Cyclooxygenase-2 Activation Mediates the Proangiogenic Effect of Nitric Oxide in Colorectal Cancer

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ABSTRACT
Purpose: Up-regulation of both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) enzymes has been reported in colorectal cancer. We aimed at evaluating the possible interaction between the nitric oxide and COX-2 pathways, and its effect on promoting tumor angiogenesis.

Experimental Design: Expression of iNOS, COX-2, vascular endothelial growth factor (VEGF), and CD31 was analyzed in tumor samples and corresponding normal mucosa obtained from 46 surgical specimens. We also evaluated iNOS activity, prostaglandin E2 (PGE2), cyclic GMP and cyclic AMP production in the same specimens. Nitrite/nitrate levels, and PGE2 and VEGF production were assessed in HCT116 and HT29 colon cancer cell lines after induction and selective inhibition of the two enzyme pathways.

Results: A significant correlation was found between iNOS and COX-2 immunohistochemical expression. PGE2 production significantly correlated with iNOS activity and cGMP levels. A significant correlation was also found among PGE2 production, microvessel density, and VEGF expression. Coinduction of both iNOS and COX-2 activities occurred after lipopolysaccharide (LPS) and epidermal growth factor (EGF) treatment in HCT116 and HT29 cells. Inhibition of iNOS by 1400W significantly reduced both LPS- and EGF-induced PGE2 production. Treatment with LPS, EGF, and arachidonic acid significantly increased VEGF production in the iNOS-negative/COX-2-positive HT29 cells. This effect was completely reversed by treatment with the selective COX-2 inhibitor celecoxib.

Conclusions: Our data showed a prominent role of nitric oxide in stimulating COX-2 activity in colorectal cancer. This interaction is likely to produce a cooperative effect in promoting angiogenesis through PGE2-mediated increase in VEGF production.

INTRODUCTION
The formation of new blood vessels, i.e., angiogenesis, is necessary to supply oxygen and nutrients to solid tumors that are ≥2 mm in diameter (1). This phenomenon is the result of the activation of several signaling pathways by the promotion of proangiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, transforming growth factor β, and platelet-derived growth factor (2). In colorectal cancer, two enzymatic pathways have emerged recently as being closely involved in tumor angiogenesis: one is the cyclooxygenase (COX) pathway and the other is the nitric oxide synthase (NOS) pathway.

COX is a rate-limiting enzyme in the biosynthesis of prostanoids from arachidonic acid (AA). Two isoforms of COX have been identified (3). COX-1 is considered a housekeeping gene and is constitutively expressed in most normal tissues, whereas COX-2 is rapidly induced by cytokines and tumor promoters (4). COX-2 is up-regulated in most human tumor cells (5) and the antitumor properties of nonsteroidal anti-inflammatory drugs, i.e., COX inhibitors, have been demonstrated by epidemiological studies and in some experimental models (reviewed in Ref. 6). One of the most important metabolites of COX-2 activity is prostaglandin E2 (PGE2), which is produced in large amounts in colorectal tumors (7, 8) and can induce the production of angiogenic factors in many cell types (9–11).

NO is produced by three different isoforms of NOS: the neural and the endothelial NOSs are constitutively expressed and Ca2+ dependent, whereas the inducible isoform or inducible NOS (iNOS) is Ca2+ independent and can be expressed in response to proinflammatory agents (12, 13). iNOS produces high and sustained concentrations of nitric oxide (NO), whereas the NO produced by the other two isoforms is characterized by low, transient levels (14). Several studies have shown increased expression of iNOS in several types of tumors such as gynecological, head and neck, breast, central nervous system, and colorectal cancer (reviewed in Ref. 15).

A large body of evidence suggests that both COX-2 and iNOS may play pivotal roles in cancer development by promoting angiogenesis in animal model and human tumors (16–21). We have shown recently that VEGF is one of the most important proangiogenic factors involved in the modulation of both COX-2- and iNOS-mediated angiogenesis in human colorectal cancer (8, 22). It has been also demonstrated that VEGF-mediated angiogenesis is dependent on NO production (23) and...
requires the activation of the NO/cyclic GMP (cGMP) pathway within the endothelial compartment (24). Moreover, prostaglan-
dins are known to activate adenylate cyclase and, thus, increase
the intracellular levels of cyclic AMP (cAMP; Ref. 25). Cyclic
AMP-dependent signaling is responsible for the induction of
angiogenesis through the activation of VEGF in several types of
cultured cells (26–29).

Previous reports have shown that NO may be involved in
stimulating prostaglandin biosynthesis through the activation of
COX-2 in many cell systems, especially in inflammatory models
(30–31). One of the possible mediators of this effect is per-
oxynitrite, the coupling product of nitric oxide and superoxide
(32). Only a few data exist regarding the possible role of the
interaction between COX-2 and iNOS activities in carcinogene-
sis. A coexpression of these two enzymes and its involvement in
the process of angiogenesis has been demonstrated in hepa-to-
cellular carcinoma (33), non-small cell lung carcinoma (34),
head and neck cancer (35), and breast and colon cancer (36, 37).

The aims of this study were to investigate the iNOS and
COX-2 pathways in human colorectal cancer and to determine
whether any correlation exists between the expression/activity
of these two enzymes. The interaction between iNOS and
COX-2, and its possible role in stimulating VEGF production
were also evaluated in two human colon cancer cell lines with
different iNOS and COX-2 constitutive expression.

