Cyclooxygenase-2 Expression Correlates with Tumor Neovascularization and Prognosis in Human Colorectal Carcinoma Patients

Reiko Masunaga,[1] Hitoshi Kohno, Dipok Kumar Dhar, Satoshi Ohno, Muneaki Shibakita, Syoichi Kinugasa, Hiroshi Yoshimura, Mitsuo Tachibana, Hirofumi Kubota, and Naofumi Nagasue

Second Department of Surgery, Shimane Medical University, Izymo 693-8501, Japan

ABSTRACT

The role of cyclooxygenase-2 (COX-2) in tumor neovascularization of human colorectal carcinoma is yet to be delineated. One hundred colorectal carcinoma specimens were evaluated for COX-2 expression and CD34-stained microvessel density (MVD) by immunohistochemical methods. The relationships between COX-2 expression and clinicopathological feature of the patients, MVD, and survival time were analyzed. Increased COX-2 expression was significantly correlated with pathologically unfavorable findings such as tumor size (>3.0 cm), tumor differentiation (poor, moderate > well differentiated), number of metastatic lymph nodes (≥4), and Dukes' stage (Dukes' B, C, and D). Larger number of microvessels congregated around the COX-2-expressing area, and the Spearman rank correlation test showed a strong correlation between COX-2 expression and tumor MVD ($P < 0.0001$). Patients with COX-2-positive tumors had a significantly ($P = 0.037$, by log-rank test) shorter survival time than those with negative tumors did. In the multivariate analysis, however, only Dukes' stage and number of metastatic lymph nodes remained as independent prognostic factors. Augmented tumor neovascularization may be one of the several effects of COX-2 responsible for poor prognosis in human colorectal carcinoma patients.

INTRODUCTION

Several previous studies indicated that NSAIDs[2] can prevent the development of colorectal carcinoma in humans. The major target of NSAIDs is COX, which catalyzes the conversion of arachidonic acid to prostaglandin H_2, the common precursor for all prostanoids (1). Thus far, two COX isozymes have been identified, the constitutive COX-1 and inducible COX-2. COX-1 is expressed in normal intestine, but its expression is not altered in intestinal tumors. In contrast, COX-2 is undetectable in normal intestine (2), and its expression is significantly increased in up to 85% of colorectal adenocarcinomas (3, 4). Similarly, an elevated COX-2 expression has been detected in several other carcinomas including esophageal carcinoma (5), hepatocellular carcinoma (6), and gastric carcinoma (7), in which COX-2 expression is correlated with poor prognostic outcome.

Tumor growth is dependent on angiogenesis (8), and several studies have indicated that higher MVD in colorectal carcinoma is associated with poor patient prognosis (9, 10). Recent evidence suggests that COX-2 contributes to neovascularization and may support vasculature-dependent solid tumor growth and metastasis in animal experiments (11) and in *in vitro* studies (12). However, the relationship between COX-2 expression and tumor neovascularization in colorectal carcinoma is yet to be delineated.

In this study, we investigated the correlation of COX-2 expression with tumor MVD, clinicopathological characteristics, and prognosis in colorectal carcinoma patients.

MATERIALS AND METHODS

Patients and Tissues. One hundred colorectal carcinoma patients of different Dukes' stages (25 from each of the Dukes' A, B, C, and D stages) were selected randomly from patients operated on between 1990 and 1999 in the Second Department of Surgery, Shimane Medical University. None of them had any preoperative radiochemotherapy. Fifty-eight patients had postoperative chemotherapy, and one patient had radiotherapy. Five patients who had died of other diseases than colorectal carcinoma were excluded from survival analysis. The mean follow-up period was 4.5 years.

Immunohistochemical Stainings. The representative sections containing both the normal mucosa and tumor tissue were selected for this study. A Universal Immuno-enzyme Polymer method was used for immunostaining. Briefly, slides were deparaffinized, rehydrated, and treated with 3% H_2O_2 for 15 min to quench endogenous peroxidase activity. Nonspecific bindings were blocked by treating slides with normal rabbit serum for 30 min. The slides were incubated with mouse monoclonal antibodies against COX-2 (Cayman Chemical Co., Ann Arbor, MI; dilution 1:300, for 14 h at 4°C; Ref. 13) and CD34 (CD34 Class II; DAKO A/S Glostrup, Denmark; dilution 1:50; for 1.5 h at room temperature; Ref. 14). Next, slides were incubated with labeled polymer [N-Histofine Simple Stain PO(M); Nichirei Co., Tokyo, Japan] for 30 min at room temperature. Color development was done with the peroxidase

Received 4/25/00; revised 8/1/00; accepted 8/1/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Second Department of Surgery, Shimane Medical University, Enyacho 89/1, Izumo 693-8501, Japan. Phone: 81-853-202232; Fax: 81-853-202229; E-mail: nigeka30@shimane-med.ac.jp.

