Int. J. Cancer: **91,** 894–899 (2001)

Publication of the International Union Against Cancer

TUMOR INVASIVENESS AND LIVER METASTASIS OF COLON CANCER CELLS CORRELATED WITH CYCLOOXYGENASE-2 (COX-2) EXPRESSION AND INHIBITED BY A COX-2–SELECTIVE INHIBITOR, ETODOLAC

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Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to reduce the risk and mortality of colorectal cancer (CRC). Although the exact mechanisms remain unclear, the inhibition of cyclooxygenase (COX) by NSAIDs appears to abort, if not prevent, CRC carcinogenesis or metastatic tumor progression. The aim of our study was to investigate the association between COX-2 expression and CRC tumor cell invasiveness. The differences in immunoblot-detectable COX-2 protein contents in primary CRCs, metastatic hepatic lesions and corresponding normal mucosa from the same individual were evaluated in 17 patients. Three different colon cancer cell lines, SW620, Lovo, HT-29 and a metastatic variant of HT-29, HT-29/Inv3, were employed to evaluate COX-2 expression and prostaglandin E_2 (PGE_2) production in relation to their invasive abilities *in vitro*. The effects of a COX-2–selective inhibitor, etodolac, on cell proliferation and invasive activity were also determined. The results showed that 15 of 17 (88%) metastatic CRC cells from the liver and 14 of 17 (82%) primary CRC tissue exhibited much higher levels of COX-2 than corresponding adjacent normal mucosa from the same patient. Among those patients with relatively high COX-2 expression in the primary tumors, almost all exhibited even higher levels of COX-2 in their hepatic metastases. Among the 4 colon cancer cell lines, HT-29/Inv3 manifested the highest COX-2 expression, PGE_2 production and *in vitro* invasive activity. The selective COX-2 inhibitor, etodolac, could especially exert cytotoxicity and markedly suppress the invasive property and PGE_2 production, although not the COX-2 protein level, in HT-29/Inv3 cells. Our results imply that COX-2 expression may be associated with the invasive and metastatic properties of CRC tumor cells.

Key words: *tumor invasiveness; COX-2 inhibitor; colon cancer; liver metastases*

Some NSAIDs are among the most commonly used pharmaceuticals worldwide. From a plethora of well-known therapeutic effects on thermo-regulation and anti-inflammation, certain individual compounds have also been noted to have cancer prevention properties, particularly against gastrointestinal cancers.[1] Evidence from both animal experiments and large-scale epidemiological studies points toward NSAIDs being potential chemopreventive agents for CRC.[2–5] In patients with FAP, administration of sulindac resulted in regression and simultaneously prevented recurrence of polyps, the precursor lesion of colon cancer.[6,7] Epidemiological studies further ascertained that regular intake of aspirin or other NSAIDs not only has the potential to prevent the occurrence of CRC, but also may improve the prognosis in terminal CRC patients.[8]

COX, also known as PGHS, is a key enzyme in catalyzing the conversion of AA to PGs and other eicosanoids. Two isoforms of COX, COX-1 and COX-2, have been identified and isolated by gene cloning. COX-1 is expressed constitutively in a number of cell types and tissues such as gastric mucosa, generating PGs for normal physiological function. In contrast, COX-2 is an early gene which is rapidly induced by a variety of agents, including LPS, cytokines, growth factors and tumor promoters. Many studies have consistently observed that mRNA and protein levels of COX-2, but not COX-1, are markedly elevated in rodent colonic tumors that develop after carcinogenic treatment[9] and in most human colorectal adenocarcinoma tumors.[10] Similar observations were made in adenomas from $B6^{Min} \times 129$ *Min* mice.[11] In addition, PGE_2 is also significantly increased in human colon cancer cells, with the capability of inhibiting programmed cell death (or apoptosis) possibly by up-regulation of Bcl-2 expression.[12] Moreover, inhibition of COX-2 could markedly suppress the number and size of intestinal polyps in APC delta 716 "knockout" mutant mice, indicating that COX-2 expression may play a pivotal role in colorectal carcinogenesis.[13] There has been clinical confirmation of a significant inverse relationship between patient survival and increased COX-2 expression by immunohistochemical study of surgical specimens.[14,15]

Metastasis is the major cause of death in colorectal adenocarcinoma. The invasive potential of tumor cells portends for further metastases. Proteolysis of the basement membrane, consisting of laminin, fibronectin, type IV collagen and proteoglycan, is an important step in tumor invasion.[16] It is a fact that the activity of MMPs is markedly increased in COX-2–over-expressing Caco-2 colon cancer cells.[17] Increased invasiveness and PGE_2 production can be reversed by treatment with sulindac sulfide, drawing a direct link between COX-2 and activation of MMPs. Murata *et al.*[18] indicated that COX-2 over-expression might enhance lymphatic invasion and metastases in human gastric carcinoma.

