

# Cyclooxygenase-2 Activity Altered the Cell-Surface Carbohydrate Antigens on Colon Cancer Cells and Enhanced Liver Metastasis

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## ABSTRACT

Cyclooxygenase-2 (COX-2) was recently reported (M. Tsujii and R. N. DuBois, *Cell*, 83: 493-501, 1995) to affect the metastatic potential of cells. Previous studies (M. Fukuda, *Cancer Res.*, 56: 2237-2244, 1996) indicated that sialyl Lewis antigen expression is correlated with hematogenous metastasis of colon cancer. In the present study, we investigated the interaction between COX-2 activity, expression of sialyl Lewis antigens, *in vitro* cancer cell adhesion to endothelial cells, and *in vivo* metastatic potential. Effects of COX-2 activity and prostaglandin E<sub>2</sub> on cell adhesion, expression of sialyl Lewis antigens, and glycosyltransferase genes were determined in Caco-2-m (COX-2 low level), Caco-2-COX-2 (programmed to overexpress COX-2), and HT-29 (COX-2 high level) cells. Metastatic spread of these cells to the liver was also investigated. Caco-2-COX-2 cells had increased SPan-1 levels and increased adherence to endothelial cells via SPan-1 compared with Caco-2-m cells. HT-29 cells expressed sialyl Lewis a and adhered to endothelial cells via sialyl Lewis a. Treatment with a COX-2 inhibitor, celecoxib, decreased SPan-1 and sialyl Lewis a expression and adherence to endothelial cells.  $\beta$ 3Gal-T5 and ST3Gal III and IV expression was inhibited by celecoxib and was enhanced by prostaglandin E<sub>2</sub> treatment. Caco-2-COX-2 and HT-29 cells metastasized to the liver, whereas Caco-2-m cells did not. Pretreatment with celecoxib reduced the metastatic potential as well as anti-sialyl Lewis antibodies. Our results indicate a direct link between COX-2 and enhanced adhesion of carcinoma cells to endothelial cells, and enhanced liver metastatic potential via accelerated production of sialyl Lewis antigens. COX-2 inhibitors may suppress metastasis.

## INTRODUCTION

NSAIDs<sup>2</sup> are currently being evaluated for their effectiveness as chemopreventive and chemotherapeutic agents (1, 2). COX is a major target of NSAIDs and the inducible cyclooxygenase, COX-2, is up-regulated in gastrointestinal cancers (3, 4). Therefore, it is likely that COX-2 has an important role in gastrointestinal carcinogenesis. We previously reported (5) that overexpression of COX-2 leads to phenotypic changes involving increased adhesion to the extracellular matrix and inhibition of apoptosis in rat intestinal epithelial cells, which could enhance their tumorigenic potential. Constitutive expression of COX-2 can also lead to alterations in the invasive potential of colorectal cancer cells (6), and COX-2 may be involved in tumor angiogenesis (7). Several reports recently suggested that COX-2 expression has an important role in hematogenous metastasis of colon

carcinomas to the liver (8). The precise mechanisms underlying the role of COX-2 in this process are unknown.

The liver is a target organ of metastasis from gastrointestinal malignancies. Among the various steps in hematogenous metastasis to the liver, the initial adhesion of tumor cells to endothelial cells has a crucial role (9). To resist the substantial wall shear stress exerted by blood flow, metastasizing colon carcinoma cells have to form adhesive contacts with endothelial cells, which prevents detachment and the colon carcinoma cells ability to reenter the circulation (10). Adhesion of circulating cells to endothelial cells is mediated by a variety of cell adhesion molecules. Membrane-anchored selectins and carbohydrates initiate tethering and rolling of flowing tumor cells on endothelial cells during metastasis. Cell surface glycoproteins have essential roles in maintaining the function as well as the structure of cells (11). Among carbohydrates of these glycoproteins, sLe<sup>a</sup> and sLe<sup>x</sup> are human tumor-associated antigens. Tumor makers sLe<sup>a</sup> and sLe<sup>x</sup> are also ligands for E-selectin (12, 13). Highly metastatic colonic carcinoma cells bind more strongly to E-selectin expressed on activated human endothelial cells than do low-metastatic counterparts (14). Poorly metastatic tumor cells expressing low levels of sLe<sup>x</sup> adhere more strongly to E-selectin after being genetically engineered to increase the amount of sLe<sup>x</sup> (15). These studies strongly suggest that the abundance of sLe<sup>a</sup> and sLe<sup>x</sup> is a key factor in metastatic spread.

The aim of the present study was to investigate the effect of constitutive COX-2 expression in human colon carcinoma cells on adherence to endothelial cells and the metastatic potential of these cancer cells. In the present study, using colon cancer cell lines that are programmed to express low and high levels of COX-2 and sialyl Lewis antigens (Caco-2 and HT-29 cell lines, respectively), we investigated the influence of COX-2 expression, COX-2 specific inhibition, and a COX-2 product, PGE<sub>2</sub>, on *in vitro* adhesion to endothelial cells, expression of sialyl Lewis antigens and glycosyltransferases, and *in vivo* metastatic potential. COX-2 expression or PGE<sub>2</sub> treatment induced the expression of some glycosyltransferases and type-I sialyl Lewis antigens, leading to enhanced tumor-cell adhesion to endothelial cells and to liver metastasis. Celecoxib, a COX-2-specific inhibitor, suppressed these phenotypic changes induced by COX-2 activity.

## MATERIALS AND METHODS

**Reagents.** PGE<sub>2</sub> was purchased from Cayman Chemical (Ann Arbor, MI). Celecoxib, a COX-2 specific inhibitor, was a kind gift from Searle (St. Louis, MO). Antimouse MAbs against sLe<sup>a</sup> (MAB2095) sLe<sup>x</sup> (MAB2096) were purchased from Chemicon International, Inc. (Temecula, CA). Anti-SPan-1 MAb was kindly provided by Dr. Hirakawa (Osaka City University, Osaka, Japan) and Dainabot Co. Ltd. (Tokyo, Japan).