MATERIALS AND METHODS

Patients. Tissue samples were obtained from 46 consec-
tutive patients (20 males; 26 females; median age, 66 years; age
range, 46–81 years) who had undergone surgical resections for
primary sporadic colorectal tumors at the Department of Gen-
eral Surgery, University of Florence. None of the patients had
taken any nonsteroidal anti-inflammatory drugs for at least 3
months before surgery. All of the patients had been thoroughly
informed about the study and gave written consent for the
investigation in accordance with the ethical guidelines of our
University. Nine tumors were located in the proximal colon (up
to the splenic flexure), 20 in the distal colon, and 17 in the
rectum. Forty tumors were adenocarcinomas, and 6 were clas-
sified as colloid. Adenocarcinomas were classified as well dif-
ferentiated (well differentiated), moderately differentiated
(moderately differentiated), and poorly differentiated (poorly
differentiated). Tumors were classified into four
stages according to the American Joint Committee on Cancer
staging system (38), stage I (T1–T2, N0, M0; n = 5); stage II
(T3–T4, N0, M0; n = 19); stage III (any T, N1–2, M0; n = 18);
and stage IV (any T, any N, M1; n = 4). Cancer tissue (from
the edge of the tumor) and adjacent normal mucosa (at least 10 cm
from the tumor) were excised from each surgical specimen. The
samples were washed in PBS. They were then flash frozen in liquid
nitrogen for Northern blot analysis, frozen at −80°C for Western
blot analysis, and frozen at −20°C for iNOS activity, and
cGMP, PGE2, and cAMP production evaluation until processing.
Paraffin-embedded tissue sections were cut from tumor
blocks for immunohistochemical analysis.

Immunohistochemistry. Four-μm-thick sections were cut
from formalin-fixed and paraffin-embedded tissue blocks,
and processed as described previously (8). Immunohistoche-
chemical staining was performed using the streptavidin-biotin
method. Staining for CD31, an endothelial antigen, was used to
highlight the endothelial cells and determine microvessel den-
sity (MVD). The following antibodies were used: a monoclonal
mouse antibody (JC 70; Dako, Milan, Italy) for CD31 at a 1:10
dilution, at room temperature for 30 min; an affinity-purified
rabbit polyclonal antibody (A-20; Santa Cruz Biotechnology,
Santa Cruz, CA) for VEGF at a 1:100 dilution, at room tem-
perature for 1 h; a rabbit polyclonal antibody for iNOS (Biomol,
Plymouth Meeting, PA) at 1:600 dilution, at 4°C overnight;
and an affinity-purified goat polyclonal antibody (C-20; Santa Cruz
Biotechnology) for COX-2 at a 1:40 dilution, at room temper-
ature for 1 h. Tissue sections, treated with nonimmune rabbit
serum in place of the primary antibodies, were used as negative
controls.

Evaluation of Immunostaining and Microvessel Count-
ing. The immunostained specimens were independently eval-
uated by two blinded investigators. The extent of iNOS, COX-2,
and VEGF staining was recorded using a three-grade system,
based on the percentage of stained tumor epithelial cells: grade
0 = 0–10%; grade 1 = 11–70%; grade 2 = >70%.

MVD was evaluated according to the method first de-
scribed by Weidner et al. (39). The entire tumor section was first
carefully scanned at low magnification (×100) to find the areas
that showed the most intense neovascularization, i.e., the highest
density of CD31-positive cells (hot spots). The hot spots were
included in the MVD counts, but only if they had been in the
stroma surrounded by malignant cells and if they had not been
found within any areas of granulation tissue, such as those near
the surface of ulcerated tumors. Individual microvessels in each
hot spot were then counted in a single ×250 field (Fig. 1A). Any
immunoreactive endothelial cell or endothelial cell cluster that
was clearly separated from the adjacent microvessels was con-
sidered as a single countable vessel. No vessel lumina or RBCs
were used to define a microvessel. The occasionally found
CD31-positive lymphocytes, macrophages, and plasma cells
were excluded on the basis of the staining pattern and/or cell
morphology. MVD in each tumor was expressed as the mi-
crovessel count of the hot spot with the highest number of
microvessels.

Cell Culture and Reagents. Experiments were per-
formed on the HCT116 and HT29 human colon cancer cell lines
(both were a gift from Claudia Casini Raggi, Department of
Clinical Physiopathology, University of Florence). The HCT116
cells constitutively express iNOS (40) but not the COX-2 gene
(37). The HT29 cells constitutively express COX-2 (37) but not
the iNOS gene (41). The cells were cultured in DMEM (Bio-
whittaker, Petit-Rechain, Belgium) supplemented with 10%
FCS (Poo Biological Industries, Logan, United Kingdom), 100
units/ml of penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml
amphotericin B at 37°C in 5% CO2.

Lipopoly saccharide (LPS), epidermal growth factor (EGF),
and AA were purchased from Sigma Chemical Co. (Milan,
Italy). 1400W, a selective inhibitor of iNOS, and the NO donor
sodium nitroprusside (SNP) were purchased from Tocris (Cook-
son, United Kingdom). Celecoxib, a selective inhibitor of
COX-2, was the kind gift of Pharmacia & Upjohn (Milan, Italy).

Cell Viability Assay. The 3-(4,5-dimethylthiazol-2-yl)-
2,5-dipheny l tetrazolium bromide assay was used to determine
cell viability. 3-(4,5-Dimethylthi azol-2-yl)-2,5-diphenyltetrazo-
lium bromide is a yellow-colored tetrazolium salt that is taken up and cleaved only by metabolically active cells, which reduce it to a colored, water-insoluble formazan salt. The solubilized formazan product can be quantified via absorbance at 570 nm, which is measured using a 96-well-format spectrophotometer. The absorbance correlates directly with the cell number. Cells were plated at $1.5 \times 10^4$ cells/well in 100 µl volume in 96-well plates and grown for 24 h in DMEM supplemented with 10% FCS. Different concentrations of test drugs or DMSO 0.1% were added to the wells. Cells were then incubated with 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) at 37°C for 2 h. The tetrazolium crystals were solubilized by the addition of 100 µl SDS in 0.01 N HCl. After overnight incubation at 37°C, the absorbance was measured at 570 nm using a 96-well spectrophotometric plate reader (TECAN, Ges, m.b.H., Salzburg, Austria). Results were expressed as the mean ± SE of eight wells.

