Celecoxib Treatment Alters the Gene Expression Profile of Normal Colonic Mucosa

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Abstract

A clinical trial was recently conducted to evaluate the safety and efficacy of a selective inhibitor of cyclooxygenase-2 (celecoxib) in hereditary nonpolyposis colon cancer patients. In a randomized, placebo-controlled phase I/II multicenter trial, hereditary nonpolyposis colon cancer patients and gene carriers received either celecoxib at one of two doses or placebo. The goal was to evaluate the effects of these treatment arms on a number of endoscopic and tissue-based biomarker end points after 12 months of treatment. As part of this trial, we analyzed gene expression by cDNA array technology in normal descending (rectal) colonic mucosa of patients before and after treatment with celecoxib or placebo. We found that treatment of patients with celecoxib at recommended clinical doses (200 and 400 mg p.o. bid), in contrast to treatment with placebo, leads to changes in expression of >1,400 genes in the healthy colon, although in general, the magnitude of changes is <2-fold. Twenty-three of 25 pairs of colon biopsies taken before and after celecoxib treatment can be classified correctly by the pattern of gene expression in a leave-one-out cross-validation. Immune response, particularly T- and B-lymphocyte activation and early steps of inflammatory reaction, cell signaling and cell adhesion, response to stress, transforming growth factor-β signaling, and regulation of apoptosis, are the main biological processes targeted by celecoxib as shown by overrepresentation analysis of the distribution of celecoxib-affected genes across Gene Ontology categories. Analysis of possible cumulative effects of celecoxib-induced changes in gene expression indicates that in healthy colon, celecoxib may suppress the immune response and early steps of inflammation, inhibit formation of focal contacts, and stimulate transforming growth factor-β signaling. (Cancer Epidemiol Biomarkers Prev 2006; 15(7):1382–91)

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) inhibit colorectal carcinogenesis and decrease the risk of death from colorectal cancer as shown in several epidemiologic studies (reviewed in refs. 1-4). Many of the NSAIDs inhibit the activity of cyclooxygenases (COX), which are involved in the synthesis of prostaglandins from arachidonic acid (5). Two major isoforms of COX have been identified: COX-1 is constitutively expressed in most tissues and considered to be responsible for synthesis of prostaglandins that mediate normal physiologic functions, including maintenance of colonic mucosal integrity. COX-2 is usually not detectable under normal conditions in most tissues but can be induced by a wide variety of growth stimulatory and proinflammatory factors. COX-2 is thought to be mainly involved in inflammation, regulation of cell growth, apoptosis, and angiogenesis. However, it has also been shown that COX-2 is expressed constitutively in several tissues, including healthy gastric mucosa (6). It is implicated in the maintenance of mucosal integrity in the healthy stomach as well (review ref. 7).

Several lines of evidence suggest that COX-2 plays an important role in carcinogenesis. It has been shown that the concentration of prostaglandin E_2, a major product of the prostaglandin synthesis pathway controlled by COX-1 and COX-2, is higher in various human and animal tumors compared with adjacent normal tissue (8-11), and that some prostaglandins (including prostaglandin E_2) stimulate growth of colon tumor cells (12, 13). Importantly, prostaglandin levels in tumor tissues correlate with increased expression of COX-2 but not COX-1 (10, 14, 15). In addition to stimulation of cell proliferation, prostaglandin E_2 enhances cell motility and angiogenesis and inhibits apoptosis and immune surveillance, phenomena that can be important for carcinogenesis (2, 16, 17). Prostaglandins act through prostanoid receptors, and homozygous deletion of prostaglandin E_2 receptor EP_4 reduces formation of aberrant crypt foci (ACF), preneoplastic colon lesions, in azoxymethane-treated mice (18), whereas knockout of the genes encoding prostaglandin E_2 receptors EP_1 and EP_2 decreases the number and size of intestinal polyps in mice (19, 20) and inhibits polyp-related angiogenesis (21). Expression of COX-2 is increased in 40% of colon adenomas and in about 90% of adenocarcinomas (11, 14).