The effect of COX-2 inhibitors on colon cancer carcinogenesis has been the subject of much investigation.[2,9] Relatively less is known about COX-2 protein level and enzyme activity in different colon cancer cells in relationship to their invasive potentials, and about clinical significance of changes in COX-2 in the process of colon cancer metastasis. Our study was designed to address these specific questions by using paired surgical specimens from primary tumors, hepatic metastases and the corresponding normal mucosa from the same CRC patients in Taiwan, and by using various colon cancer cell lines with different metastatic potentials. Our results revealed that COX-2 expression, PG production emanating thereof, is positively correlated with tumor cell invasive-

Abbreviations: AA, arachidonic acid; bFGF, basic fibroblast growth factor; COX-2, cyclooxygenase 2; CRC, colorectal cancer; ECL, enhanced chemiluminescence; EIA, enzyme immunoassay; FAP, familial adenomatous polyposis; HRP, horseradish peroxidase; LPS, lipopolysaccharide; MEM, minimal essential medium; MMP, metalloproteinase; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; PGE_2, prostaglandin E_2; PGHS, prostaglandin G/H synthase; PVDF, polyvinylidene fluoride; VEGF, vascular endothelium growth factor.

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Received 24 May 2000; Revised 17 October 2000; Accepted 3 November 2000

ness. We also show that a COX-2–selective inhibitor, etodolac, although it does not affect the COX-2 protein level, could inhibit PGE_2 production and suppress tumor invasiveness.

MATERIAL AND METHODS

Colon cancer cell lines

Human SW620, Lovo, HT-29 colorectal carcinoma cell lines (ATCC, Rockville, MD) and a selected variant clone of HT-29, HT-29/Inv3, were grown in MEM (Sigma, St. Louis, MO). This was supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Life Technologies, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO_2. HT-29/Inv3 cells were originally isolated by selecting rare variants of HT-29 cells that rapidly penetrated a layer of Matrigel (Sigma). After expansion, the HT-29 variants were injected intra-splenetically in nude mice, which subsequently formed metastatic tumor nodules in the liver. Tumor cells were again removed and the Matrigel invasion procedure was repeated. All cell lines were regularly tested and assured to be free from Mycoplasma infection.

Immunoblot analysis

Surgical specimens from 17 CRC patients who underwent resection of both the primary and subsequent liver metastases at the Veterans General Hospital-Taipei between 1997 and 1999 were analyzed. Tissue samples of the primary tumor (T), hepatic metastasis (M) and corresponding normal mucosa (N) were rinsed in sterile normal saline and snap frozen in liquid nitrogen immediately after surgical removal. A portion was also fixed in formalin for pathological verification. Frozen tissues were homogenized in lysis buffer (50 mM TRIS-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 1 mM diethyldithiocarbamic acid, 1 mM EDTA, 1% Tween 20, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) on ice. The lysates were sonicated and centrifuged at 10,000 × g for 20 min. The monolayer of human HT-29, SW620, Lovo, HT-29/Inv3 and etodolac-treated HT-29/Inv3 colon cancer cells was harvested with a scraper, washed twice in PBS by centrifugation and suspended in buffer C (50 mM TRIS-HCl, 4 mM EDTA, 2 mM EGTA, 10 mM DTT, 1 mM PMSF), briefly sonicated and centrifuged at 100,000 × g for 1 hr. The supernatant was used for immunoblot analysis. An equal amount (60 μg) of protein was separated by electrophoresis in 10% SDS polyacrylamide gel and transferred onto PVDF membranes (Amersham, Arlington Heights, IL). These were then reacted with the specific primary antibodies in 3% BSA overnight at 4°C (COX-2 antibody [1:250] from Transduction Laboratories [Lexington, KY] and COX-1 and CDC2 antibodies [0.5 μg/ml] from Santa Cruz Biotechnology [Santa Cruz, CA]), followed by a 1 hr incubation with HRP-conjugated secondary anti-mouse antibody (Amersham). Specific protein bands were visualized using an ECL detection system (Amersham). CDC2 protein, which is relatively constant in these cells, was employed as an internal control. The relative amounts of COX-2 were quantitated by using densitometry and expressed as the ratio of COX-2 in primary tumors, hepatic metastases with that in normal mucosa.

Determination of PGE_2 production

PGE_2 was measured in the culture media taken from human HT-29, SW620, Lovo, HT-29/Inv3 and etodolac-treated HT-29/Inv3 colorectal carcinoma cells by competitive EIA, according to the manufacturer's instructions (Assay Designs, Ann Arbor, MI). Briefly, 1×10^6 cells per well were plated on 6-well dishes and incubated overnight. HT-29/Inv3 cells were prereated with or without 0.5 mM of etodolac for 1 hr. For measuring PGE_2 production, the monolayer cells were incubated with fresh medium containing 10 μM of AA for 1 hr. The harvested medium was centrifuged at 500 × g for 5 min (4°C) to remove the floating cells and the supernatant was frozen at -20°C until use for PGE_2 determination. The monolayer cells were used for immunoblot analysis of the COX-1, COX-2 and CDC2 contents.

In vitro *cell invasion assay*

In vitro cell invasion assay was performed as described previously.[19] Human HT-29, SW620, Lovo and HT-29/Inv3 colorectal carcinoma cells were treated with either 0.5 mM etodolac or medium only for 24 hr. PVP-free polycarbonate filter, 8 μm in pore size (Nuclepore; Corning Costar, Acton, MA), was coated with Matrigel (1.05 mg/ml) for 1 hr at 37°C. Cells (5×10^4) were placed on the top well of the Boyden chamber (Corning Costar). After 8 hr of incubation at 37°C in 5% CO_2, the filter was fixed in 4% paraformaldehyde and stained with hematoxylin. Cells on the upper filter surface were carefully removed and cells on the lower filter surface were counted under light microscope. All experiments were performed in triplicate and repeated twice.