**Cell Proliferation Assay.** HCT116 and HT29 cell proliferation was determined by the [3H]thymidine incorporation assay. Briefly, $10^4$ cells were seeded in 24-well plates and left to grow to subconfluence. After a 24-h incubation in steady-state medium (i.e., medium supplemented with 0.1% FCS), the medium was substituted with medium containing 10% FCS, added or not with test drugs. At the end of the incubations, the cells were pulsed for 4 h with 0.5 µCi [methyl-3H]thymidine (specific activity, 50 Ci/mmol) per well. The medium was then removed, and DNA was precipitated with cold 3% trichloroacetic acid and extracted with 1 ml of 0.3 N NaOH. The recovered radioactivity was measured in a β counter (model 1900 TR; Packard Tri-Carb, Zürich, Switzerland). The experiments were performed in quadruplicate, and the values were expressed as dpm/well (mean ± SE).

**Measurement of Nitrite/Nitrate Production.** The amount of nitrites was determined spectrophotometrically by the Griess reaction (42). The HCT116 and HT29 cells were seeded in 24-well plates. When the cells were subconfluent, they were washed twice with culture medium and incubated in 2 ml of DMEM without FCS for 24 h. The medium was then removed and the cells were incubated in 1.5 ml of DMEM without FCS for an additional 3 h. After two washes with culture medium, only the subconfluent cells were subjected to 3-h of serum starvation. Inhibitors (1 µmol/liter 1400W or 10 µmol/liter celecoxib) were added 30 min before LPS (10 µg/ml), EGF (100 ng/ml), SNP (10 µmol/liter), or AA (100 µmol/liter) adminis-

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Fig. 1 CD31, vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 immunostaining. Successive sections of a representative cancer specimen with a high density of CD31-positive endothelial cells (A) and classified as grade 2 for VEGF (B), iNOS (C), and COX-2 (D) immunostaining. VEGF, iNOS, and COX-2 immunoreactivity was detected within the same tumor epithelial cells. Hematoxylin counterstain; original magnification ×250.
tation. After 24 h of incubation, the supernatants were collected and allowed to react with Griess reagent (aqueous solution of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H$_3$PO$_4$) to form a stable chromophore that absorbed at a wavelength of 546 nm in the presence of nitrate reductase (276 mU) and NADPH, 40 μmol/liter. Determinations were performed in triplicate. Protein concentration was determined according to the method described by Lowry et al. (43). BSA was used as the standard. The values were obtained by comparison with standard concentrations of sodium nitrite and expressed as net amounts (pmol) of nitrites per μg protein.

**PGE$_2$ Measurement.** Supernatants of the HCT116 and HT29 cells were prepared as described above. Tissue fragments (normal control mucosa and tumor edge sample tissues) were homogenized at 0–4°C in the presence of 10 μmol/liter indomethacin so as to prevent PG production during the procedure and then centrifuged at 600 × g. Five hundred μl supernatants of the cells and tissue homogenates were used for PGE$_2$ determination using a competitive enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI) according to the method described by Pradelles et al. (44). Protein concentration in the tissue samples and in the cells was determined according to the method described by Lowry et al. (43). BSA was used as the standard. Determinations were performed in triplicate. PGE$_2$ values were expressed as μg/mg protein in the tissue samples and pg/μg protein in the cells.

**Determination of VEGF Production.** An ELISA method was used to quantitate the secreted VEGF in the culture medium of the HCT116 and HT29 cells. The cells were seeded in 24-well plates. After 24 h they were washed in serum-free medium and then incubated in 1 ml of medium 1% fetal bovine serum in the absence (controls) or presence of 1400W or 10−6 M celecoxib for 6 days at 37°C. The supernatants were assayed for VEGF production using the Human VEGF ELISA kit according to the manufacturer’s protocol (Biosource International, Nivelles, Belgium). Protein concentration in the cells was determined according to the method described by Lowry et al. (43). BSA was used as the standard. Determinations were performed in triplicate. VEGF values were expressed as pg/μg protein.

**Assay for NOS Activity.** Fragments of tissue were homogenized at 0–4°C in buffer containing 0.32 mol/liter sucrose, 20 mmol/liter HEPES (pH 7.2), 0.5 mol/liter EDTA, and 1 mmol/liter DTT. Total NOS activity was measured in tissue homogenates by the [1H]arginine conversion assay, as described previously (45). The activity of the calcium-cammodulin-independent isofor (iNOS) was identified in the supernatant of the tumor homogenates by measuring the enzymatic activity in buffer without calcium, and containing 1 mmol/liter EGTA and the calmodulin inhibitor trifluoperazine (100 μmol/liter) as reported previously (45). Protein concentration in the tissue samples was determined according to the method described by Lowry et al. (43). BSA was used as the standard. Determinations were performed in triplicate. NOS activity was expressed as pmol of [1H]citrulline formed per min per mg protein.

**Measurement of cGMP and cAMP Content.** cGMP and cAMP were measured in the aqueous phase of tissue homogenates extracted from 10% trichloroacetic acid with 0.5 mol/liter tri-n-octylamine dissolved in 1,1,2-trichlorotrifluoro-ethane. cGMP and cAMP were measured with commercially available radioimmunooassay kits (Amersham, Buckinghamshire, England). The assay is based on the competition between unlabelled nucleotides, and $^{125}$IcGMP and $^{125}$IcAMP for binding to a limited quantity of an antibody raised with a high specificity to cGMP and cAMP (46, 47). Determinations were performed in triplicate. cGMP values were expressed as pmol/min/mg protein and cAMP values were expressed as pmol/min/μg protein.