Several observational, case-control and, recently, double-blinded placebo-controlled studies have shown a protective effect of NSAIDs on sporadic colorectal cancer occurrence and mortality; compelling evidence was obtained in clinical trials for patients with familial adenomatous polyposis (FAP) syndrome (reviewed in refs. 2, 22-26). It was shown in several clinical trials that nonspecific NSAIDs aspirin and sulindac

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and the COX-2-specific inhibitor celecoxib reduce the number and size of colonic polyps in FAP patients (24, 27, 28). Particularly, it was found in a large, double blinded, placebo-controlled study that treatment of FAP patients for 6 months with 400 mg of celecoxib (twice daily) reduced the mean number of colorectal polyps by 28%, with no sign of adverse side effects (29).

The pathway of colorectal carcinogenesis in FAP patients is initiated by the loss of the wild-type allele of the *APC* gene and leads to abnormal activation of the Wingless/Wnt signaling pathway and to chromosomal instability (30-32). This pathway is considered to account also for ~85% of sporadic colorectal cancer. Another major pathway of colorectal carcinogenesis is recognized by microsatellite instability and mostly pseudodiploid karyotypes of colorectal cancer and is thought to be responsible for the remaining 15% of sporadic colorectal cancer (33, 34) and for most of the colorectal cancer in patients with hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome (35-37). HNPCC is an autosomal dominant syndrome characterized by a relatively small number of adenomas, development of colorectal cancer at a relatively young age, frequent occurrence of extracolonic tumors, and predominance of proximal colorectal cancer relative to distal colorectal cancer (38). Patients with HNPCC carry a germ line mutation in one allele of any several DNA mismatch repair genes (most frequently in *hMSH2* or *hMLH1*, but in rare cases, in *hPMS1*, *hPMS2*, and *hMSH6*). It remains unknown whether NSAIDs may be useful as cancer preventive agents in HNPCC.

Celecoxib, a selective COX-2 inhibitor, was approved by the Food and Drug Administration for prevention of colon cancer in patients with FAP, and a clinical trial was recently conducted to evaluate the safety and efficacy of celecoxib in HNPCC patients. In a randomized, placebo-controlled phase I/II multicenter trial, HNPCC gene carriers received either celecoxib at one of two doses or placebo. The goal was to evaluate the effects of these treatment arms on a number of endoscopic and tissue-based biomarker end points after 12 months of treatment (39). As part of this trial, we studied gene expression in normal colonic mucosa of HNPCC patients and gene carriers to determine if there are differences in gene expression profiles at baseline and after treatment of these patients with celecoxib.

Materials and Methods

Methods of RNA extraction and amplification, cDNA labeling and microarray hybridization, data acquisition, and statistical analysis were described in detail in our publication on differences of gene expression in proximal and distal colon (40) and will be only outlined here.

Patients and Biopsy Samples. All HNPCC patients were known to carry germ line mutations in the *hMLH1* or *hMSH2* genes or have a family history of colorectal cancer with microsatellite instability, and were enrolled during a multi-institutional (M.D. Anderson Medical Center; National Medical Navy Center, National Cancer Institute; Creighton University; and University of Toronto) ''Phase I-II multiple-dose safety and efficacy study of a selective inhibitor of cyclooxygenase-2 (celecoxib) in HNPCC patients and carriers.'' Study design and dose selection were based on the results of previous clinical trial in FAP patients (29). Patients were randomized to receive celecoxib at 200 mg p.o. bid, 400 mg p.o. bid, or placebo p.o. bid for 12 months. Protocols used in the study were approved by Institutional Review Boards of the National Cancer Institute and respective clinical performance sites (M.D. Anderson Medical Center; National Naval Medical Center, National Cancer Institute; Creighton University; and University of Toronto). Before enrollment, informed consent was obtained from all of the patients. Standard pinch biopsies of normal colonic mucosa were taken during colonoscopy at the beginning of the study (baseline or pretreatment samples) and 12 months after celecoxib or placebo treatment (posttreatment samples), flash frozen in liquid nitrogen, and stored at −80°C. More than 80% of the cells in standard colon pinch biopsies were colonic crypt cells as determined by H&E staining. Although we have studied almost all available pretreatment biopsies from descending (rectum) and ascending colon (from 50 patients; ref. 40), we decided to analyze posttreatment biopsies only from descending (rectum) colon of randomly selected patients, and it seemed, after code disclosure, that we have data for both pretreatment and posttreatment biopsies of 39 patients. All but one patient have germ line mutations in hMLH1, hMSH2, or PMS1; 14 patients were treated with placebo and 25 with celecoxib.