**Cell Culture.** Caco-2 and HT-29 cells derived from human colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS), 1% antibiotics and antimycotics (Life Technologies, Inc., Grand Island, NY), in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. HUVECs were obtained from Kurabou Co.

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<sup>2</sup> The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; sLe<sup>a</sup>, sialyl Lewis a; sLe<sup>x</sup>, sialyl Lewis x; PG, prostaglandin; MAb, monoclonal antibody; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; IL, interleukin; RT, reverse transcription;  $\beta$ 1,3GnT,  $\beta$ 1,3-N-acetylglucosaminyltransferase; GalT, galactosyltransferase; ST3Gal,  $\alpha$ 2,3-sialyltransferase; Fuc-T, fucosyltransferase; core2 GnT,  $\beta$ 1,6-N-acetylglucosaminyltransferase.

(Osaka, Japan) and were maintained in Daigo's T medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2 ng/ml of recombinant basic fibroblast growth factor (Takeda Pharmaceutical Co., Osaka, Japan) and 10% FBS. In the studies of the effects of celecoxib, the cells were cultured for 48 h in DMEM supplemented with 10% FBS in the presence of 10  $\mu$ M celecoxib. For investigation of the effect of PGE<sub>2</sub>, after 48-h starvation, the cells were treated by various concentration of PGE<sub>2</sub> for 24 h.

**Stable Transfection.** A 2.1-kb fragment containing the open reading frame for the rat COX-2 gene was isolated and cloned into the eukaryotic expression vector pCB6. This vector was constructed so that transcription of the cDNA was controlled by the cytomegalovirus promoter. This vector also contains a neomycin-resistant gene that allows for selection of transfected cells by the addition of G418 to the cell culture medium. Caco-2 cells obtained from the American Type Culture Collection were transfected using LipofectAMINE (Life Technologies, Inc.), as described previously (16), and cultured in DMEM containing 1.5 mg/ml G418 (Sigma Chemical Co.), supplemented with 10% FBS. Five independently derived controls and COX-2 transfected cell lines were characterized and found to have similar phenotypes.

**PGE<sub>2</sub> Measurements.** PGE<sub>2</sub> was measured by ELISA (Cayman Chemical). PGE<sub>2</sub> is the major metabolite of arachidonic acid metabolism in Caco-2, Caco-2-COX-2, and HT-29 cells. These measurements were made in triplicate and repeated in three experiments.

**Adhesion of Cancer Cells to Endothelial Cells.** HUVECs were maintained in a collagen-coated dish with E300 medium in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. HUVECs at passages 2 through 5 were plated at a density of 1 × 10<sup>5</sup> cells/well 24 h before the assay and were grown to a confluent monolayer in six-well plates. The HUVECs were stimulated with 1 ng/ml of IL-1 $\beta$  (Otsuka Pharmaceutical Co., Tokushima, Japan) for 4 h before the assay. The medium was replaced with DMEM dissolved in 1% BSA and incubated for 30 min. After preincubation, 100  $\mu$ l of [<sup>3</sup>H]thymidine-labeled Caco-2-m, Caco-2-COX-2, or HT-29 cell suspension (1 × 10<sup>6</sup> cells/ml, 0.5  $\mu$ Ci/ml) were added and incubated with rotation (90 rpm) for 30 min at 37°C. To the HUVECs in six-well plates, the tumor cells (1 × 10<sup>5</sup> cells/well) were plated and incubated. After gently washing three times with PBS [137 mM NaCl, 8.10 mM Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub> (pH7.4)] to remove unattached cells, the attached cells were solubilized in 0.1 N NaOH, and the radioactivity was measured. The attaching potential was estimated by the ratio of attaching cell counts:totalcounts applied to the culture. For the celecoxib-treated group, cancer cells were treated with 10  $\mu$ M celecoxib for 48 h before labeling. For inhibition assays using antibodies, tumor cells were preincubated with anti-sLe<sup>a</sup> (50  $\mu$ g/ml), sLe<sup>x</sup> (100  $\mu$ g/ml), or SPan-1 (100  $\mu$ g/ml) antibody for 30 min at 37°C before the adhesion assay.

**Flow Cytometry Analysis and ELISA for Sialyl Lewis Antigen Expression.** Flow cytometric analysis was performed using FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The indirect immunofluorescence method was applied to stain the tumor cells with anti-sLe<sup>a</sup>, anti-sLe<sup>x</sup>, or anti-SPan-1 MAb at 10  $\mu$ g/ml as the primary antibody (incubated for 30 min at room temperature), followed by the addition of FITC-labeled antimouse immunoglobulin (Silenus Lab., Hawthorn, Australia) as the secondary antibody. For ELISA, cells were washed in PBS, harvested with a rubber scraper, washed once again in PBS, and pelleted by centrifugation. Cell pellets were solubilized by brief sonication in 20 mM HEPES buffer (pH 7.2) containing 2% Triton X-100. After determination of the protein concentration with a Micro BCA protein assay reagent kit (Pierce, Rockford, IL), solubilized protein (10  $\mu$ g) from each sample was subjected to ELISA using the same antibodies as were used in the FACScan. Samples were measured in triplicate, and this experiment was repeated five times.