**Western Blot Analysis.** Tumors and normal mucosa samples were processed as described previously (8). SDS-PAGE was performed using 8% and 5% acrylamide for the separating gel and the stacking gel, respectively. Proteins were transferred onto nitrocellulose membranes (Pierce, Chemical Co., IL). Blots were blocked with Blocker BSA 5% in PBS (Pierce, Chemical Co.) and incubated overnight at 4°C with a human polyclonal antibody for iNOS (Chemicon International, Inc., Temecula, CA) and a human polyclonal antibody for COX-2 (Cayman Chemical, Ann Arbor, MI) at 1:1000 dilution. Blots were additionally incubated with secondary antibodies conjugated with horseradish peroxidase for 2 h at room temperature and, finally, incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.) for 5 min and then exposed to CL-Xposure Film (Pierce, Chemical Co.). The HCT116 colon cancer cell line served as a positive control for iNOS (40). The HT29 colon cancer cell line served as a positive control for COX-2 (37). The cells were processed and analyzed as described previously (48).

**Northern Blot Analysis.** Total RNA from tumor and normal mucosa specimens was extracted using the guanidium isothiocyanate method, and analysis was performed as described previously (8). As iNOS and COX-2 cDNA probes, we used PCR product fragments obtained by amplification of sequences obtained from NIH-GenBank. The primers used were iNOS sense: 5′-GTCTTGGTCGAAGCTGGCT-3′ and iNOS antisense: 5′-CAAAGCTTGTACCTCGCA-3′, which gave rise to a 633-bp product; COX-2 sense: 5′-TACAGGTCTCCATTGCACCC-3′ and COX-2 antisense: 5′CGCAACAGGAGTACTGACCTTC-3′, which gave rise to a 644-bp product. These products were then purified using the QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA). Each probe was labeled with α-[32P]dCTP, using a random priming reaction (Boehringer Mannheim, Mannheim, Germany). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as an internal control to adjust for differences in the amount of RNA loaded in each lane. It was obtained by a PCR using the following primer, GAPDH sense: 5′-CCATGGAGAAGCTGGGG-3′ and GAPDH antisense: 5′-CAGAGTTGGTCACTGGATACC-3′, which gave rise to a 196-bp product. We realized three identical filters loaded with the same RNA amount. Each membrane was first hybridized with an investigative probe (i.e., iNOS and COX-2) and then with the GAPDH probe. The filters were exposed to Kodak X-ray film (Eastman Kodak, Rochester, NY) for autoradiography for 1–6 days at −80°C. The level of expression in the tissue samples was assessed using a GS-670 Imaging Densitometer (Bio-Rad, Hercules, CA). The densitometric percentage of the autoradiographic signal was evaluated according to Pertschuk et al. (49). To exclude possible densito-
metric signal errors due to background and/or other aspecific phenomena, the intensity of mRNA expression was compared with that of a control gene, i.e., GAPDH. The HCT116 colon cancer cell line served as a positive control for iNOS (40). The HT29 colon cancer cell line served as a positive control for COX-2 (37). The cells were processed and analyzed as described previously (48).

Statistical Analysis. MVD values, iNOS activity, and nitrite/nitrate, cGMP, PGE₂, and cAMP production were expressed as mean values ± SE. The relationships among MVD, iNOS activity, cGMP levels, PGE₂ production, cAMP levels, and iNOS, COX-2, and VEGF staining were evaluated using the Spearman correlation coefficients (rₛ). Incorporation of [³H]thyminidine and nitrite/nitrate, PGE₂, and VEGF production in the cell lines were compared using the paired-value Wilcoxon test or the Mann-Whitney test, as appropriate. Differences in iNOS activity, cGMP levels, PGE₂ production, and cAMP levels in tumor and normal mucosa specimens, and in tumors with and without metastases were analyzed using the Mann-Whitney test. Differences in MVD according to the tumor stage were analyzed using the Mann-Whitney test. Statistical analysis was performed using Stata Statistical Software (release 5.0; Stata Corporation, College Station, TX). All of the Ps resulted from the use of two-sided statistical tests; Ps < 0.05 were considered statistically significant.

RESULTS
iNOS and COX-2 Pathways in Human Colorectal Cancer. Some of the tumor samples analyzed in this study were included in previous reports (8, 22). iNOS activity and cGMP levels were significantly higher in the cancer specimens than in the normal mucosa (22.86 ± 1.21 versus 10.04 ± 0.38, P < 0.0001; 16.70 ± 0.91 versus 7.14 ± 0.37, P < 0.0001, respectively). These parameters were also significantly related to the tumor stage: they were higher in tumors with lymph node and/or distant metastases than in those tumors without metastases (26.50 ± 1.97 versus 19.68 ± 1.18, P = 0.002; 18.59 ± 1.40 versus 15.14 ± 1.12, P = 0.04, respectively). iNOS immunostaining was observed mainly in the cytoplasm of the tumor epithelial cells: 11 tumors (23.9%) were grade 0, 13 (28.3%) were grade 1 and 22 (47.8%) were grade 2 (Fig. 1C). iNOS was also expressed by tumor-infiltrating inflammatory cells. Normal mucosa specimens were negative for iNOS staining. Western blotting confirmed the up-regulation of iNOS protein expression in the neoplastic tissue when compared with the adjacent normal mucosa (Fig. 2A). Northern blot analysis confirmed higher mRNA levels for iNOS in the tumor specimens when compared with the corresponding normal mucosa (Fig. 2B).

PGE₂ production and cAMP content were significantly higher in the cancer specimens than in the normal mucosa (9.64 ± 0.74 versus 3.15 ± 0.22, P < 0.0001; 39.69 ± 2.02 versus 15.70 ± 0.49, P < 0.0001, respectively). These parameters were also significantly higher in tumors with lymph node or distant metastases than in those tumors without metastases (11.01 ± 1.06 versus 8.37 ± 0.99, P = 0.01; 44.89 ± 7.25 versus 34.92 ± 1.76, P = 0.03, respectively). Cytoplasm staining for COX-2 was found mainly in the tumor epithelial cells: 15 tumors (32.6%) were grade 0, 25 (54.3%) were grade 1, and 6 (13.1%) were grade 2 (Fig. 1D). COX-2 protein was also expressed by tumor-infiltrating inflammatory cells and endothelial cells. Western blotting confirmed the up-regulation of COX-2 protein expression in the cancer specimens when compared with the corresponding normal mucosa (Fig. 2A). Using Northern hybridization analysis, up-regulation of COX-2 mRNA levels was demonstrated in the tumor specimens when compared with the corresponding normal mucosa (Fig. 2B).