Measurement of etodolac-induced cytotoxicity

Human HT-29, SW620, Lovo and HT-29/Inv3 cells were plated 2×10^3 per well in 96-well plates and incubated overnight. Medium was changed to contain 0, 0.25, 0.5, 1.0 and 2.5 mM of etodolac (Wyeth-Ayerst Research, Philadelphia, PA). Seventy-two hours later, 50 μl of 0.5% MTT in PBS was added to the 100 μl medium in each well. After incubation at 37°C for 2 hr, the (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) medium was removed and 100 μl of DMSO was added to extract the blue formazan precipitate. The color reaction was measured using an ELISA reader (Bio-Rad, Hercules, CA). Cell viability was simultaneously evaluated by trypan blue exclusion staining.

Statistical evaluation

COX-2 expression levels were evaluated by paired 2-tailed *t*-test with 95% confidence interval. Significance was determined if $p < 0.05$. The statistical package used was SPSS, release 8.0.

RESULTS

COX-2 expression in primary, metastatic liver tumors of CRC patients and their corresponding normal mucosa

To determine the significance of COX-2 expression in tumor invasion and metastasis in the clinical scenario, the expression level of COX-2 protein in primary, metastatic liver tumors, as well as the corresponding normal mucosa of 17 CRC patients, was evaluated by immunoblotting analysis. The 17 patients included 4 females and 13 males, ranging in age from 50 to 83 years old. There were 13 T3 and 4 T4 tumors, 16 of 17 had lymph node metastases and histological differentiation was moderate in 13 and poor in 4 cases. Primary tumors were located at the sigmoid (8), ascending (3), transverse (1), descending colon (1) and rectum (4). A relatively high level of COX-2 protein was detected in 15 of 17 (88.2%) liver CRC metastatic tumors and in 14 of 17 (82.4%) primary tumors in comparison to the corresponding normal mucosa from the same patient (Figs. 1*a*,*b*). The mean COX-2 expression level of either primary tumor (19,150 ± 4,205) or metastatic liver tumor (33,665 ± 6,222) is significantly higher than that of the corresponding normal mucosa (9,861 ± 3,248) with a *p* value of 0.001 and 0.01, respectively. In patients with a relatively high COX-2 level in the primary tumor, almost all (92.9%) exhibited an even higher COX-2 level in their hepatic metastases. Seventy-five percent (3 of 4) of patients with poorly differentiated tumors and 84.6% (11 of 13) of patients with moderately differentiated tumors had a high COX-2 level in their primary tumor.

Correlation of COX expression and PGE_2 synthesis with invasive potential in different colon cancer cell lines (HT-29, SW620, Lovo and HT-29/Inv3)

To determine the possible significance in an experimental model, 3 established human colon carcinoma cell lines, HT-29, SW620 and Lovo, were examined for COX protein levels as well