**Expression of a Series of Glycosyltransferase mRNAs.** Caco-2-m, Caco-2-COX-2, and HT-29 cells cultured in DMEM containing 10% FBS were treated with celecoxib (10  $\mu$ M) for 6 h. For investigation of the effect of PGE<sub>2</sub>, the cells were starved for 48 h and treated with PGE<sub>2</sub> (0.2  $\mu$ M) for 6 h. Total cellular RNA was isolated using ISOGEN (Nippon Gene Co., Toyama, Japan), and cDNAs were synthesized with an oligo(dT) primer from 3  $\mu$ g of DNase I-treated total RNA using a Superscript preamplification system for first-strand cDNA synthesis (Invitrogen Japan K. K., Tokyo, Japan). The sequences of primers used for PCR are given in Table 1. PCR was performed in a GeneAmp PCR system 2400 (PE Applied Biosystem, Roissy, France) on 1  $\mu$ l of the sample cDNA in a final volume of 25  $\mu$ l containing 25 pmol of sense and antisense primers, and 2 units Taq DNA polymerase (AmpliTaq Gold; Perkin-Elmer, Wellesley, MA) under the following conditions: 1 cycle at 95°C

Table 1 Oligonucleotide primers used for RT-PCR analysis

| RNA             | Primer sets <sup>a</sup>  | Size of PCR products |
|-----------------|---|----------------------|
| core2 GnT       | F: 5'-ACTCGAAACACCTCTCTTTTCTGGC-3'<br>R: 5'-GGTCAGTGTTTTAAATGTCTCCAAAG-3' | 459                  |
| $\beta$ 1,4GalT | F: 5'-AAGCAGAACCCAAATGTGAAGATG-3'<br>R: 5'-GGCGGAGATATAGACATGCCTC-3'      | 527                  |
| $\beta$ 3Gal-T5 | F: 5'-ACCACCAGCAGTGCAGCGGAAAC-3'<br>R: 5'-GCCACGATCCTCCTGAAGAGGCA-3'      | 554                  |
| ST3Gal I        | F: 5'-TATGGCCTGAGATAGACAGTAC-3'<br>R: 5'-GATCCGGATTTTATTGATGGAGGC-3'      | 543                  |
| ST3Gal II       | F: 5'-GAGAACATGGATCTCCACCCG-3'<br>R: 5'-GGCTGGTGTAGATCTGGACC-3'           | 483                  |
| ST3Gal III      | F: 5'-ATGGAGGCGTCTTCCCAACAAG-3'<br>R: 5'-ATGCGAACGGTCTCATAGTAGTG-3'       | 529                  |
| ST3Gal IV       | F: 5'-TTGAACAATGCCCCAGTCTGG-3'<br>R: 5'-TCTTGGGAGACATTATGGCCGTGAC-3'      | 503                  |
| Fuc-T III       | F: 5'-ATGGATCCCTGGGTGCAGCCAAAG-3'<br>R: 5'-TCAGGTGAACCAAGCCGCTATGCT-3'    | 1088                 |

<sup>a</sup> F, forward; R, reverse.

for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min. A 10- $\mu$ l aliquot of the RT-PCR mixture was subjected to electrophoresis in a 2% agarose gel and the bands were visualized by ethidium bromide staining.

**Liver Metastasis.** Six-week-old male BALB/c nu/nu nude mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). All of the mice were maintained under specific pathogen-free conditions at the Center for Animal Experimentation, Osaka University Graduate School of Medicine. Regular laboratory food and tap water for drinking were freely available. Mice were anesthetized with ethyl ether. The abdominal wall was incised and the spleen exposed. Viable Caco-2-m, Caco-2-COX-2, or HT-29 cells, 1 × 10<sup>7</sup> in 100  $\mu$ l PBS were injected into the spleen. In the celecoxib-treated group, these cells were preincubated with 10  $\mu$ M celecoxib for 48 h and then injected into the spleen. In the antibody-treated groups, the cells were preincubated with MAb against SPan-1 (100  $\mu$ g/ml) or sLe<sup>a</sup> (50  $\mu$ g/ml) for 60 min before inoculation. The mice were killed 12 weeks after inoculation of the cancer cells to the spleen. The liver was removed and checked for the presence of hepatic metastasis.

## RESULTS

**PGE<sub>2</sub> Production.** The concentration of PGE<sub>2</sub> in culture medium of each cell line treated with or without celecoxib is shown in Fig. 1. The highest level of COX-2 metabolite in all of the cell lines was PGE<sub>2</sub>. The level of PGE<sub>2</sub> in Caco-2-COX-2 cells was elevated by ~7.5-fold compared with Caco-2-m cells. HT-29 cells produced the same amount of PGE<sub>2</sub> as Caco-2-COX-2 cells did. Celecoxib at 5  $\mu$ M inhibited PGE<sub>2</sub> production by 25% of control Caco-2-COX-2 and HT-29 cells, and with 10  $\mu$ M or more, PGE<sub>2</sub> production was significantly inhibited to the levels produced by COX-2-m cells.

**Effect of COX-2 Activity and PGE<sub>2</sub> in Colon Cancer Cells on Adhesion to Endothelial Cells.** To quantify the adherence of colon cancer cells to endothelial cells, Caco-2, Caco-2-COX-2, and HT-29 cells were assayed for adhesion to HUVECs (Fig. 2). Each cell line was tested for adhesion to 1 ng/ml IL-1 $\beta$ -stimulated HUVECs. None of the cell lines adhered to untreated HUVECs, and adherence of tumor cells to IL-1 $\beta$ -stimulated HUVECs was significantly blocked by anti-E-selectin antibody (data not shown), which suggested that E-selectin was involved in these cell-cell interactions. As shown in Fig. 2A, the adhesion of Caco-2-COX-2 cells to IL-1 $\beta$ -stimulated HUVECs was significantly increased, compared with Caco-2-m cells. Anti-SPan-1 MAb significantly inhibited the adhesion, although anti-sLe<sup>a</sup> or anti-sLe<sup>x</sup> MAb did not. HT-29 adhered to stimulated HUVECs, and the adhesion of HT-29 was inhibited by anti-sLe<sup>a</sup> MAb, but not by anti-sLe<sup>x</sup> or anti-SPan-1 MAb. Celecoxib inhibited cancer cell adhesion to stimulated HUVECs in a dose-dependent manner. Adhesion of the cells that were grown in serum-free condi-

