2 may play an important role in the developing neovasculature [16] and therefore might be a potential therapeutic target against tumor angiogenesis [16]. However, the mechanism governing COX-2-induced angiogenesis remains largely unclear. Our initial task of investigating surgical specimens indeed revealed a positive association among COX-2 immunoreactivity, VEGF expression, and GC vascularization. Results were consistent with earlier reports [27–30]. Additionally, we found that the association between COX-2 and MVD was substantially influenced by VEGF through a stepwise regression analysis.

Although studies have discovered some upstream factors capable of stimulating COX-2 expression in cancer cells [12], the downstream effecter molecules by which COX-2 promotes angiogenesis are largely unknown. Our clinical data implied that COX-2 may induce VEGF to promote GC angiogenesis. This hypothesis was further supported by our *in vitro* angiogenesis assessments on HUVECs. First in the experimental study, we examined the effect of COX-2 overexpression on GC angiogenesis by assessing HUVEC growth and tube formation, which are both generally accepted as indicators of *in vitro* angiogenesis. We found that GC cells increased HUVEC proliferation in a COX-2 dose-dependent manner. Meanwhile, COX-2-overexpressing GC cells effectively promoted HUVEC tube formation arising from endothelial cell migration. Our results also showed that proliferation and tube formation of HUVECs were both prohibited either by the COX-2 inhibitor or by an anti-VEGF antibody. The results support COX-2 being an angiogenic factor in GC. Second, we compared six angiogenesis-associated factors among wild-type AGS, AGS/pcDNA3, and AGS/COX-2, which revealed that VEGF gene expression markedly increased. This result was consistent with the HUVEC experiments that blockage of VEGF effectively inhibited HUVEC proliferation and network formation induced by

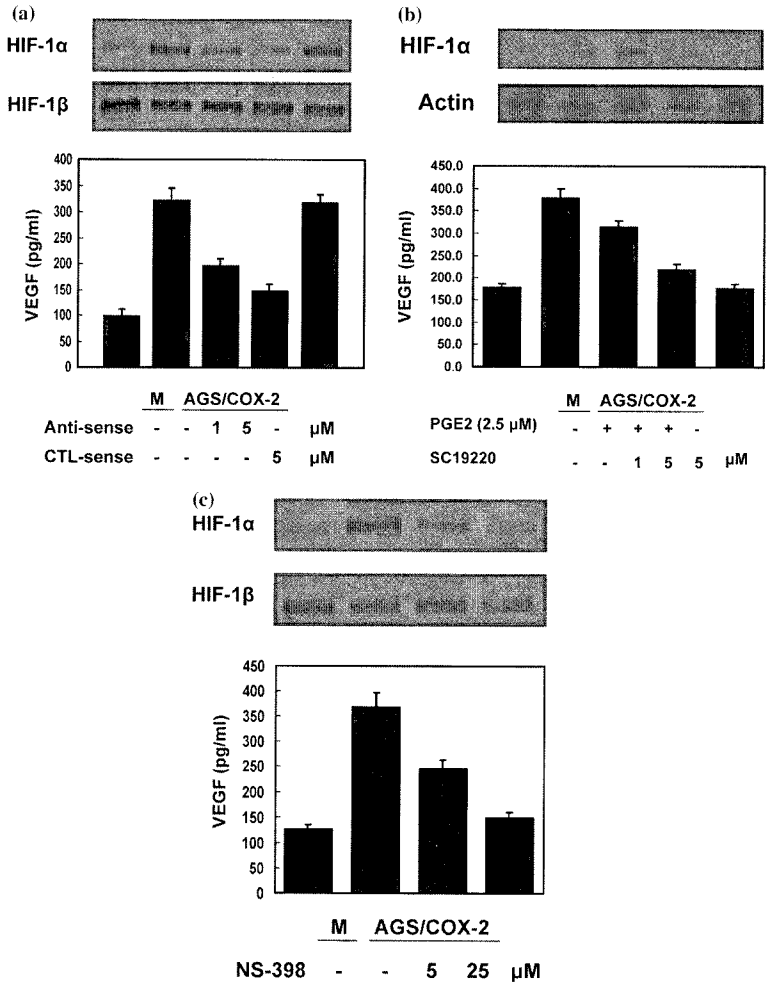

Figure 6. Effect of COX-2 on HIF-1 accumulation and VEGF production. (a) Western blot analysis confirmed that the HIF-1α protein markedly accumulated in AGS/COX-2. HIF-1α was reduced in a dose-dependent response to antisense HIF-1α transfection but not in response to control-sense (CTL-sense) HIF-1α transfection. The ELISA determination showed that VEGF levels changed as HIF-1α did, i.e., VEGF was markedly increased in AGS/COX-2 cells but reduced in a dose-dependent manner by antisense HIF-1α. (b) To determine the COX-2/PGE2/HIF-1α/VEGF pathway, we investigated the effects of exogenous PGE2 stimulation and blocking of the PEG2 EP1 receptor with SC19220. The results confirmed that AGS cells increased HIF-1α and VEGF proteins in response to PGE2, and this effect was reversed by SC19220 in a dose-dependent manner. (c) If AGS/COX-2 was pretreated with NS-398, a COX-2 inhibitor which blocks PGE2 production, both HIF-1α protein and VEGF production, which were increased by COX-2 overexpression, were reduced in a dose-dependent response to NS-398.