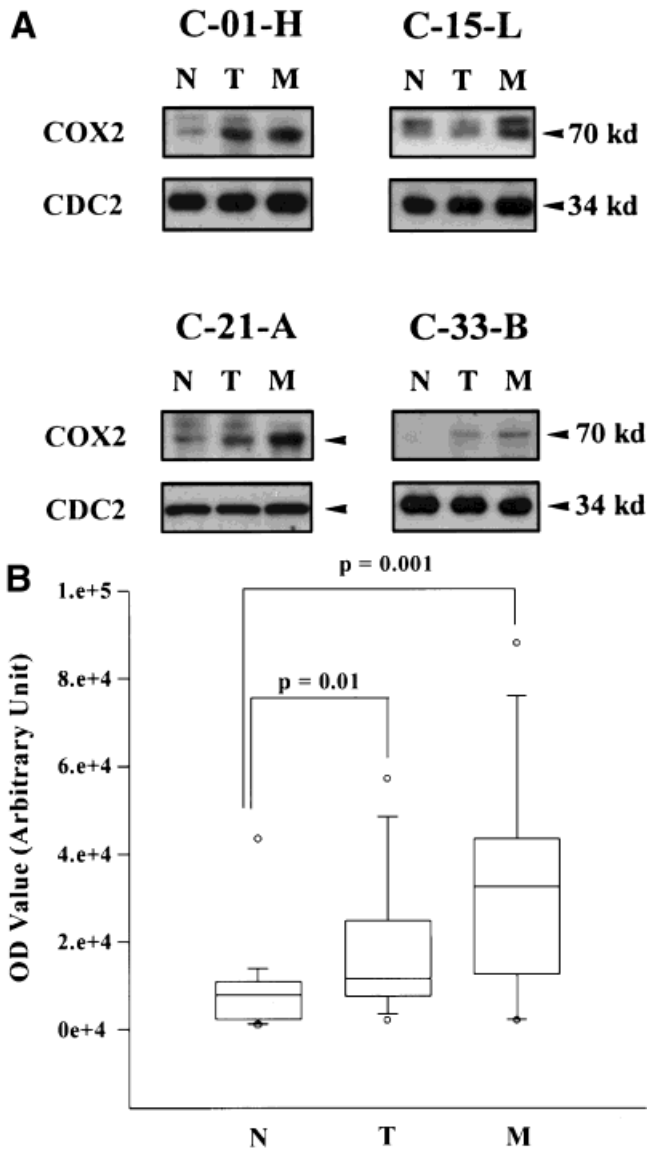

FIGURE 1 – COX-2 expression in paired clinical samples of colorectal carcinoma. (*a*) Immunoblot analysis of COX-2 protein in 4 paired clinical samples (C-01-H, C-15-L, C-21-A and C-33-B) containing primary tumor (T), hepatic metastasis (M) and adjacent normal mucosa (N). Cdc2 protein level was used as a control. (*b*) Comparative COX-2 protein levels of tissue from normal, primary tumor and hepatic metastatic tumor of individual patients. The COX-2 expression level of each sample was quantitated by densitometric measurement. Comparisons of COX-2 protein levels of either primary tumor (T) or hepatic metastasis (M) with their corresponding normal mucosa (N) were analyzed by paired *t*-test.

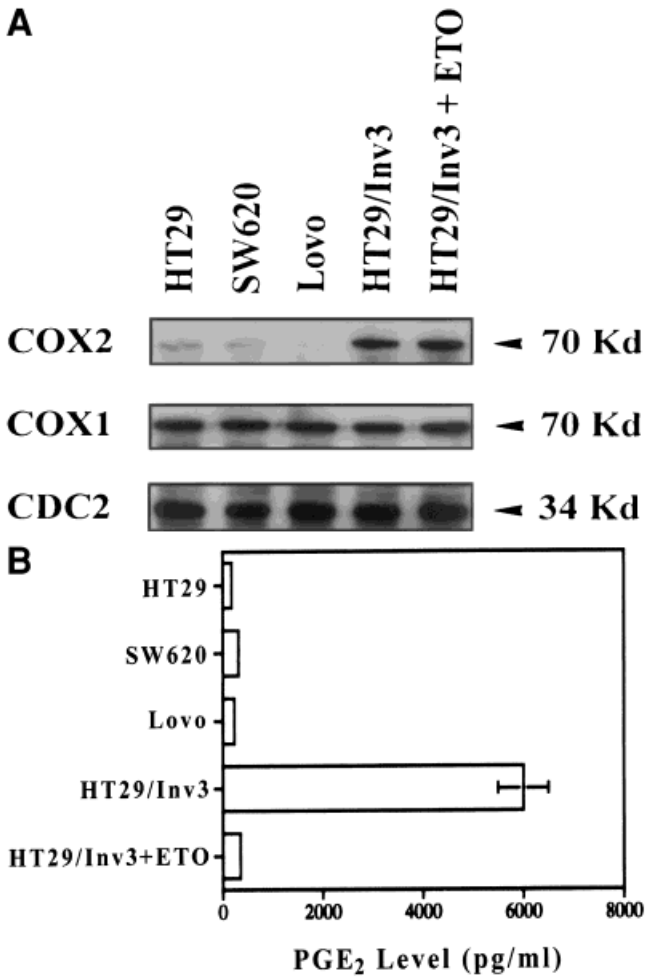

FIGURE 2 – Contents of COX proteins and PGE_2 production in colorectal carcinoma cell lines. (*a*) Immunoblot analysis of COX-1 and COX-2 proteins in HT-29, SW620, Lovo, HT-29/Inv3 and HT-29/Inv3 treated with etodolac. Cdc2 protein was used as a control. (*b*) PGE_2 production measured by EIA in 1 hr culture media taken from HT-29, SW620, Lovo, HT-29/Inv3 and HT-29/Inv3 treated with 0.5 mM etodolac. Measurements of PGE_2 production by the cells were performed in the presence of AA (10 μM) in the medium. Strong suppression of PGE_2 production by etodolac was noted in HT-29/Inv3 cells.

as for PGE_2 synthesis. A variant highly metastatic cell clone of HT-29, HT-29/Inv3, was also examined for comparison. Immunoblot analysis revealed that COX-2 content was highest in HT-29/Inv3 cells, considerably lower in HT-29 and SW620 cells and barely detectable in Lovo cells. In contrast, COX-1 protein content was not significantly different among these CRC cell lines including HT-29/Inv3 (Fig. 2*a*). As shown in Figure 2*b*, the increased COX-2 content in HT-29/Inv3 cells was accompanied by a significant increase in PGE_2 synthesis (6 ng/ml). This could be markedly reduced by treatment of the cells with 0.5 mM of the selective COX-2 inhibitor, etodolac, 1 hr before and during the time of PGE_2 production. The high COX-2 protein level of HT-29/Inv3 cells, however, was not affected by the brief etodolac treament (Fig. 2*a*).