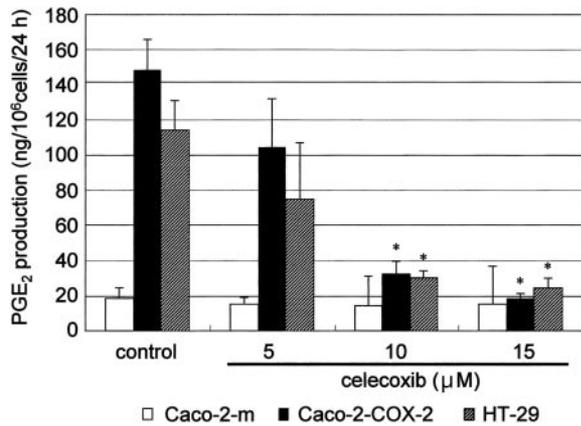


Fig. 1. COX-2 activity of Caco-2-m, Caco-2-COX-2, and HT-29 cells. ELISA for PGE<sub>2</sub> using supernatants from Caco-2-m, Caco-2-COX-2, and HT-29 cells, pretreated with celecoxib at various concentrations for 48 h. \*,  $P < 0.01$ , significant suppression of PGE<sub>2</sub> production by celecoxib pretreatment compared with untreated control by Student's  $t$  test. Results are from one experiment with five replicate samples and are representative of three experiments.

tions for 48 h was reduced (Fig. 2B). When the serum-starved cells were treated with PGE<sub>2</sub> for 24 h, however, the adhesion of Caco-2-m and HT-29 cells was increased by 4- and 2.5-fold, respectively, whereas there was no significant change in the adhesion of Caco-2-COX-2 cells. Our results suggest that SPan-1 and sLe<sup>a</sup> are involved in the adhesion of Caco-2-m and HT-29 cells enhanced by PGE<sub>2</sub> treatment, because these antibodies suppressed the adhesion. These results indicate PGE<sub>2</sub>, produced by COX-2, up-regulates SPan-1 and sLe<sup>a</sup> expression in Caco-2 cells and HT-29 cells, respectively.

**Effect of COX-2 Activity in Colon Cancer Cells on Sialyl Lewis X Antigen Expression.** The results of flow cytometric analysis and ELISA of sialyl Lewis antigens of Caco-2 and HT-29 cells cultured in DMEM/10% FBS with or without 10 μM celecoxib are shown in Figs. 3A and 4A, respectively. Neither Caco-2-m nor Caco-2-COX-2 cells expressed sLe<sup>a</sup>. Both Caco-2-m and Caco-2-COX-2 cells expressed sLe<sup>x</sup>, and there was no significant difference between the two cell lines. Celecoxib did not have a significant effect on sLe<sup>x</sup> expression in Caco-2-m or Caco-2-COX-2 cells. Caco-2-COX-2 cells express more SPan-1 than do Caco-2-m cells, and 10 μM celecoxib significantly inhibited SPan-1 expression in Caco-2-COX-2 cells. In HT-29 cells, sLe<sup>a</sup> expression was detected using FACScan and ELISA, and 10 μM celecoxib significantly inhibited its expression. Regarding SPan-1 expression in HT-29 cells, there was no significant difference between control and celecoxib-treated group, although the data from FACScan suggested that SPan-1 expression was slightly suppressed by 10 μM celecoxib treatment. sLe<sup>x</sup> expression was detected, but celecoxib did not have a significant effect on the levels.

**Effect of PGE<sub>2</sub> in Colon Cancer Cells on Sialyl Lewis X Antigen Expression.** The results from FACScan and ELISA of the cells treated with or without PGE<sub>2</sub> after 2-day starvation in serum-free media are shown in Figs. 3B and 4B, respectively. PGE<sub>2</sub> (0.2 μM) significantly induced SPan-1 expression in Caco-2-m cells, although it did not have a significant effect on sLe<sup>x</sup> expression. In Caco-2-COX-2 cells, there was no significant change in SPan-1 or sLe<sup>x</sup> expression before or after PGE<sub>2</sub> treatment. The PGE<sub>2</sub> concentration in the culture medium of Caco-2-COX-2 cells was ~0.35 μM, and the effect of PGE<sub>2</sub> was maximal in Caco-2-COX-2 cells. Neither Caco-2-m nor Caco-2-COX-2 cells expressed sLe<sup>a</sup> even after PGE<sub>2</sub> treatment. In HT-29 cells, PGE<sub>2</sub> induced sLe<sup>a</sup> expression, although it did not have a significant effect on sLe<sup>x</sup> or SPan-1 levels.