COX-2-overexpressing GC cells. In our study, increases in bFGF and IL-8 transcripts were modest but not as significant as the increase in VEGF seen in AGS/COX-2. Results indicated that bFGF, IL-8, and/or other factors not examined in our study may also play a role in COX-2-related angiogenesis, but further studies are needed to address this issue. Because VEGF functions to promote the majority of processes involved in angiogenesis, including endothelial proliferation and migration, vascular lumen formation, and vascular permeability [31, 32], it is suggested that VEGF induction may be one of the main strategies by which COX-2 increases GC angiogenesis.

Under most conditions, hypoxia induces VEGF production through upregulating HIF-1 [33]. HIF-1α dimerizes with HIF-1β and then binds to the hypoxic response element of the target genes, including VEGF [34]. As the most important regulator of VEGF expression, however, HIF-1α is not only controlled by the oxygen concentration but also regulated by several hypoxia-independent

mechanisms, including transition metals, nitric oxide, reactive oxygen species, growth factors, and mechanical stress [35]. Jones et al. reported that NSAIDs increased the expression of the von Hippel Lindau tumor suppressor, which targets proteins for ubiquitination and leads to reduced accumulation of HIF-1α, and this results in suppression of VEGF expression [36]. Surprisingly, we found that concomitant upregulation of HIF-1α and VEGF occurred in COX-2-overexpressing GC cells. We hypothesized that PGE2 might play a role in this process because PGE2 is well known for mediating a vast array of effects by COX-2. Our additional work provided further evidence supporting the link among PGE2, HIF-1α, and VEGF by showing that HIF-1α and VEGF were simultaneously increased by exogenous PGE2 stimulation and reversed by blocking the PGE2 receptor. Moreover, VEGF production was markedly reduced in those cells treated either by the selective COX-2 inhibitor, NS-398 [37], which inhibits excess PGE2 production but does not affect the physiological level of PGE2 [38], or by the antisense HIF-1α, which blocks HIF-1α protein production, respectively. Earlier studies investigating the relationship among COX-2, HIF-1, and VEGF were few. Those studies reported controversial results in different types of cells. A study in prostate cancer cells found that NSAIDs reduced HIF-1 protein levels, but the inhibitory effect might be COX-2-independent [39]. Another study on a lung cancer cell line investigated the role of HIF-1α in interleukin-1β-induced inflammation, and results showed that an HIF-1α transcriptional inhibitor, NSC-609699 [40], suppressed VEGF expression induced by COX-2 [41]. In benign cells, COX-2 was found to mediate the hypoxic effect on HIF-1 activation and VEGF production in retinal cells and hepatic stellate cells, respectively [42, 43]. Collectively, COX-2 may increase angiogenesis through oxygen-independent mechanisms in addition to its role as a hypoxia effecter to increase VEGF expression. Our work supports the COX-2/PGE2/HIF-1/VEGF pathway possibly playing an important role in GC angiogenesis, by which chronic inflammation might be capable of contributing to GC development.

The support for COX-2 as a molecular target for cancer prevention and treatment comes from multiple lines of evidence [12]. Epidemiological studies have shown that NSAIDs reduce the risk of developing malignancies, including colorectal cancer [44]. Animal studies have also shown that NSAIDs protect against the formation of tumors [45]. Currently, many clinical trails are underway to evaluate selective COX-2 inhibitors as agents for preventing or treating human cancers [12]. Our clinical study demonstrated that the correlation between COX-2 and tumor angiogenesis predominated in certain subsets of GC, indicating that diverse mechanisms may govern the differential effects of COX-2 on angiogenesis and also that careful selection of patients is mandatory for the application of COX-2 inhibitors as an anti-GC agent in the future.

One feature of our clinical study was that GCs with *H. pylori* infection exhibited a positive correlation between COX-2 and tumor angiogenesis but those without *H. pylori* infection did not. This result might indicate that inflammation associated with *H. pylori* infection enhances GC neovascularization through COX-2 upregulation. On the other hand, although our earlier research found that the multifunctional inflammatory cytokine, IL-6, can promote VEGF and angiogenesis in GC [46], the present study showed that IL-6 does not mediate GC angiogenesis induced by COX-2.

In summary, our work supports the COX-2/PGE2/HIF-1/VEGF pathway possibly being important in GC angiogenesis. Further research to elucidate other important factors or mechanisms by which COX-2 promotes tumor angiogenesis is needed to shed more light on the use of COX-2 inhibitors in cancer chemoprevention and treatment.

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