Cross-Talk between the iNOS and COX-2 Pathways: Correlation with Tumor Angiogenesis. The immunohistochemical expression of iNOS significantly correlated with that of COX-2 (rₛ = 0.44; P = 0.001). Tumor epithelial cells expressing iNOS stained positive for COX-2 in the majority of the examined tumor sections (Fig. 1, C and D). The immunohistochemical findings were confirmed by Western and Northern blot analysis: the majority of the carcinomas that showed up-regulation of the iNOS protein and gene also had high levels of the
COX-2 protein and mRNA (Fig. 2, A and B). PGE₂ production correlated significantly with iNOS activity (rₙ = 0.60; P < 0.0001; Fig. 3) and cGMP levels (rₙ = 0.57; P < 0.0001; Fig. 4). A significant correlation was also found between cAMP levels and cGMP production (rₙ = 0.59; P < 0.0001).

VEGF staining was found in the cytoplasm and the membranes of the tumor epithelial cells: 10 tumors (21.6%) were grade 0, 18 (39.2%) were grade 1, and 18 (39.2%) were grade 2 (Fig. 1B). No immunoreactivity for VEGF was detected in the normal mucosa cells. When all of the cases were considered, the mean value of MVD was 29.0 (±2.4). The expression of iNOS, COX-2, and VEGF significantly correlated with MVD (rₙ = 0.31, P = 0.02; rₙ = 0.54, P < 0.0001; rₙ = 0.67, P < 0.0001, respectively). A significant correlation was found between iNOS and VEGF expression (rₙ = 0.44; P = 0.001), as well as between COX-2 and VEGF staining (rₙ = 0.54; P < 0.0001).

PGE₂ production correlated significantly with MVD (rₙ = 0.33; P = 0.01; Fig. 5) and VEGF immunostaining (rₙ = 0.25; P = 0.04). We also found a significant association between cAMP levels and MVD (rₙ = 0.26; P = 0.03). No significant correlations were found between functional parameters of the NO pathway and the angiogenic markers. MVD and iNOS, COX-2, and VEGF expressions did not significantly differ in metastatic (stage III and IV) and nonmetastatic (stage I and II) tumors.

**Effects of 1400W and Celecoxib on Cell Viability.** The cytotoxicity of the iNOS inhibitor 1400W and the COX-2 inhibitor celecoxib against the HCT116 and HT29 cells was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 1400W had similar cytotoxic effects against the HCT116 and HT29 cells with significant loss of viability at concentrations of ≤10 μmol/liter (the IC₅₀ was 25 μmol/liter for HCT116 cells and 18 μmol/liter for HT29 cells; Fig. 6A). Treatment of these cells with 1400W at concentrations of ≤1 μmol/liter had no effect on cell viability (Fig. 6A).

Treatment of the HCT116 cells with celecoxib concentrations of ≤25 μmol/liter had no effect on cell viability. When ≥50 μmol/liter celecoxib was used, a decrease in cell viability was observed (IC₅₀ = 83 μmol/liter; Fig. 6B). Treatment of the HT29 cells with ≤10 μmol/liter celecoxib had no cytotoxic effect, whereas treatment with ≥25 μmol/liter celecoxib resulted in a decrease in cell viability (IC₅₀ = 63 μmol/liter; Fig. 6B).

Subsequent experiments were performed using noncytotoxic doses of 1400W (1 μmol/liter) and celecoxib (10 μmol/liter).

**Effects of 1400W and Celecoxib on Cell Proliferation.** We examined the effect of 1 μmol/liter 1400W and 10 μmol/liter celecoxib on the proliferation of the HCT116 and HT29
cells by measuring the incorporation of [3H]thymidine to assess DNA synthesis. Neither 1400W nor celecoxib at the indicated concentrations had any effect on cell proliferation of the HCT116 cells (122.7 concentrations had any effect on cell proliferation of the HCT116 and HT29 cells with 1 μmol/liter 1400W and 10 μmol/liter celecoxib had no effect on cell viability. HCT116, HT29).

Fig. 6 HCT116 and HT29 cell viability after treatment with vehicle (DMSO) or with the indicated amounts of 1400W (A) and celecoxib (B). Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values represent OD570 treated/OD570 control (DMSO), and are expressed as mean values of eight replicate wells/treatment condition; bars, ±SE. Treatment of the HCT116 and HT29 cells with 1 μmol/liter 1400W and 10 μmol/liter celecoxib had no effect on cell viability. HCT116, HT29.

Effects of Coinduction of the iNOS and COX-2 Pathways by LPS and EGF Stimulation in HCT116 and HT29 Cell Lines. Unstimulated release of nitrite/nitrate was significantly higher in the HCT116 cells than in the HT29 ones (14.3 ± 1.5 versus 9.2 ± 1.2; P = 0.04). LPS and EGF are known to be potent inducers of both iNOS and COX-2 enzyme (48). After incubation of the HCT116 and HT29 cells with LPS and EGF, we found a significant increase in nitrite/nitrate and PGE2 concentrations in the supernatants (Tables 1 and 2).

Effects of Endogenous NO on COX-2 Activation. To examine the effects of endogenous NO on the COX-2 pathway by LPS and EGF, the iNOS positive/COX-2 negative HCT116 cells were treated with LPS and EGF in the presence of 1400W: a marked reduction in both nitrite/nitrate and PGE2 production was observed (Table 1). The same treatment in the presence of celecoxib significantly reduced PGE2 production but did not affect nitrite/nitrate levels (Table 1). Incubation of the HCT116 cells with AA did not affect nitrite/nitrate levels and caused a slight increase in PGE2 production. This increase was reversed by the treatment with celecoxib (Table 1).