**Effect of COX-2 Activity in Colon Cancer Cells on Glycosyltransferases to Form sLe<sup>a</sup>.** The synthesis of sLe<sup>x</sup> and sLe<sup>a</sup> epitopes at the reducing terminal ends of the carbohydrate chains require a set of several glycosyltransferases ( $\beta$ 1,3GnT,  $\beta$ 1,4GalT, ST3Gal, and  $\alpha$ 1,3Fuc-T) for sLe<sup>x</sup> synthesis and ( $\beta$ 1,3GnT,  $\beta$ 1,3GalT, ST3Gal, and  $\alpha$ 1,4Fuc-T) for sLe<sup>a</sup> synthesis. The gene encoding a  $\beta$ 1,3GnT, which is required for the synthesis of type I and type II chains, has not yet been cloned. The gene encoding a  $\beta$ 1,4GalT has been cloned (17). ST3Gal I-IV are suggested to be involved in carbohydrate synthesis and carcinogenesis in many tissues (18). A  $\beta$ 1,3Gal-T,  $\beta$ 3Gal-T5, and Fuc-T III are the most probable candidates responsible for the synthesis of the type I Lewis antigens (19). The sLe<sup>x</sup> and sLe<sup>a</sup> antigens in colorectal carcinoma tissue are carried mainly by mucins with *O*-linked glycosylated chains (20). core2 GnT catalyzes the transfer of GlcNAc residue of Gal $\beta$ 1,3-GalNAc-Ser/Thr with a  $\beta$ 1,6 linkage to synthesize one of the branched structures, the core 2 structure, at the root of the mucin *O*-glycans. To identify the key enzymes responsible for the apparent SPan-1 or sLe<sup>a</sup> antigen expression that was enhanced by COX-2 expression and inhibited by celecoxib, the quantities of the eight glycosyltransferase transcripts determined using RT-PCR were compared. As shown in Fig. 5A, there was no difference in the expression of core2 GnT or  $\beta$ 1,4GalT between control and celecoxib-treated groups, although their RT-PCR products were present in Caco-2 and HT-29 cells. As shown in Fig. 5B, core2 GnT or  $\beta$ 1,4GalT levels were not different between control and PGE<sub>2</sub>-treated groups.

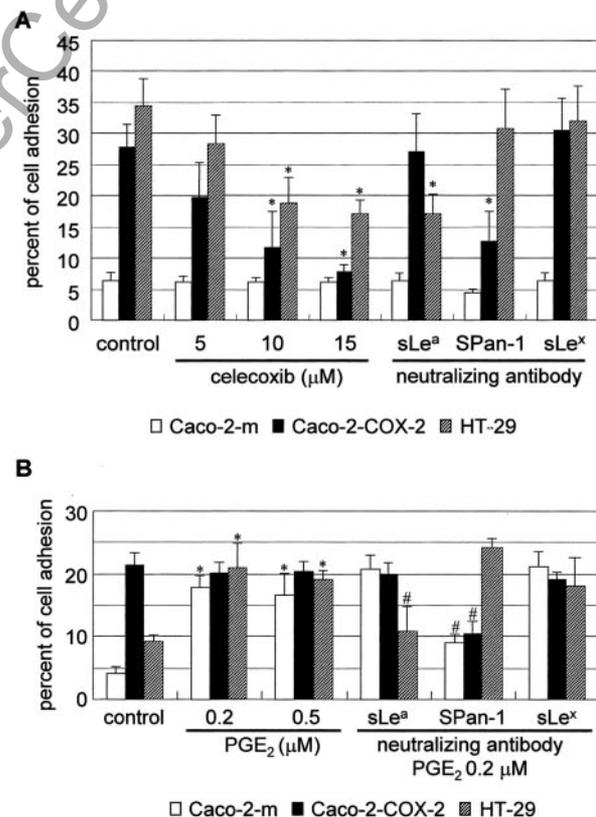


Fig. 2. Adhesion assay of cancer cells to a monolayer of HUVEC. In A, Caco-2-m, Caco-2-COX-2, or HT-29 cells were treated with celecoxib for 2 days and then labeled with [<sup>3</sup>H]thymidine. In B, Caco-2-m, Caco-2-COX-2, or HT-29 cells were starved for 48 h, treated with PGE<sub>2</sub> for 24 h, and then labeled with [<sup>3</sup>H]thymidine. Cancer cells ( $1 \times 10^5$  cells/well) were incubated with IL-1 $\beta$ -stimulated HUVECs for 30 min at 37°C with rotation (90 rpm). After the nonadhering cells were washed out, the cpm of the cells on the plates were counted. For inhibition of cell adhesion, MAb against sLe<sup>a</sup>, SPan-1, or sLe<sup>x</sup> was preincubated with cancer cells for 30 min before application of the monolayer of endothelial cells. \*,  $P < 0.01$ , significant suppression or enhancement of adhesion compared with controls. #,  $P < 0.01$ , significant inhibition in adhesion compared with 0.2-μM PGE<sub>2</sub>-treated groups by Student's  $t$  test.