Effect of COX-2 inhibition on invasion of COX-2–over-expressing colon cancer cells

The COX-2–over-expressing HT-29/Inv3 cell line, when examined by the *in vitro* Matrigel cell invasion assay, exhibited a highly invasive character (Figs. 3*a*,*b*). Under the same incubation conditions, the number of HT-29/Inv3 cells that invaded the Matrigel membrane layer was 200- to 250-fold higher than those of the other 3 colon cnacer cell lines with low or undetectable COX-2 expression (Fig. 3*a*). The effect of the COX-2 inhibitor (etodolac) on tumor cell invasiveness was also examined by the *in vitro* assay using Matrigel membrane matrix. HT-29/Inv3 cells were cultured either with or without the COX-2 inhibitor, etodolac, at a concentration of 0.5 mM for 24 hr. Etodolac treatment exerted a strong inhibitory effect on HT-29/Inv3 cells, causing a 27- to 31-fold decrease in the number of cells that penetrated the Matrigel layer (Figs. 3*a*,*b*). In contrast, no such effects of etodolac on tumor

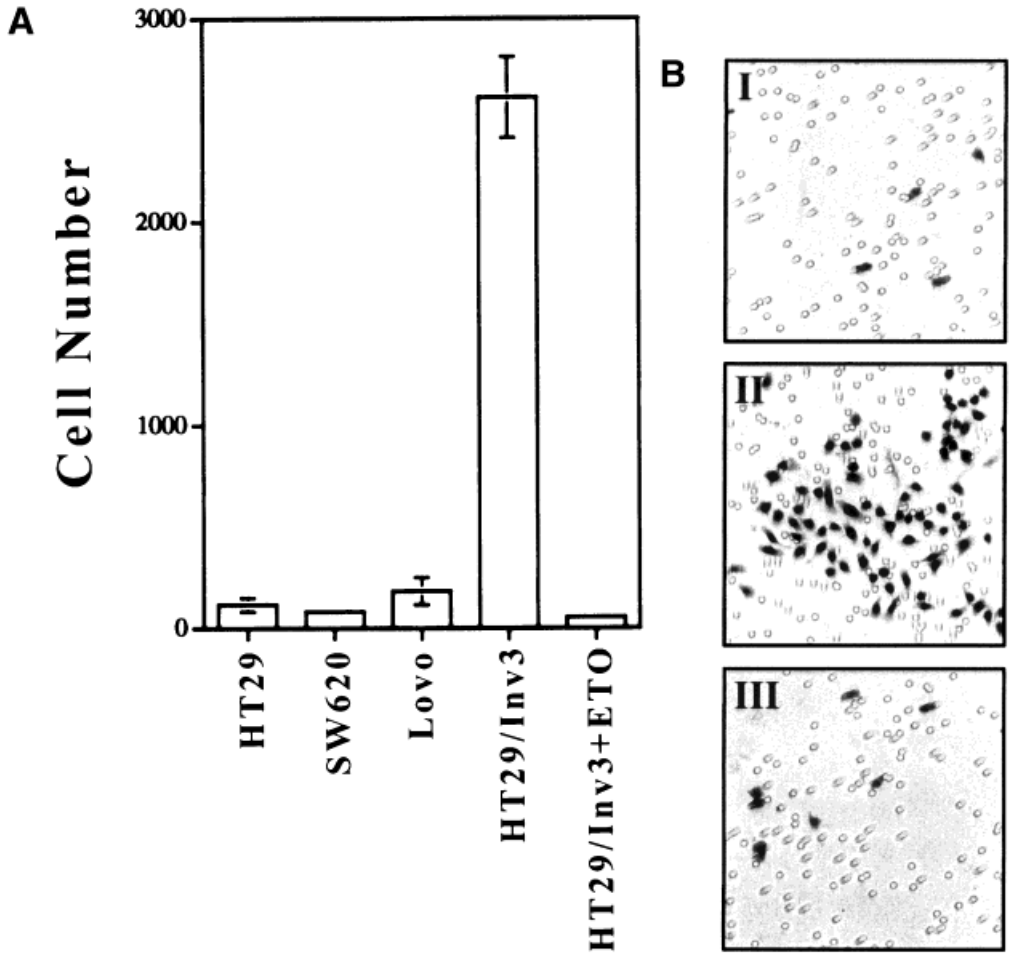

FIGURE 3 – Measurements of invasive potential in colorectal carcinoma cell lines. (*a*) Matrigel assay of HT-29, SW620, Lovo, HT-29/Inv3 and HT-29/Inv3 treated with etodolac. Cells penetrating Matrigel-coated PVP-free PC filters (8 μm) in the Boyden chamber were counted. Experiments were performed in triplicate and repeated twice. (*b*) Microscopic pictures of HT-29 (top), HT-29/Inv3 (middle) and etodolac-treated HT-29/Inv3 (bottom) for cells that had passed through the Matrigel filter (100× magnification).

invasiveness were obvious in SW620 or Lovo colon cancer cells which contained much less COX-2 enzyme activity (data not shown).

Cytotoxicity of selective COX-2 inhibitor in different human colon cancer cell lines

In the above *in vitro* experiments, especially by prolonged treatment with etodolac, cell growth inhibition was often observed which varied among SW620, Lovo, HT-29 and HT-29/Inv3 cells. Therefore, these 4 different human colon cancer cell lines were grown in MEM containing 0, 0.25, 0.5, 1.0 and 2.5 mM of etodolac. Viable cells were measured by the MTT assay. The effect of etodolac on cell viability was not obvious at 24 hr, but began to emerge around 36–48 hr. At 72 hr, all 4 cell lines showed cell growth inhibition in a dose-dependent manner, with considerable variation in susceptibility to etodolac cytotoxicity (Fig. 4). The estimated IC_{50}s, or concentrations of etodolac causing 50% inhibition, were 0.5 mM in HT-29/Inv3 cells, 1.75 mM in Lovo cells, 1.88 mM in SW620 cells and 1.88 mM in HT-29 cells. This indicated that HT-29/Inv3 was more susceptible than others to the selective COX-2 inhibitor, NSAID. The time course and the drug dose suggest that the effects of etodolac on PGE_2 synthesis or on the invasive activity of HT-29/Inv3 cells could not have resulted from cytotoxicity.