Effects of Exogenous NO on COX-2 Activation. To examine the effect of exogenous NO on the COX-2 pathway, the iNOS negative/COX-2 positive HT29 cells were treated with the NO donor SNP. A significant increase in PGE2 production after stimulation with SNP was observed (Table 2). The activity of SNP was confirmed by an increase in nitrite/nitrate concentration in the supernatant (Table 2). iNOS inhibition with 1400W did not affect the SNP-mediated PGE2 increase, whereas the administration of celecoxib significantly reduced the SNP-mediated PGE2 production (Table 2).

Effects of Coinduction of the iNOS and COX-2 Pathways on VEGF Production. To examine the effect of the iNOS and COX-2 pathways on tumor angiogenesis, we evaluated VEGF production in the HCT116 and HT29 cells. An unstimulated release of VEGF was present in both of the cell lines. Incubation with LPS, EGF, and AA significantly increased VEGF levels in the HT29 cells but had no effect on the HCT116 cells (Tables 1 and 2). Treatment of the HT29 cells with LPS and EGF in the presence of 1400W reduced the LPS- and EGF-stimulated increase in nitrite/nitrate, PGE2, and VEGF production. The same treatment in the presence of celecoxib reduced PGE2 and VEGF production but did not affect nitrite/nitrate levels (Table 2). Simultaneous treatment of the HT29 cells with 1400W and celecoxib after LPS/EGF stimulation had a cooperative effect in reducing nitrite/nitrate, PGE2, and VEGF production (Table 2). Stimulation of the HT29 cells with SNP caused an increase in VEGF production, which was reversed by the treatment with celecoxib (Table 2). Incubation of the HT29 cells with AA did not affect nitrite/nitrate levels, and caused a significant increase in PGE2 and VEGF production (Table 2). The AA-mediated increase in VEGF was reversed by the treatment with celecoxib (Table 2).

DISCUSSION
We have demonstrated recently that both the iNOS and COX-2 genes are up-regulated in human colorectal cancer (8, 22). In the present study, we showed that the immunohistochemical expression of iNOS correlated significantly with that of COX-2 and that these two enzymes were coexpressed within the same tumor epithelial cells. Moreover, most tumors with high levels of iNOS protein and mRNA showed an overexpression of the COX-2 gene. We also found a significant correlation be-
The NO donor SNP to the iNOS-negative/COX-2-positive HT29 cell center of COX-2. These data are in contrast with those reported on head and neck squamous cell carcinoma.

Salvemini et al. (30) demonstrated that both endogenous and exogenous NO plays a critical role in the release of PGE2 by direct activation of COX in macrophages. The same authors also showed that the effects of NO on COX activity are independent of GMP levels and suggested that the pathway underlying such activation may involve an interaction of NO at the iron-heme center of COX-2. These data are in contrast with those reported by Gallo et al. (35) on head and neck squamous cell carcinoma.

**Table 1** Effects of iNOS\(^a\) and COX-2 inhibition on nitrite/nitrate (pmol/µg protein), PGE\(_2\) (pg/µg protein), and VEGF (pg/µg protein) production after LPS, EGF, and AA stimulation of HCT116 cells\(^b\)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Nitrite/nitrate production</th>
<th>PGE(_2) production</th>
<th>VEGF production</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>61.6 ± 2.9</td>
<td>9.2 ± 1.2</td>
<td>781.7 ± 103.4</td>
</tr>
<tr>
<td>1400W (1 µmol/liter)</td>
<td>51.5 ± 4.7</td>
<td>14.9 ± 1.0</td>
<td>749.3 ± 71.6</td>
</tr>
<tr>
<td>Celecoxib (10 µmol/liter)</td>
<td>56.1 ± 3.8</td>
<td>9.2 ± 0.4</td>
<td>797.7 ± 82.6</td>
</tr>
<tr>
<td>LPS (10 µg/ml)</td>
<td>250.6 ± 13.1(^c)</td>
<td>132.6 ± 12(^c)</td>
<td>552.6 ± 13.7</td>
</tr>
<tr>
<td>LPS + 1400W</td>
<td>111.6 ± 9.8(^d)</td>
<td>79.7 ± 4.0(^d)</td>
<td>—</td>
</tr>
<tr>
<td>LPS + Celecoxib</td>
<td>231.1 ± 6.6</td>
<td>46.8 ± 4.3(^d)</td>
<td>—</td>
</tr>
<tr>
<td>EGF (100 ng/ml)</td>
<td>203.9 ± 10.6(^d)</td>
<td>167.4 ± 7.4(^d)</td>
<td>697.8 ± 89.0</td>
</tr>
<tr>
<td>EGF + 1400W</td>
<td>114.6 ± 9.5(^d)</td>
<td>133.0 ± 3.5(^d)</td>
<td>—</td>
</tr>
<tr>
<td>EGF + Celecoxib</td>
<td>182.8 ± 6.8</td>
<td>57.1 ± 1.7(^d)</td>
<td>—</td>
</tr>
<tr>
<td>AA (100 µmol/liter)</td>
<td>72.8 ± 2.9</td>
<td>73.9 ± 1.4(^d)</td>
<td>789.5 ± 79.2</td>
</tr>
<tr>
<td>AA + Celecoxib</td>
<td>70.9 ± 1.6</td>
<td>23.3 ± 1.9(^d)</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) iNOS, Inducible nitric oxide synthase; COX, cyclooxygenase; PG, prostaglandin; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; EGF, epidermal growth factor; AA, arachidonic acid.

\(^b\) Results are expressed as means ± SE of three tests per treatment.