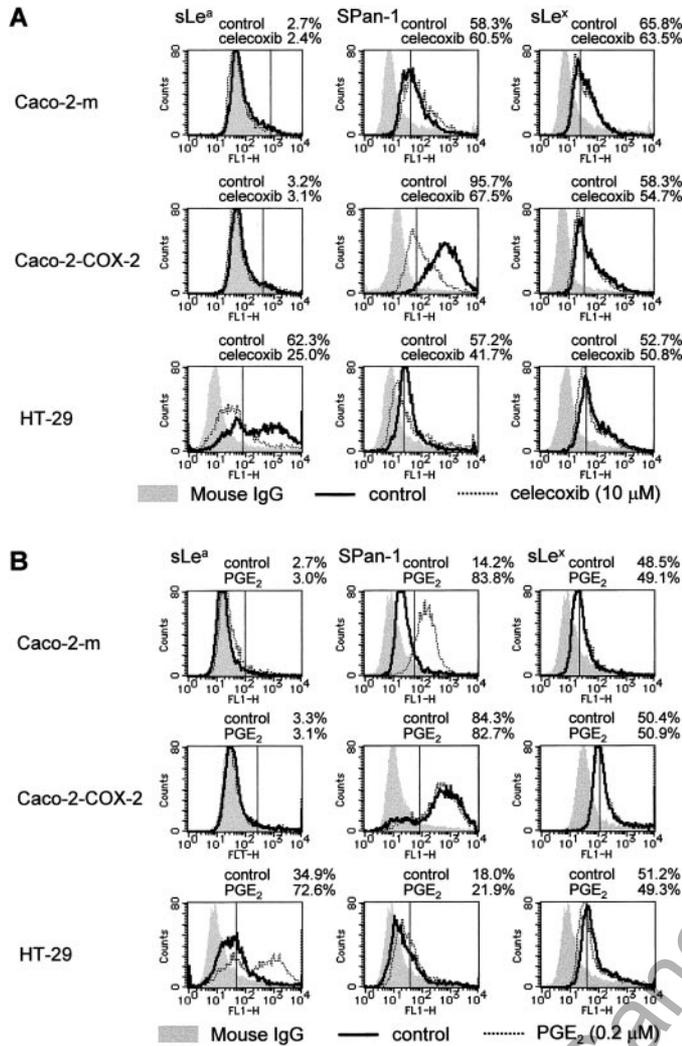


Fig. 3. Flow cytometric analysis of cell surface carbohydrates on colon cancer cells. In A, Caco-2-m, Caco-2-COX-2, and HT-29 cells, cultured in DMEM containing 10% FBS, were treated with celecoxib (10 μM) for 48 h. In B, the cells, starved for 48 h, were treated with PGE<sub>2</sub> for 24 h. Cells were stained with primary (MAbs) and secondary antibodies, and analyzed by flow cytometry. Shaded peaks in A and B, the results of staining with mouse IgG for the first antibody as a negative control. Black lines, control conditions without celecoxib or PGE<sub>2</sub>. Dotted lines, the pattern with 10 μM celecoxib or 0.2 μM PGE<sub>2</sub>. Samples were measured in triplicate, and this experiment was repeated three times.

β3Gal-T5, ST3Gal III, and ST3Gal IV expression, which was detected in all cell lines, was inhibited by celecoxib and induced by PGE<sub>2</sub> treatment or COX-2-programmed expression. ST3Gal I was expressed in all of the cell lines examined. COX-2-programmed expression or PGE<sub>2</sub> treatment reduced ST3Gal I expression, whereas celecoxib up-regulated its expression. ST3Gal II expression was not detected in any cell line. Fuc-T III expression was not detected in Caco-2-m or Caco-2-COX-2 cells. Although Fuc-T III expression was present in HT-29 cells, celecoxib or PGE<sub>2</sub> had no effect.

**Effect of COX-2 Activity on Liver Metastasis.** The number of metastatic foci after the intrasplenic injection of tumor cells is shown in Fig. 6. Caco-2-m cells did not produce gross tumors in the livers of nude mice. Caco-2-COX-2 cells were highly metastatic to the liver after inoculation to the spleen. Pretreatment with MAb against SPan-1 inhibited the number of metastatic foci. HT-29 cells also demonstrated the capacity to metastasize to the liver after inoculation of cancer cells into the spleen. Pretreatment with anti-sLe<sup>a</sup> MAb markedly inhibited the metastasis of HT-29 cells to the liver. Celecoxib pretreatment significantly inhibited liver metastases after injection of Caco-2-COX-2 and HT-29 cells.

DISCUSSION

Hematogenous metastasis of tumor cells to the liver is one of the major causes of death in patients with colorectal cancer. Recent studies indicate an involvement of COX-2 expression in colorectal tumors and in their metastatic spread to distant organs. Several steps are thought to be involved in metastasis. We previously demonstrated that COX-2 expression in colon cancer cells reduces E-cadherin levels and activates matrix metalloproteinase-2, leading to an acceleration of cellular invasion (6). We also reported that COX-2 expression, partially via PGE<sub>2</sub>, accelerates the production of angiogenic factors and enhances tumor angiogenesis (7). Each one of these effects is an important step for the development of metastatic foci in the liver. Adhesion of tumor cells to endothelial cells represents another important step in metastasis. Carbohydrates on tumor cells have an important role in the first stage of the cell adhesion cascade (tethering, rolling) by interacting with selectins (9, 10). In an effort to better understand the role of COX-2 in hematogenous metastasis, we prepared colon cancer cells programmed to express COX-2 and investigated the effect of COX-2 expression and PGE<sub>2</sub> treatment on the adhesion of tumor cells to endothelial cells, carbohydrate expression, and metastatic spread to the liver. The present study was designed to