[2] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; MVD, microvessel density.

substrate AEC (3-amino-9-ethylcarbazole). The slides were finally counterstained with Meyer's hematoxylin. A positive and negative control slide were always included in each immunostaining.

Evaluation of Staining. The slides were evaluated under a transmission light microscope by two separate investigators (R. M. and D. K. D.) in a blind manner in terms of the patient's background. For COX-2 assessment, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). Extent of staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%) according to the percentages of the positive staining areas in relation to the whole carcinoma area. The sum of the intensity and extent score was used as the final staining score (0–7) for COX-2 (5, 8). Tumors having a final staining score of >2 were considered to be positive.

For MVD assessment, specimens were examined under a light microscope, and the three most hypervascular areas were selected under low magnification. Any single endothelial cell or cluster of endothelial cells was counted as a single microvessel. MVD was expressed as the number of vessels per high-power field (×200). The mean value for three fields was regarded as the MVD for each tumor.

Double Staining of COX-2 and MVD. Double immunostaining was performed to simultaneously localize COX-2 and microvessels on several slides using a Labeled-[strept]Avidin-Biotin method as described previously (15). An affinity-purified biotinylated secondary antibody in conjunction with streptavidin-peroxidase and streptavidin-alkaline phosphatase was used. Two distinct substrate/chromogen/enzyme systems were used: 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium/alkaline phosphatase produced a dark purple color and hydrogen peroxide/3,3′-diaminobenzidine/peroxidase produced brown color. Primary antibodies were same as above.

Statistical Analysis. All statistical analyses were carried out with Statistical Analysis System software (Version 5.0, Stat View). The relationship between COX-2 expression and categorical variables was compared with the χ^2 test, or Fisher's exact probability test when appropriate. Continuous variables were compared with the Mann-Whitney *U* test. The strength of association between the COX-2 score and MVD was assessed by the Spearman rank correlation test. The Kaplan-Meier method was used to estimate survival, and differences were analyzed by the log-rank test. The Cox proportional hazards model was used for multivariate analysis of prognostic factors. $P < 0.05$ was considered to be significant.

RESULTS

Relationship between the COX-2 Expression and Clinicopathological Findings. The expression of COX-2 was positive (3–7 of the final staining score) in 76% and negative (0–2) in 24% of the tumors studied (Table 1). COX-2 was observed mainly in the tumor area. Normal colonic mucosa adjacent to the COX-2-positive tumors was not stained or occasionally stained weakly for COX-2 (Fig. 1*A*). The associations between clinicopathological variables and COX-2 expression are shown in Table 1. There was no significant association between COX-2 expression and patient age ($P = 0.24$), sex ($P = 0.21$), and tumor location ($P = 0.79$). On the other hand, there were significant differences between COX-2 expression and various pathological characteristics, including tumor size ($P = 0.047$), tumor differentiation ($P = 0.012$), number of metastatic lymph nodes ($P = 0.006$), and Dukes' stage ($P = 0.0002$). The average number of MVD was almost double in COX-2-positive tumors compared with COX-2-negative tumors(86.0 ± 37.3 *versus* 44.4 ± 19.3, respectively) with a significant statistical difference ($P < 0.0001$).

Table 1 Correlation of clinicopathological findings with COX-2 expression

Clinical feature	Case no.	COX-2 expression[a] Negative ($n = 24$)	Positive ($n = 76$)	*P*
Age (yr)[b]				
≤65	50	15	35	0.24
>65	50	9	41	
Sex				
Male	59	11	48	0.21
Female	41	13	28	
Tumor size (cm)				
<3.0	21	9	12	0.047
≥3.0	79	15	64	
Location				
Right	25	5	20	0.79
Left	75	19	56	
Differentiation				
Well	45	17	28	0.012
Moderate	51	6	45	
Poor	4	1	3	
No. of metastatic lymph nodes				
≤3	83	24	59	0.006
≥4	17	0	17	
Dukes' stage				
A	25	14	11	0.0002
B	25	2	23	
C	25	5	20	
D	25	3	22	
MVD		44.4 ± 19.3	86.0 ± 37.3	<0.0001[c]

[a] "Negative" and "Positive" mean 0–2 and 3–7 of the final score, respectively.
[b] Cut-off value is the median value.
[c] Mann-Whitney *U* test.