\(^c\) Denotes significant increase compared to unstimulated cells (\(P < 0.05\); Wilcoxon test).

\(^d\) Denotes significant inhibition compared to LPS, EGF, and AA treatment (\(P < 0.05\); Wilcoxon test).

**Table 2** Effects of iNOS\(^a\) and COX-2 inhibition on nitrite/nitrate (pmol/µg protein), PGE\(_2\) (pg/µg protein), and VEGF (pg/µg protein) production after LPS, EGF, SNP, and AA stimulation of HT29 cells\(^b\)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Nitrite/nitrate production</th>
<th>PGE(_2) production</th>
<th>VEGF production</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>26.0 ± 0.1</td>
<td>14.3 ± 1.5</td>
<td>811.3 ± 81.8</td>
</tr>
<tr>
<td>1400W (1 µmol/liter)</td>
<td>22.9 ± 2.8</td>
<td>15.3 ± 1.2</td>
<td>808.1 ± 67.4</td>
</tr>
<tr>
<td>Celecoxib (10 µmol/liter)</td>
<td>23.1 ± 0.8</td>
<td>12.4 ± 0.7</td>
<td>731.2 ± 19.9</td>
</tr>
<tr>
<td>LPS (10 µg/ml)</td>
<td>96.7 ± 4.2(^c)</td>
<td>101.9 ± 5.3(^c)</td>
<td>1156.0 ± 91.2</td>
</tr>
<tr>
<td>LPS + 1400W</td>
<td>46.9 ± 3.1(^c)</td>
<td>72.2 ± 2.0(^c)</td>
<td>805.0 ± 37.6(^d)</td>
</tr>
<tr>
<td>LPS + Celecoxib</td>
<td>85.6 ± 2.5</td>
<td>53.3 ± 6.3(^d)</td>
<td>501.3 ± 15.1(^d)</td>
</tr>
<tr>
<td>LPS + 1400W + Celecoxib</td>
<td>34.4 ± 0.2(^d)</td>
<td>42.6 ± 6.7(^d)</td>
<td>450.6 ± 47.2(^d)</td>
</tr>
<tr>
<td>EGF (100 ng/ml)</td>
<td>83.1 ± 1.6(^d)</td>
<td>167.6 ± 5.7(^d)</td>
<td>1132.9 ± 67.9</td>
</tr>
<tr>
<td>EGF + 1400W</td>
<td>29.6 ± 1.2(^c)</td>
<td>51.7 ± 0.92(^c)</td>
<td>687.7 ± 29.5(^d)</td>
</tr>
<tr>
<td>EGF + Celecoxib</td>
<td>83.9 ± 1.4</td>
<td>48.7 ± 1.1(^d)</td>
<td>801.3 ± 42.2(^d)</td>
</tr>
<tr>
<td>EGF + 1400W + Celecoxib</td>
<td>26.2 ± 0.4(^d)</td>
<td>34.7 ± 5.0(^d)</td>
<td>568.3 ± 42.7(^d)</td>
</tr>
<tr>
<td>SNP (10 µmol/liter)</td>
<td>149.8 ± 1.8(^c)</td>
<td>95.1 ± 4.1(^d)</td>
<td>1255.0 ± 64.9(^d)</td>
</tr>
<tr>
<td>SNP + 1400W</td>
<td>132.8 ± 3.3</td>
<td>94.5 ± 2.2</td>
<td>1103.2 ± 57.7</td>
</tr>
<tr>
<td>SNP + Celecoxib</td>
<td>144.9 ± 0.6</td>
<td>54.9 ± 1.3(^d)</td>
<td>675.2 ± 50.4(^d)</td>
</tr>
<tr>
<td>AA (100 µmol/liter)</td>
<td>29.1 ± 1.3(^d)</td>
<td>118.5 ± 4.1(^d)</td>
<td>1033 ± 58.6(^d)</td>
</tr>
<tr>
<td>AA + Celecoxib</td>
<td>32.8 ± 1.6</td>
<td>45.6 ± 1.9(^d)</td>
<td>719.0 ± 38.9(^d)</td>
</tr>
</tbody>
</table>

\(^a\) iNOS, Inducible nitric oxide synthase; COX, cyclooxygenase; PG, prostaglandin; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; EGF, epidermal growth factor; SNP, sodium nitroprusside; AA, arachidonic acid.

\(^b\) Results are expressed as means ± SE of three tests per treatment.

\(^c\) Denotes significant increase compared to unstimulated cells (\(P < 0.05\); Wilcoxon test).

\(^d\) Denotes significant inhibition compared to LPS, EGF, SNP, and AA treatment (\(P < 0.05\); Wilcoxon test).
These authors found that the increase in COX-2 mRNA levels and PGE₂ production induced by the NO donor S-nitroso-acetylpenicillamine in the A-431 and SCC-9 cancer cells are suppressed by the guanylate cyclase inhibitor methylene blue. Our findings seem to confirm the possible role of cGMP in COX activation and PGE₂ release. Indeed, we found that cGMP levels were significantly correlated with both PGE₂ production and cAMP levels in human colorectal cancer specimens. Another possible molecular mechanism by which NO can activate COX involves peroxynitrite, the coupling product of the superoxide anion and NO. Landino et al. (32) demonstrated that peroxynitrite activates the activity of COX by serving as a hydroperoxide substrate for the peroxidase activity of the enzyme. Nitrotyrosine is the stable product of the action of peroxynitrite on tyrosine-containing proteins and is, thus, considered a marker of peroxynitrite. In a previous study (22), we showed that iNOS and nitrotyrosine are coexpressed within the same colorectal tumor epithelial cells. Taken together, all of these data strongly suggest a modulation of the COX-2 pathway by NO in human colorectal cancer.