DISCUSSION

Substantial evidence from epidemiological surveys, clinical observations and animal model studies has strongly implied that NSAIDs have the potential of preventing carcinogenesis[20,21] and positively ameliorating the poor prognosis of some CRC patients.[22] The anti-neoplastic effect of NSAIDs was presumably mediated through inhibition of the COX enzyme, particularly COX-2.[13,23] Two issues should be considered with regard to the NSAID effect, chemoprevention of carcinogenesis initiation and subsequent inhibition of neoplastic progression. Relatively less is known about tumor invasion and dissemination. We have therefore initiated a systematic study to address the issue of CRC tumor invasion and metastasis. The purpose of our study is to determine COX protein content in surgical specimens of normal mucosa, primary tumors and liver metastases from the same patient. We also determine the functional activities of COX-2 as well as selective COX-2 inhibition *in vitro* culture of human colon cancer cell lines and a selected highly invasive variant. In general, our results support the hypothesis that functional expression of COX-2 may enhance the invasive and metastatic potential of CRC cells.

Previous studies have observed an increased COX-2 expression in 80% of colorectal carcinomas and in 40% of pre-malignant adenomas, suggesting a relationship to neoplastic progression.[23,24] Two recent studies have specifically addressed the issue of tumor invasion and COX-2 levels in human colorectal carcinomas. Fujita

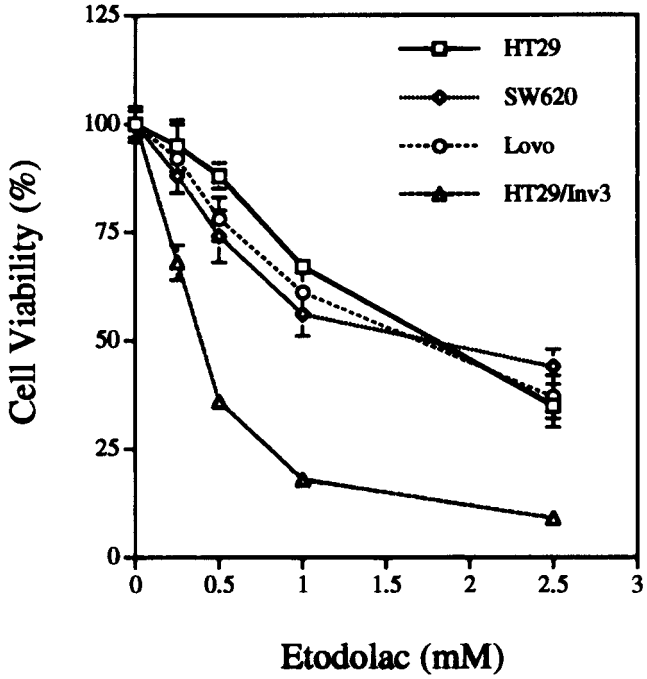

FIGURE 4 – Cytotoxicity of etodolac on colorectal carcinoma cell lines. Cell viability of HT-29, SW620, Lovo and HT-29/Inv3 using MTT assay after exposure to varying concentrations of etodolac (0, 0.25, 0.5, 1.0 and 2.5 mM). Note that HT-29/Inv3 was more susceptible to etodolac than HT-29 and the other cell lines. The means and standard errors of 3 separate experiments carried out in quadruplicate are depicted.

et al.[14] found that higher COX-2 levels were correlated with larger tumor size and deeper invasion in 43 primary colorectal carcinomas. Examining COX-2 content by specific immunochemical staining of tumor tissue sections for 76 patients with colorectal carcinomas at various staging and 14 normal colon biopsy samples, Sheehan *et al.*[15] detected no COX-2 staining in normal colon controls, weak staining in normal mucosa juxtaposing the tumor and varying degrees of COX-2 staining in tumor cells. After detailed analysis, they found the association of COX-2 staining with more advanced Duke's stage, larger tumor size and particularly lymph node involvement. In our study, we utilized specific immunoblotting procedures to estimate COX-2 protein levels in adjacent normal mucosa, primary tumors and liver metastatic tumors in fresh-frozen specimens from individual CRC patients. Although the number of patients was small and the COX-2 measurement at best semi-quantitative, the results have confirmed Fujita *et al.*-Sheehan *et al.*'s previous findings of high COX-2 expression in primary CRC tumor tissues. The results also revealed a new observation that liver metastatic CRC tumors tend to have still higher COX-2 protein levels than the primary tumors from the same patients. In this regard, our finding is consistent with observations that PGE_2 levels, a COX enzyme-catalyzed product, were raised in CRC patients with liver or lung metastases.[25]