Correlation between COX-2 Expression and MVD. A statistically significant (Spearman correlation test, $P < 0.0001$, $\rho = 0.67$) correlation was observed when MVD was plotted against COX-2 score for individual cases by simple linear regression (Fig. 2). As shown by the double immunohistochemical staining, in the immediate vicinity of strong COX-2-expressing tumors large numbers of discrete blood vessels were detected (Fig. 1*B*).

Univariate and Multivariate Analyses of Prognostic Variables. Prognostic variables were analyzed by the log-rank test (Table 2). Statistically significant differences in survival were observed according to Dukes' classification (A/B *versus* C/D, $P < 0.0001$), metastatic lymph node number (≤3 *versus* ≥4, $P < 0.001$), COX-2 expression (negative *versus* positive, $P = 0.037$; Fig. 3*A*) and MVD (≤100 *versus* >100, $P = 0.007$; Fig. 3*B*). After multivariate analysis with the Cox proportional hazards model, only Dukes' classification and number of met-

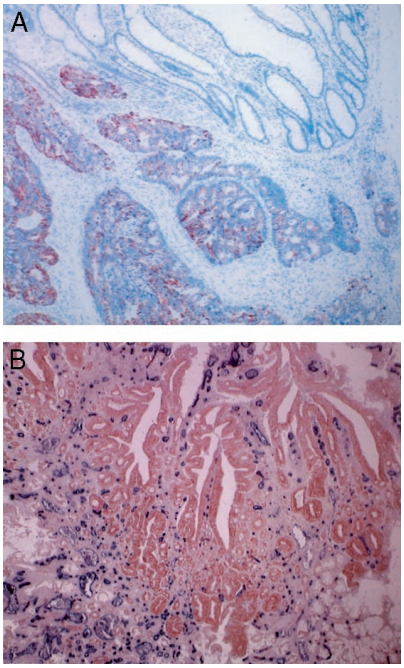

Fig. 1 *A*, immunohistochemical staining for COX-2 in a representative section of colorectal carcinoma is shown (*red* by 3-amino-9-ethylcarbazole shows positive staining and *blue* by Meyer's hematoxylin shows counterstain). COX-2 expression was restricted mainly in the tumor areas. Normal colonic mucosa adjacent to the COX-2-positive tumor was not stained or occasionally stained weakly for COX-2 (×40). *B*, immunohistochemical double staining for COX-2 (*brown* by 3,3′-diaminobenzidine) and CD34 (*dark purple* by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium). In the immediate vicinity of strong COX-2-expressing tumor, large numbers of discrete blood vessels were stained for CD34 (×40).

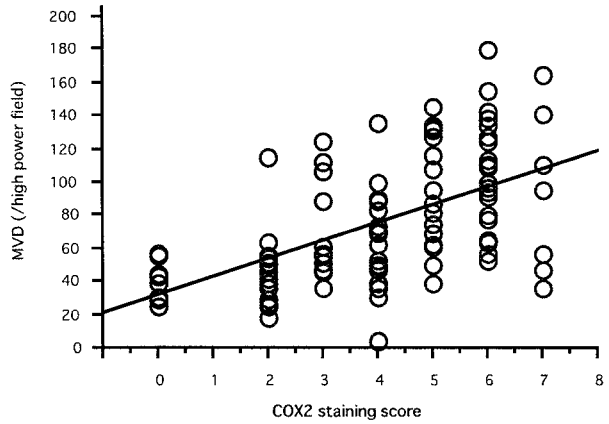

Fig. 2 Number of MVD was plotted against the COX-2 score for individual cases by simple linear regression. A significant statistical ($P < 0.0001$, $\rho = 0.67$) correlation between COX-2 expression and MVD was observed in most of the tumors.