Increasing evidence links iNOS and COX-2 with tumor angiogenesis. Jenkins et al. (19) showed that the human colon adenocarcinoma DLD-1 cells transfected with iNOS have a faster growth rate and higher blood vessel density in nude mice than do tumors seeded from the parental cell line. Increased iNOS activity in head and neck cancer is associated with elevated cGMP levels and correlates with tumor vascularization (21). Ziche et al. (24) showed that VEGF produced by tumor cells requires a functional endothelial NO/cGMP pathway to promote angiogenesis. Even angiogenesis promoted by COX-2 seems to be mediated by the induction of VEGF. PGE₂ activates E-prostanoid receptors, and signaling via EP₂ and EP₄ receptors leads to increased levels of cAMP (29). It has been reported that a cAMP-dependent pathway is responsible for the up-regulation of VEGF in many cultured cells (10, 11, 26–29). We have demonstrated recently that the overexpression of both iNOS and COX-2 is significantly correlated with intratumor MVD and VEGF expression in colorectal cancer (8, 22). Coexpression of iNOS and COX-2, and its possible role in controlling tumor angiogenesis have been shown previously in hepatocellular carcinoma (33), non-small cell lung carcinoma (34), head and neck squamous carcinoma (35), and breast and colon cancer (36). In the present study, we addressed the hypothesis that the activation of COX-2 and, thus, the production of PGE₂, might act as possible mediators of the proangiogenic effect of NO in colorectal cancer. We found that the PGE₂ levels in human cancer specimens significantly correlated with MVD and VEGF expression. Even cAMP levels were associated significantly with MVD. On the other hand, the products of the iNOS pathway did not appear to correlate with any of the angiogenic markers that we had investigated. These findings suggest a more direct involvement of COX-2 activity than that of iNOS in the induction of tumor angiogenesis. Our in vitro data seem to confirm the hypothesis that the proangiogenic role of NO is mainly mediated by enhancing COX-2 activity and less importantly by its direct effect on the production of angiogenic factors such as VEGF. Coinduction of iNOS and COX-2 activities with LPS and EGF had different effects on VEGF production in the HCT116 and in the HT29 cell lines. We did not find any increase in VEGF production in the iNOS-positive/COX-2-negative HCT116 cells, whereas the iNOS-negative/COX-2-positive HT29 cells showed a marked increase in VEGF levels. Moreover, the LPS-, EGF-, SNP-, and AA-stimulated production of VEGF in the HT29 cells was reversed by the COX-2 inhibitor celecoxib. This finding suggests that the NO-promoted angiogenesis through VEGF stimulation in colorectal cancer is mainly mediated by COX-2 activity and, thus, PGE₂ production. However, the finding that PGE₂ levels after LPS and EGF stimulation were similar in the HCT116 and HT29 cells gave rise to the question of what molecular mechanism could be responsible for the difference in VEGF production in the two cell lines. Eibl et al. (50) recently provided a possible explanation. These authors found that both exogenous and endogenous PGE₂ stimulates VEGF production in COX-2-positive but not in COX-2-negative pancreatic cancer cells. No differences in the expression and activity (i.e., intracellular cAMP levels) of the EP₂ receptor were found in the two types of cell lines. On the other hand, the authors demonstrated that differences in the signaling pathways downstream from cAMP are responsible for the differential effect of PGE₂ on VEGF production in COX-2-positive and -negative cell lines.

It is remarkable that both the stimulation of the HCT116 cells with LPS/EGF and the simultaneous inhibition of the iNOS/COX-2 pathways in the HT29 cells after the same stimulation did not correlate with any increase in VEGF synthesis. Studies published previously have shown that LPS and EGF can stimulate VEGF synthesis in inflammatory or tumor experimental models, respectively (51, 52). Although the present study did not address the mechanisms by which LPS and EGF stimulate VEGF production, our findings suggest that COX-2 activation and PGE₂ production might have a relevant role even in mediating LPS- and EGF-promoted angiogenesis.

Fig. 7 summarizes the molecular mechanisms that might underlie the proangiogenic effect mediated by iNOS and COX-2 activation in the HT29 cells. Our results support the hypothesis that the blockade of one or both of these enzymes can exert an antiangiogenic effect by decreasing VEGF production in colon cancer cells. However, it should be pointed out that this potential antiangiogenic ability of the iNOS and COX-2 inhibitors is likely to be effective only in tumor cells that have high levels of activity of these two inducible enzymes.

We have reported previously that both iNOS activity/cGMP levels and PGE₂ production are related to tumor stage (8, 22). In the present study, we found that even cAMP levels were significantly higher in metastatic tumors than in nonmetastatic ones. On the contrary, the extent of either iNOS or COX-2 immunostaining of the neoplastic epithelial cells did not reveal any association with tumor stage. It has been demonstrated that even the cells of the stromal compartment within colorectal tumors (namely, endothelial cells, fibroblasts, macrophages, and other inflammatory cells) express both iNOS and COX-2 (53). It might be suggested that the total amount of NO and PGE₂ within the tumor microenvironment, irrespective of the cell types by which they are synthesized, is the crucial determinant in tumor growth and invasiveness. This might explain why only the products of iNOS and COX-2 activity are associated with tumor staging. These data give additional support to the hypothesis that cross-talk between the NO and COX-2 pathways can
proceed a cooperative effect in the metastatic behavior of colorectal tumors.

In conclusion, our data showed a prominent role of NO in stimulating COX activity in colorectal cancer. This interaction is likely to produce a cooperative effect in promoting tumor angiogenesis through an increase in VEGF production. However, the proangiogenic effect seems to be mainly mediated by COX-2 activity and may be effective only in the presence of a functional signaling pathway downstream from cAMP. Collectively, these data point to the dual inhibition of the iNOS and COX-2 enzymes as a possible therapeutic tool in the treatment of colorectal cancer.

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Cyclooxygenase-2 Activation Mediates the Proangiogenic Effect of Nitric Oxide in Colorectal Cancer

Fabio Cianchi, Camillo Cortesini, Ornella Fantappiè, et al.


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