COX-2 studies of tumor tissues may be complicated by heterogeneity of tumor cell populations and by dilution of normal cells. To further explore the therapeutic implications of increased COX-2 expression in the liver metastatic CRC tumor cells, we turned to *in vitro* studies with established cell lines from human CRC. In view of the possibility that long-term *in vitro* culture may have altered the original pathophysiological properties, including COX-2 expression, in these established CRC cell lines, we used the Matrigel membrane to select for rare HT-29 cell variants that could rapidly invade the membrane. Some of these highly invasive variant HT-29 clones showed high COX-2 levels. In particular, the HT-29/Inv3 variant clone expressed considerably higher COX-2 levels as well as PGE_2 synthesis than the original HT-29, Lovo and SW620 cells (Figs. 2*a*,*b*). Although the reason for the high COX-2 expression remains unknown, these variant HT-29 cell clones were useful for the study of NSAID effects, which may be similar but distinct from the Caco-2 cell that has been transduced with COX-2 to increase invasive capability.[17] Using the highly invasive and highly COX-2–expressing HT-29/Inv3 cells, we have found that 2 to 24 hr treatment with the selective COX-2 inhibitor, etodolac, can inhibit PGE_2 synthesis (Fig. 2*b*), while ameliorating the invasive potential (Fig.3*a*), although COX-2 protein levels remained high (Fig. 2*a*). The results clearly indicate that the enzymatic activity, rather than the enzyme protein content per se, of COX-2 is essential for the invasive potential of HT-29/Inv3 cells.

Many NSAIDs were found to inhibit cell division and alter the cell cycle distribution in cultured colon cancer cells.[26] Tumor cells were arrested in G0/G1 phase, finally undergoing apoptosis. In addition, the effects of NSAIDs on the cell cycle were also accompanied by a reduction in certain cyclin-dependent kinases, such as $p34^{cdc2}$ and $p33^{cdk2}$.[27] The inhibition of colon cancer cell growth by NSAIDs was mainly mediated through inhibition of COX expression. COX-2 inhibitors have been demonstrated to inhibit the growth of intestinal cancer cells either in culture or as xenografts in athymic nude mice.[28] In our study, there is a clear dose-dependent inhibitory effect of a selective COX-2 inhibitor, etodolac, on CRC tumor cell growth. As shown in Figure 4, the high COX-2–expressing colon cancer cell line, HT-29/Inv3, was much more susceptible to etodolac killing (IC_{50}: 0.50 mM) than the other CRC cell lines (IC_{50}: ~1.88 mM). This cytotoxic effect of etodolac was most likely mediated through selective COX-2 inhibition. However, repeated treatment with NSAIDs could cause apoptosis in S/KS cells with undetectable COX-2 expression as well as in COX-2–expressing HT-29 cells.[29] Thus, it remains to be determined if cytotoxicity with or without COX-2–mediated action could account for the chemopreventive effect of a selective COX-2 NSAID. It also needs to be determined if the mechanisms of cytotoxicity in HT-29/Inv3 cells at low doses of etodolac would be the same as that in the established cell lines of Lovo, SW620, HT-29 or S/KS at relatively high doses of this selective COX-2 inhibitor NSAID.

Tumor metastasis is a complex multi-factorial and multi-step process. Many factors, including the invasive potential of tumor cells, host immunity or the angiogenesis milieu at the metastatic loci, will influence the establishment of metastatic lesions. In this regard, MMPs are known to play important roles in tumor invasion and metastasis by degradation of the type IV collagen contained in the basement membrane.[16,30] We have made attempts to determine possible MMP activity changes in these CRC cell lines with different invasive and metastatic activities and to assess the effect of etodolac treatment. With the use of gelatin gel electrophoresis zymogram to demonstrate MMP-2 and MMP-9, no definite difference in the level of MMPs could be demonstrated, due to sensitivity of the method. Secretion and cellular contents of MMP-9 and MMP-2 in HT-29/Inv3 cells were decreased 2- to 20-fold following etodolac treatment, although complicated by the cytotoxic effect in our experiments (data not shown). This aspect requires further investigation. Regarding angiogenesis, COX-2 inhibitors have also been known to reduce angiogenesis driven by bFGF, VEGF and carrageenan in the rat model.[31] Studies[17,32] using CRC cells transfected with the COX-2 gene have demonstrated the increased metastatic ability of CRC cells with COX-2 over-expression, which may also regulate angiogenesis in favor of tumor growth and metastatic spread. Taking an opposite approach of selecting invasive HT-29/Inv3 variant cells, we have reached the same conclusion of an apparent correlation between COX-2 expression and malignant progression. It would be of interest to further investigate if HT-29/Inv3 cells are capable of regulating angiogenesis, as has been observed by Tsujii *et al.*[17,32] Furthermore, we have found that etodolac treatment of HT-29/Inv3 cells inhibited COX-2–associated PGE_2 synthesis, Matrigel invasion and MMP activity. It also induced apoptosis through a distinct caspase-10–mediated cascade of caspase activation (Wei et al., unpublished data).

In conclusion, COX-2 expression was increased not only in the primary CRC but also in the hepatic metastases with even higher expression. Colon cancer cells with high COX-2 expression might exhibit a more invasive phenotype, which could be abrogated by a selective COX-2 inhibitor. Future clinical trials should consider incorporation of a COX-2 inhibitor with the potential benefit for patient survival.