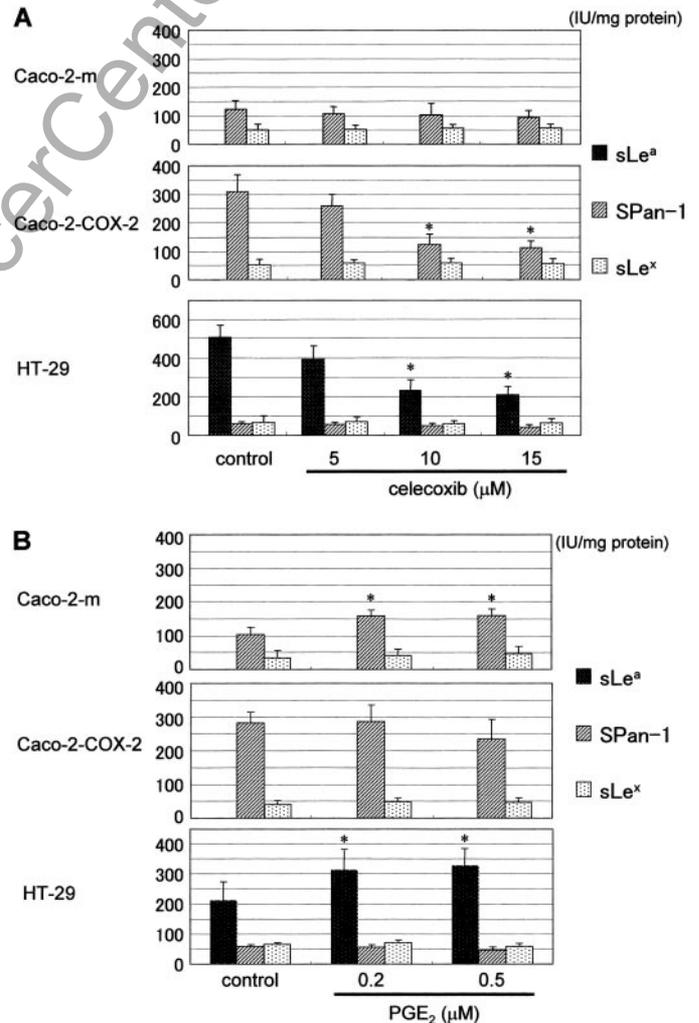


Fig. 4. Production of sLe<sup>a</sup>, Span-1, and sLe<sup>x</sup> in Caco-2-m, Caco-2-COX-2, and HT-29 cells. In A and B, the cells were prepared the same as in Fig. 3, A and B. ELISA was performed for each carbohydrate, using cell lysates of the cells and probing with specific antibodies. \*, P < 0.01, significant increase or decrease in carbohydrate production by celecoxib or PGE<sub>2</sub> treatment compared with controls by Student's *t* test.

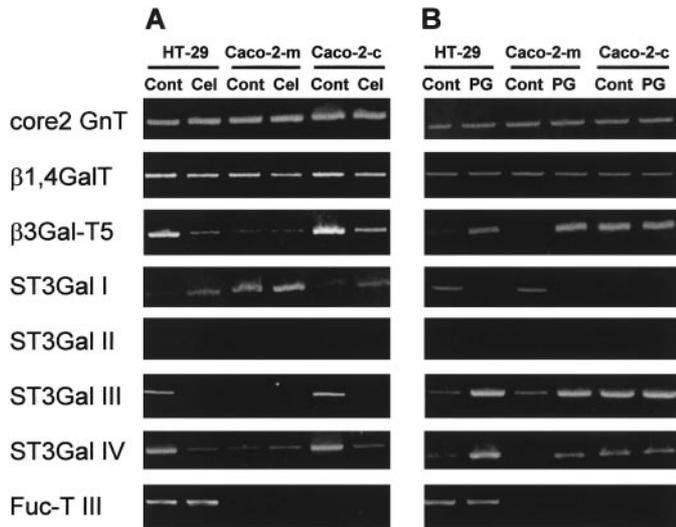


Fig. 5. RT-PCR analysis of glycosyltransferases. In A, Caco-2-m, Caco-2-COX-2 (*Caco-2-c*), and HT-29 cells, cultured in DMEM containing 10% FBS, were treated with celecoxib (10  $\mu$ M) for 6 h. In B, the cells were starved for 48 h and then treated with PGE<sub>2</sub> for 6 h. After treatment, 3  $\mu$ g of total RNA was prepared, and cDNA was obtained. After the PCR reaction, aliquots of the products were electrophoresed on 2% agarose gel in the presence of ethidium bromide. *Cont*, the group under the control condition. *Cel*, the celecoxib-treated group.

evaluate the role of COX-2 in the early stages of liver metastasis, *i.e.*, attachment to endothelial cells. Therefore, celecoxib treatment was initiated just before intrasplenic injection. In a preliminary study, PGE<sub>2</sub> synthesis recovered soon after the discontinuation of celecoxib treatment, and resistance to apoptosis, angiogenic factor production, and matrix metalloproteinase-2 activation was reduced by celecoxib and restored within 3 to 6 h after removal of the drug. Therefore, it appears that the cells pretreated with celecoxib recovered to levels equivalent to those of nontreated cells in mice within 6 h. The present study indicates that COX-2 activity may regulate expression of sialyl Lewis antigens, resulting in enhancement of liver metastasis via accelerated adhesion to endothelial cells.

The present study demonstrates that sLe<sup>a</sup> and SPan-1 might be involved in tumor cell adhesion to endothelial cells and liver metastasis, whereas sLe<sup>x</sup> might not. Cumulative data indicate that sialylation of the terminal structure of the carbohydrate antigens (sialyl Lewis antigens) on the tumor cell surface confer an adhesion and metastasis-prone phenotype, based on clinicopathological and experimental studies (21). sLe<sup>a</sup> and SPan-1 on the surface of colorectal cancer constitute a major ligand for E-selectin on activated endothelial cells, and the sLe<sup>a</sup> (SPan-1)-E-selectin system contributes to the establishment of hematogenous metastasis (22, 23). These findings are consistent with our results. On the other hand, sLe<sup>x</sup> is also recognized by the endothelial selectins that mediate metastasis of tumor cells (12). Both cell lines (Caco-2 and HT-29) were strongly positive for sLe<sup>x</sup>. Our results, however, indicated that anti-sLe<sup>x</sup> antibodies did not have an inhibitory effect on the adhesion to endothelial cells. Carbohydrate ligands involved in the adhesion of cancer cells to endothelial cells differ, corresponding to the type of cancer. sLe<sup>a</sup> has a functional role that mediates the adhesion of cancer cells to endothelial cells in colon carcinoma, and the adhesion of cancer cells to endothelial cells that is mediated by sLe<sup>x</sup> is weaker than that mediated by sLe<sup>a</sup> (22, 24), although coexpression of sLe<sup>a</sup> and sLe<sup>x</sup> is common.