RNA Extraction and Amplification. Total RNA was isolated from flash-frozen specimens (two standard pinch biopsies per one RNA sample), homogenized with a Disposable Generator and Micro-H Omni Homogenizer in lysis buffer RLT (Qiagen, Chatsworth, CA), and purified using Qiagen's RNeasy Mini kit columns (Qiagen) according to the manufacturer's instructions. mRNA was amplified according to a modified Eberwine's protocol (41). SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD) was used for first-strand cDNA synthesis, starting with 5 μg of total RNA. First-strand cDNA synthesis was done with an oligo(dT_{24}) primer containing 5′-T7 promoter-primer. The second strand was synthesized with a mixture of RNase H, DNA Polymerase, and DNA Ligase. Purified cDNA was transcribed *in vitro* with T7 MEGAscript kit (Ambion, Austin, TX) according to the manufacturer's instructions, and antisense RNA (aRNA) was purified using RNeasy mini spin columns (Qiagen). This and similar protocols of linear aRNA amplification are shown to retain the RNA ratio characteristics of unamplified RNA especially when starting with a relatively large amount (in our case, 5 μg) of total RNA (42-44). We have also used total RNA isolated from HCT116 cells for RNA amplification and found good correlation (Pearson correlation coefficient was >0.9) for gene expression data when arrays were hybridized with total RNA and aRNA samples, which were independently amplified using 5 μg of the total RNA.

cDNA Labeling and Microarray Hybridization. Fluorescent-labeled cDNA was synthesized by reverse transcription of colon aRNA and human testis aRNA (prepared from total testis RNA as described for colon aRNA; Clontech, Inc., Palo Alto, CA) with Superscript II reverse transcriptase (Invitrogen, San Diego, CA) and random oligonucleotide primers in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ), respectively. For each hybridization experiment, 5 μg of colon aRNA and 6 μg of testis aRNA (common reference) were used to prepare a mixture of labeled cDNAs. Purified labeled colon and testis cDNAs were dissolved in hybridization buffer containing 3.7 × saline-sodium phosphate-EDTA, 2.5 × Denhardt's solution (Sigma, St. Louis, MO), 0.5 μg/μL of poly$(A)_{40\text{-}60}$ (Amersham Pharmacia Biotech) and Human C_0t1 DNA (Invitrogen), 0.125 μg/μL yeast tRNA (Sigma), and 10% SDS in TE buffer.

Microarrays containing cDNA clones (from Research Genetics, Inc., Huntsville, AL) spotted on lysine-coated glass slides were obtained from the Advanced Technology Center (National Cancer Institute). Detailed information on printed cDNA can be found on the mAdb web site (http://nciarray.nci.nih.gov/). Microarrays contain 9,128 sequence-verified cDNAs, among which 7,102 represent named genes and 1,179 expressed sequence tag clusters, including 8,556 cDNAs with UniGene cluster ID (mapping to 7,777 unique UniGene clusters).

After hybridization at 65°C for 16 to 18 hours with 40 μL of hybridization mixture, slides were washed at room

temperature in 2 × SSC-0.1% SDS (5 minutes), 2 × SSC (2 minutes), 1 × SSC (1 minute), 0.2 × SSC (30 seconds) and dipped in 0.05% SSC. Slides were dried by centrifugation at 150 × *g* for 5 minutes at room temperature.