Table 2 Univariate and multivariate analyses of prognostic variables

	Univariate	Multivariate	
	P	*P*	Relative risk
Dukes' stage (A,B *vs.* C,D)	<0.0001	0.009	4.13
No. of metastatic lymph nodes (≤3 *vs.* ≥4)	<0.001	0.018	2.64
COX-2 expression (negative *vs.* positive)	0.037	NS[a]	1.47
MVD (≤100 *vs.* >100)	0.007	NS	1.21

[a] NS, not significant.

astatic lymph nodes retained their strength as independent predictors of survival. Although both COX-2 expression and MVD were unable to predict prognosis independently, COX-2 had a better predictability of prognosis than MVD (relative risk, 1.47 *versus* 1.21, respectively).

DISCUSSION

Colorectal cancer is one of the deadliest malignancies with a grim prognosis and accounted for an estimated 55,000 deaths in the United States alone in 1998 (16). A similar trend of increasing incidence and poor outcome have been reported in Japan also (17). Among the several new treatment strategies to treat these patients, NSAIDs have drawn much attention in recent years. NSAIDs exhibited a significant antitumor effect in animal models (18, 19) and colorectal polyps in humans (20, 21). Although the exact mechanism of this antitumor effect is not clear, it has been postulated that this effect could be partially attributable to the antiangiogenic effect through modulation of COX-2 activity (19). Results from several experimental models indicate that COX-2 expression is associated with augmentation of neovascularization (11). However, to date, it is not clear whether a similar correlation exists in the clinical setting in colorectal carcinoma patients. This is the first report showing compelling evidence that COX-2 expression in human colorectal carcinoma is responsible for enhanced tumor neovascularization.

Sheehan *et al.* (22) in a recent study concluded that higher COX-2 expression in colorectal cancer was significantly associated with more advanced disease and pathological variables, which represent poor prognosis. We concur with their findings, and we found that most of the pathological variables having a poorer outlook had significant correlation with high COX-2 expression. Tsujii *et al.* (12) found that induction of COX-2 expression in colon cancer cells produced activation of membrane-type metalloproteinase. This could explain the increased invasiveness and greater metastatic potential of COX-2-expressing tumors. COX-2 expression also had a significant impact on patient survival. Patients having positive COX-2 tumors survived for a shorter time than those with negative COX-2. A similar survival advantage for patients with negative COX-2 tumors was reported by Sheehan *et al.* (22). It has been considered that tumor invasiveness, frequent metastasis, and expression in larger tumors are responsible for the worse prognosis for patients bearing COX-2-positive tumors. Hereby, we have shown that increased tumor vascularity might be another cause of the worse prognosis in this group of patients.

The tumorigenic effects of COX-2 could be divided into two distinct streamlines: the direct effect on tumor cells and the

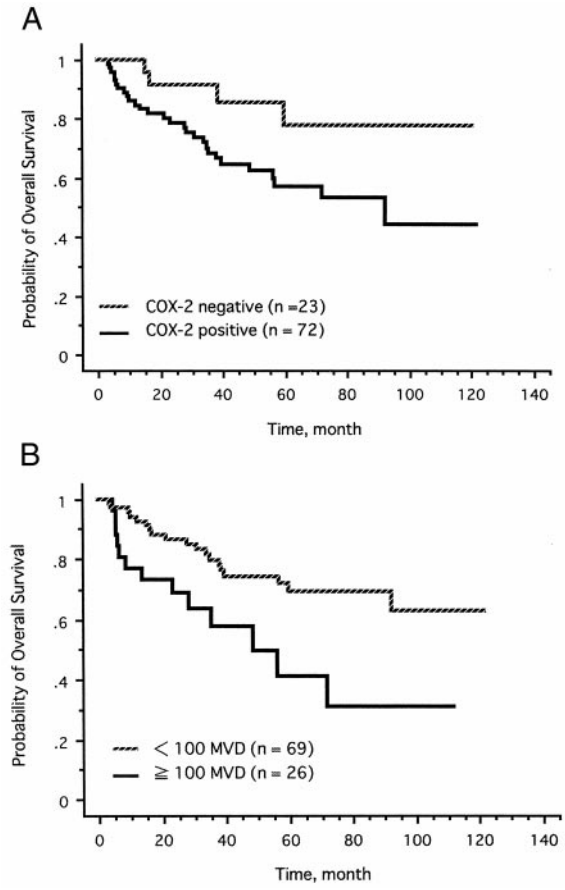

Fig. 3 *A*, the overall 10-year survival of patients with COX-2-negative and COX-2-positive after operations are shown. There was a significant difference between the two groups in survival ($P = 0.037$, by log-rank test). *B*, the overall 10-year survival of patients with low MVD and high MVD after operation are shown. There was a significant difference between the two groups in survival ($P = 0.007$).