REFERENCES

1. Thun MJ. Aspirin and gastrointestinal cancer. Adv exp Med Biol 1997;400A:395–402.
2. Kahn MJ, Morrison DG. Chemoprevention for colorectal carcinoma. Hematol Oncol Clin North Amer 1997;11:779–94.
3. Sandler RS, Galanko JC, Murray SC, Helm JF, Woosley JT. Aspirin and nonsteroidal anti-inflammatory agents and risk for colorectal adenomas [see comments]. Gastroenterology 1998;114:441–47.
4. Shiff S, Rigas, B. Nonsteroidal anti-inflammatory drugs and colorectal cancer: evolving concepts of their chemopreventive actions. Gastroenterology 1997;130:1992–8.
5. Williams CS, Smalley W, DuBois RN. Aspirin use and potential mechanisms for colorectal cancer prevention. J Clin Invest 1997;100: 1325–9.
6. DuBois RN, Giardiello FM, Smalley WE. Nonsteroidal anti-inflammatory drugs, eicosanoids, and colorectal cancer prevention. Gastroenterol Clin North Am 1996;25:773–91.
7. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med 1993;328: 1313–16.
8. Kune GA, Kune S, Watson LF. Colorectal risk, chronic illness, operations and medication: case control results from Melbourne. Cancer Res 1988;48:4399–404.
9. DuBois RN, Smalley WE. Cyclooxygenase, NSAIDs, and colorectal cancer. J Gastroenterol 1996;31:898–906.
10. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Upregulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–88.
11. Williams CS, Luongo C, Radhika A, Zhang T, Lamps LW, Nanney LB, et al. Elevated cyclooxygenase-2 levels in Min mouse adenomas. Gastroenterology 1996;111:1134–40.
12. Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res 1998;58:362–66.
13. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, et al. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996;87: 803–9.
14. Fujita T, Matsui M, Takaku K, Uetake H, Ichikawa W, Taketo MM, et al. Size- and invasion-dependent increase in cyclooxygenase 2 levels in human colorectal carcinomas. Cancer Res 1998;58:4823–26.
15. Sheehan KM, Sheahan K, O'Donoghue DP, MacSweeney F, Conroy RM, Fitzgerald DJ, et al. The relationship between cyclooxygenase-2 expression and colorectal cancer. J amer med Assoc 1999;282:1254–57.
16. Cockett MI, Murphy G, Birch ML, O'Connell JP, Crabbe T, Millican AT, et al. Matrix metalloproteinases and metastatic cancer. Biochem Soc Symp 1998;63:295–313.
17. Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc nat Acad Sci Wash 1997;94:3336–40.
18. Murata H, Kawano S, Tsuji S, Tsuji M, Sawaoka H, Kimura Y, et al. Cyclooxygenase-2 overexpression enhances lymphatic invasion and metastasis in human gastric carcinoma. Amer J Gastroenterol 1999; 94:451–55.
19. Lin MT, Wei SJ, Wing LY. Chemotactic and mitogenic activities of granulosa cells in developing follicles. Mol cell Endocrinol 1992;84: 47–54.
20. Jacoby RF, Marshall DJ, Newton MA, Novakovic K, Tutsch K, Cole CE, et al. Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. Cancer Res 1996;56:710–14.
21. Beazer-Barclay Y, Levy DB, Moser AR, Dove WF, Hamilton SR, Vogelstein B, et al. Sulindac suppresses tumorigenesis in the Min mouse. Carcinogenesis 1996;17:1757–60.
22. Thun MJ, Namboodiri MM, Heath CW Jr. Aspirin use and reduced risk of fatal colon cancer [see comments]. N Engl J Medicine 1991; 325:1593–96.
23. Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, et al. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. Proc nat Acad Sci Wash 1996;93:4816–20.
24. Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, et al. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 1995;55:3785–89.
25. Narisawa T, Kusaka H, Yamazaki Y, Takahashi M, Koyama H, Koyama K, et al. Relationship between blood plasma prostaglandin E2 and liver and lung metastases in colorectal cancer. Dis Colon Rectum 1990;33:840–45.
26. Qiao L, Hanif R, Sphicas E, Shiff SJ, Rigas B. Effect of aspirin on induction of apoptosis in HT-29 human colon adenocarcinoma cells. Biochem Pharmacol 1998;55:53–64.
27. Shiff SJ, Koutsos MI, Qiao L, Rigas B. Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis. Experimental cell Res 1996;222:179–88.
28. Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, et al. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J clin Invest 1997;99:2254–59.
29. Elder DJ, Paraskeva C. NSAIDs to prevent colorectal cancer: a question of sensitivity [editorial; comment]. Gastroenterology 1997; 113:1999–2003.
30. Albini A. Tumor and endothelial cell invasion of basement membranes. The matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. Pathol Oncol Res 1998;4:230–41.
31. Masferrer JL, Koki A, Seibert K. COX-2 inhibitors. A new class of angiogenic agents. Ann N Y Acad Sci 1999;889:84–6 (1999).
32. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells [published erratum appears in Cell 1998 Jul 24;94(2):following 271]. Cell 1998;93:705–16.