All array hybridization and pretreatment image analysis were finished before trial codes were broken.

Data Acquisition and Analysis. Microarrays were scanned with an Axon 4000 laser scanner, and pretreatment image analysis was done with GenePix Pro 3.0 Software (Axon Instruments, Inc., Sunnyvale, CA). Images were also visually inspected, and questionable spots were flagged and excluded from the analysis. Data analysis was done with the BRB-ArrayTools (version 3.2) software package developed by the Biometric Research Branch of the Division of Cancer Treatment and Diagnosis of the National Cancer Institute and The EMMES Corp. (45).

Briefly, background intensities were subtracted, and data were filtered for minimal spot intensity (100 units) in each of the two channels and for missing values (not more than in 20% of arrays). Fluorescence intensity ratio data were log transformed and normalized by subtracting the median log ratio from all log ratios on the array.

The Class comparison and Class Prediction modules of BRB-ArrayTools were used to determine if the pattern of gene expression allowed discrimination of colonic mucosa samples at baseline and after treatment of patients for 12 months with celecoxib or placebo. First, a paired *t* test was done on average differences in gene expression log ratios of pairs of samples (pretreatment and posttreatment biopsies) from the same patients, to select genes that showed univariately statistically significant (at a stated *P*, routinely at $P < 0.001$) differences in expression between baseline and posttreatment colonic mucosa samples of patients treated with celecoxib or placebo. In a case of multiple classes (in the comparison of printing batches, see Results), an *F* test was used for identification of the differentially expressed genes, which is based on comparing mean log ratios between classes relative to the variation expected in the mean differences (45). The multivariate class label permutation test was used to compute the number of false positives among selected genes (46, 47). Several multivariate classification methods available in BRB ArrayTools 3.2 (compound covariate predictor, diagonal linear discriminant analysis, nearest neighbor and nearest centroid predictors, and support vector machine) were used for classification of pretreatment and posttreatment samples from celecoxib or placebo treated patients and gave comparable results, which will be illustrated with the Compound Covariate Predictor (CCP) and Support Vector Machine Predictor (SVMP) classifiers. CCP was calculated as a linear combination of log ratio differences weighed by univariate *t* values (48). A positive sign was assigned to *t* test values for genes that show higher log ratios in posttreatment biopsies and a negative sign for genes that show higher log ratios in pretreatment biopsies. The CCP value was calculated for each pair of samples and, as a classification threshold, the sign (positive or negative) of the CCP was used for classifying. SVMP was calculated as a linear function of the log ratio differences for genes selected in a paired *t* test with weights estimated by linear kernel support vector machine algorithm to minimize the number of misclassified samples. The misclassification rate was estimated by a leave-one-out cross-validation. Specifically, a pair of samples (from one patient) was omitted, and a CCP and a SVMP were developed from scratch using the remaining samples. The cross-validation procedure includes performing *t* tests for a selection of genes on arrays for which differences in expression are significant at a stated *P* for remaining samples, recalculating the CCP, SVMP, and classification threshold for the remaining pairs of samples, and applying the new CCP, SVMP, and threshold values to classify the omitted pair of samples. This was done independently for each omitted pair of samples. The ratio of pairs of samples correctly classified in cross-validation to the total number of sample pairs yields the misclassification rate. Permutation *P* for a classifier was calculated by performing 2,000 random permutations of class labels and repeating the cross-validation procedure for each permutation. The proportion of random permutations that gave the same or smaller misclassification rate as was obtained with the true class labels is presented as a (permutation) *P* for the classifier, and $P < 0.0005$ was reported when no random permutation of the class label was found out of 2,000 permutations with the same or smaller misclassification rate as for the true class labeling.