effect on nontumor cells, such as tumor-nurturing blood vessels and immune competent cells (23). Jones *et al.* (24) also demonstrated that COX-2 inhibitors inhibited angiogenesis through direct effects on three endothelial cell lines and indicated that COX-2 was important for the direct regulation of angiogenesis in endothelial cells. Recent evidence indicates that COX-2 modulates angiogenesis either by augmenting the release of angiogenic peptides (vascular endothelial growth factor, basic fibroblast growth factor, and nitric oxide) by the tumor cells or by directly increasing production of prostaglandins (25, 26). Prostaglandins stimulate angiogenic process by endothelial cell migration and tube formation. The clinical data of this study indicate that COX-2 expression augments tumor neovacularization because a direct correlation between COX-2 expression score and MVD was observed in colorectal cancers. Using a double immunohistochemical method, we have shown that a larger number of vessels congregated in the immediate vicinity of strong COX-2-expressing tumor areas (Fig. 1*B*), whereas the reverse was true around a weak COX-2-expressing tumor area in the same tumor (data not shown). A very recent study in gastric carcinoma patients described a similar correlation between COX-2 expression and MVD (27). Apart from the tumor-associated neovacularization, it was shown in an acute exudative inflammatory model in rats that COX-2 was induced in inflammatory granuloma, and selective COX-2 inhibitors suppressed microvessel formation in such granuloma (28). Whether COX-2-induced colonic tumor neovascularization is associated with an initial inflammatory process remains to be determined.

Our results showed that COX-2 expression was associated with prognostically worse pathological variables in colorectal carcinoma and had a direct correlation with tumor MVD. Coculture of endothelial cells with tumor cells promotes COX-2-dependent endothelial motility and assembly into capillary-like structures (26). Our results showed that the relationship was materialized also in clinical colorectal cancer and suggested the effectiveness of COX-2 inhibitor for clinical chemotherapy.

REFERENCES

1. Vane, J. R., Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat. New Biol., *231:* 232–235, 1971.

2. Kargman, S., Charleson, S., Cartwright, M., Frank, J., Riendeau, D., Mancini, J., Evans, J., and O'Neill, G. Characterization of prostaglandin G/H synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. Gastroenterology, *111:* 445–454, 1996.

3. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and Dubois, R. N. Up-regulation of *cyclooxygenase 2* gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology, *107:* 1183–1188, 1994.

4. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res., *55:* 3785–3789, 1995.

5. Zimmermann, K. C., Sarbia, M., Weber, A. A., Borchard, F., Gabbert, H. E., and Schrör, K. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res., *59:* 198–204, 1999.

6. Koga, H., Sakisawa, S., Ohishi, M., Kawaguchi, T., Taniguchi, E., Sasatomi, K., Harada, M., Kusaba, T., Tanaka, M., Kimura, R., Nakashima, Y., Nakashima, O., Kojiro, M., Kurohiji, T., and Sata, M. Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor differentiation. Hepatology, *29:* 688–696, 1999.

7. Ristimäki, A., Honkanen, N., Jänkälä, H., Sipponen, P., and Härkönen, M. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res., *57:* 1276–1280, 1997.

8. Folkman, J. What is the evidence that tumors are angiogenesis dependent? J. Natl. Cancer Inst., *82:* 4–6, 1990.

9. Tanigawa, N., Amaya, H., Matsumura, M., Lu, C., Kitaoka, A., Matsuyama, K., and Muraoka, R. Tumor angiogenesis and mode of metastasis in patients with colorectal cancer. Cancer Res., *57:* 1043–1046, 1997.

10. Takebayashi, Y., Akiyama, S., Yamada, K., Akiba, S., and Aikou, T. Angiogenesis as an unfavorable prognostic factor in human colorectal carcinoma. Cancer (Phila.), *78:* 226–231, 1996.

11. Sawaoka, H., Tsuji, S., Tsuiji, M., Gunawan, E. S., Sasaki, Y., Kawano, S., and Hori, M. Cyclooxygenase inhibitors suppress angiogenesis and reduce tumor growth *in vivo*. Lab. Investig., *79:* 1469–1477, 1999.