In the present study, we demonstrated for the first time that cell surface carbohydrates are altered by COX-2 activity or PGE<sub>2</sub> treatment. Celecoxib suppressed type I Lewis antigen expression and liver metastasis. To clarify the mechanism responsible for this effect, we

investigated the relationship between COX-2 expression, celecoxib, or PGE<sub>2</sub> treatment and glycosyltransferase expressions. Sialyl Lewis epitopes produced in cancer cells are mainly carried on mucin O-glycans (20). core2 GnT is essential for the synthesis of the sialyl Lewis antigens in mucin-type oligosaccharides (25).  $\beta$ 1,4GalT is an enzyme responsible for the type II Lewis antigens, sLe<sup>x</sup> (18). COX-2 expression, celecoxib, or PGE<sub>2</sub> had no influence on the expression of these two enzymes.

The  $\beta$ 3Gal-T5 gene is the most probable candidate responsible for the synthesis of type I Lewis antigens in gastrointestinal tumor cells, and it is a key enzyme that determines the amount of type I Lewis antigens (19). Its mRNA expression was suppressed by celecoxib and induced by COX-2 expression and PGE<sub>2</sub> in Caco-2 and HT-29 cells. Because its transcriptional regulation is not yet clarified, the precise mechanism by which COX-2 activity and PGE<sub>2</sub> are involved in its regulation is still obscure.

Sialic acid is transferred to galactose in Gal $\beta$ 1-3GlcNAc/Gal $\beta$ 1,4GluNAc-R by ST3Gals, the expression of which is significantly increased in carcinoma tissue compared with that in normal mucosa (18). In the present study, COX-2 expression or PGE<sub>2</sub> treatment enhanced, and celecoxib inhibited, ST3Gal III and IV expression, whereas COX-2 expression or PGE<sub>2</sub> reduced, and celecoxib increased, ST3Gal I expression. AP-2 has a critical role in the epithelial-cell-specific transcriptional regulation of the *ST3Gal IV* gene (26). Because PGE<sub>2</sub> induces specific binding activity to CRE and AP-2 DNA elements (27, 28), COX-2 and PGE<sub>2</sub> are thought to be involved in ST3Gal IV expression via transcriptional regulation of AP-2. Among these ST3Gals, ST3Gal III (29) and ST3Gal IV (30) are responsible for the biosynthesis of sLe<sup>a</sup> and sLe<sup>x</sup> antigens, because ST3Gal III and ST3Gal IV transfer sialic acid to the precursor structures of sLe<sup>x</sup> and sLe<sup>a</sup> with good efficiency, whereas ST3Gal I and ST3Gal II are inefficient (31), and transfection of ST3Gal I or II to colon cancer cells do not alter the level of sialyl Lewis antigens (32).

Fuc-T III is required for sLe<sup>a</sup> synthesis (33), whereas Fuc-T III, V, VI, and VII are responsible for sLe<sup>x</sup> synthesis (33-36). Therefore, the absence of expression of sLe<sup>a</sup> synthesis in Caco-2 cells is attributable to a lack of Fuc-T III. In HT-29, Fuc-T III expression was detected;

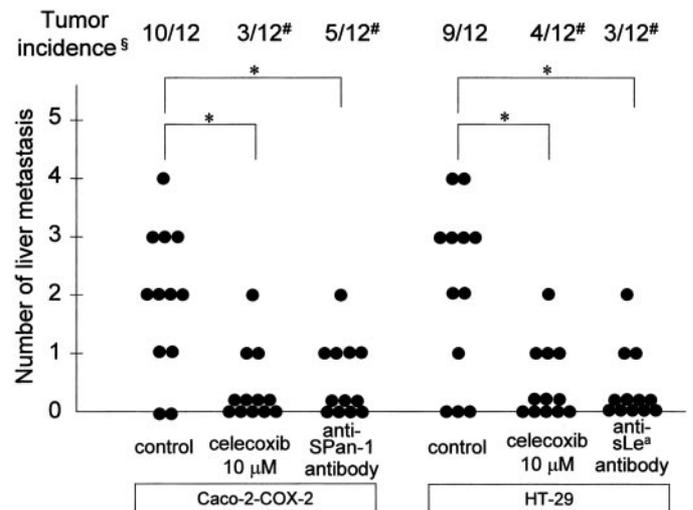


Fig. 6. Liver metastasis by Caco-2-COX-2 and HT-29 cells. The colon cancer cells were treated with celecoxib at 10  $\mu$ M for 48 h or with MAb against sLe<sup>a</sup> or SPan-1 for 60 min. Then,  $1 \times 10^7$  cells were inoculated into the spleen. The mice were killed and examined for the presence of hepatic metastasis 12 weeks after the intrasplenic inoculation. §, a ratio of mice showing tumor growth:mice given injections of tumor cells. #,  $P < 0.05$ , significant decrease in tumor incidence compared with control by  $\chi^2$  test. \*,  $P < 0.05$ , significant decrease in number of tumor nodules compared with controls by Mann-Whitney's *U* test analysis.

and COX-2 expression, celecoxib, or PGE<sub>2</sub> had no effect on its expression.

Celecoxib or PGE<sub>2</sub> treatment influenced expression, not of sLe<sup>x</sup>, but of type I sialyl Lewis antigens in Caco-2 or HT-29 cells. β1,3Gal-T5 is a key responsive enzyme for type I Lewis antigen synthesis (19) and ST3Gal III uses the type-I structure more efficiently than type II (29), although ST3Gal IV exhibits high activity with type II compared with the type I structure (30). Therefore, these results suggest that the key enzymes responsible for the augmented expression of sLe<sup>a</sup> and SPan-1 by COX-2 activity or PGE<sub>2</sub> are β3Gal-T5 and ST3Gal III.

In conclusion, we observed a direct link between COX-2 expression and alterations of cell surface carbohydrate levels via changes of glycosyltransferases, leading to an increased metastatic potential. The inhibition of COX-2 may suppress the metastatic spread of colorectal carcinoma cells to the liver, which might have important therapeutic value in humans with metastatic disease.

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