12. Tsujii, M., Kawano, S., and DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc. Natl. Acad. Sci. USA, *94:* 3336–3340, 1997.

13. Hla, T., and Neilson, K. Human cyclooxygenase-2 cDNA. Proc. Natl. Acad. Sci. USA, *89:* 7384–7388, 1992.

14. Sutherland, D. R., Marsh, J. C., Davidson, J., Baker, M. A., Keating, A., and Mellors, A. Differential sensitivity of CD34 epitopes to cleavage by *Pasturella haemolytica* glycoprotease: implication for purification of CD34-positive progenitor cells. Exp. Hematol., *20:* 590–599, 1992.

15. Nagasue, N., Dhar, D. K., Yamanoi, A., Yamaguchi, E., Udagawa, J., Yamamoto, A., Tachibana, M., Kubota, H., Kohno, H., and Harada, T. Production and release of endothelin-1 from the gut and spleen in portal hypertension due to liver cirrhosis. Hepatology, *31:* 1107–1114, 2000.

16. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1998. CA Cancer J. Clin., *48:* 6–29, 1998.

17. The Research Group for Population-based Cancer Registration in Japan (5-3). Cancer Incidence in Japan in 1992–1993: estimates based on data from population-based cancer registries. Jpn. J. Clin. Oncol., *28:* 641–647, 1998.

18. Piazza, G. A., Alberts, D. S., Hixson, L. J., Paranka, N. S., Li, H., Finn, T., Bogert, C., Guillen, J. M., Brendel, K., Gross, P. H., Sperl, G., Ritchie, J., Burt, R. W., Ellsworth, L., Ahnen, D. J., and Pamukcu, R. Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. Cancer Res., *57:* 2909–2915, 1997.

19. Boolbol, S. K., Dannenberg, A. J., Chadburn, A., Martucci, C., Guo, X. J., Ramonetti, J. T., Abreu-Goris, M., Newmark, H. L., Lipkin, M. L., DeCosse, J. J., and Bertagnolli, M. M. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. Cancer Res., *56:* 2556–2560, 1996.

20. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R., and Offerhaus, G. J. A. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N. Engl. J. Med., *328:* 1313–1316, 1993.

21. Matsuhashi, N., Nakajima, A., Fukushima, Y., Yazaki, Y., and Oka, T. Effects of sulindac on sporadic colorectal adenomatous polyps. Gut, *40:* 344–349, 1997.

22. Sheehan, K. M., Sheehan, K., O'Donoghue, D. P., MacSweeney, F., Conroy, R. M., Fitzgerald, D. J., and Murray, F. E. The relationship between cyclooxygenase-2 expression and colorectal cancer. J. Am. Med. Assoc., *282:* 1254–1257, 1999.

23. Rahman, M. A., Dhar, D. K., Masunaga, R., Yamanoi, A., Kohno, H., and Nagasue, N. Sulindac and exisulind exhibit significant antiproliferative effect and induce apoptosis in human hepatocellular carcinoma cell lines. Cancer Res., *60:* 2085–2089, 2000.

24. Jones, M. K., Wang, H., Peskar, B. M., Levin, E., Itani, R. M., Sarfeh, I. J., and Tarnawski, A. S. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. Nat. Med., *5:* 1418–1423, 1999.

25. Form, D. M., and Auerbach, R. PGE2 and angiogenesis. Proc. Soc. Exp. Biol. Med., *172:* 214–218, 1983.

26. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93:* 705–716, 1998.

27. Uefuji, K., Ichikura, T., and Mochizuki, H. Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer. Clin. Cancer Res., *6:* 135–138, 2000.

28. Katori, M., Majima, M., and Harafa, Y. Possible background mechanisms of the effectiveness of cyclooxygenease-2 inhibitors in the treatment of rheumatoid arthritis. Inflamm. Res., *27* (Suppl. 2)*:* S107–S111, 1998.

Clinical Cancer Research

Cyclooxygenase-2 Expression Correlates with Tumor Neovascularization and Prognosis in Human Colorectal Carcinoma Patients

Reiko Masunaga, Hitoshi Kohno, Dipok Kumar Dhar, et al.

Clin Cancer Res 2000;6:4064-4068.