The Class Comparison and Class Prediction algorithms use *t* statistics and *P* cutoffs for selection of genes whose changes in expression are considered significant. We also used another algorithm to determine significant changes in gene expression in colon biopsies after treatment of patients with celecoxib, significance analysis of microarrays (SAM; ref. 49), as implemented in BRB-ArrayTools (45). SAM is based on a comparison of the observed and expected (estimated through random class label permutations) sorted modified *t* statistics (relative differences) but uses an adjustable threshold, Δ (distance from expected relative differences) and (user defined) false discovery rate as cutoffs for selection of significant genes (49).

To identify biological processes that are most likely targeted in colonic mucosa of patients treated with celecoxib, we structured 1,584 cDNA clones for which changes in expression were statistically significant by SAM (see below) according to Gene Ontology categories (50, 51). Because arrays that were used in this study were printed with a relatively small ($n = 9{,}000$) and biased (toward known genes implicated mostly in tumorigenesis) set of cDNAs, we used overrepresentation analysis, in which the number of genes belonging to a Gene Ontology category that were affected by celecoxib treatment (relative to the total number of celecoxib affected genes) is compared with the number of genes printed on the array that belong to the same Gene Ontology category (relative to the total number of genes printed on the array). Two programs were used for overrepresentation analysis: Expression Analysis Systematic Explorer (EASE; ref. 52) and GoMiner (53). Fisher exact probability test is used by GoMiner to estimate the probability of getting an enrichment of a category by chance, and EASE score (derived from the Fisher test penalized by removing one affected gene from the given category and then calculating Fisher exact probability for that category; thus, a category with more genes will be favored against a category with a smaller number of genes) is used in EASE. MatchMiner (54) was used to map UniGene Cluster ID into HUGO symbols (gene symbols approved by Human Genome Organization: http://www.gene.ucl.ac.uk/nomenclature), which are used by GoMiner (see ref. 55).

Real-time PCR. One-step Taqman real-time reverse transcription-PCR was done to study expression of several cDNAs using an ABI Prism 7700 Sequence Detection System. Primers and hybridization FAM-labeled probes were selected with PrimerExpress software (Applied Biosystems, Foster City, CA) by using complete cDNA sequences that have the same UniGene cluster ID as cDNAs printed on array. Six patients (12 RNAs) were randomly selected from 11 patients that had received celecoxib at 400 mg p.o. bid. Taqman Gold reverse transcription-PCR kit (Applied Biosystems) and the manufacturer's protocol (30 minutes at 48°C for reverse transcription reaction, 10 minutes at 95°C for activation of TaqGold Polymerase, and 40 cycles consisting of 15 seconds at 95°C and 1 minute at 60°C) were used, and 50 ng of total RNA were assayed in 25 μL of one-step reverse transcription-PCR reaction mixture with gene-specific primers and probe in triplicates for

Table 1. Genes differentially expressed ($P < 0.001$) in primary and follow-up biopsies of celecoxib-treated patients

Genes*	Clone	UniGene cluster	Fold-difference, P/F †	t
Incyte EST	IncytePD:4000648		2.098	4.45
Collagen, type X, α 1 (Schmid metaphyseal chondrodysplasia)	IncytePD:4855492	Hs.179729	2.061	5.51
Dual adaptor of phosphotyrosine and 3-phosphoinositides	IncytePD:1304879	Hs.62643	1.989	6.56
Immunoglobulin superfamily, member 6	IncytePD:522893	Hs.135194	1.964	5.65
ATP-binding cassette, subfamily B (MDR/TAP), member 1	IncytePD:1931381	Hs.21330	1.938	5.48
Homo sapiens–transcribed sequences	IncytePD:4073268	Hs.133181	1.899	4.63
Homo sapiens–transcribed sequences	IncytePD:3846507	Hs.443475	1.888	4.85
Heparin-binding growth factor binding protein	IncytePD:2024613	Hs.1690	1.885	4.3
Caspase-8, apoptosis-related cysteine protease	IncytePD:2466440	Hs.243491	1.884	4.38
Platelet-activating receptor homologue	IncytePD:3879095	Hs.159545	1.882	4
Interleukin 22 receptor, α 1	IncytePD:3257185	Hs.110915	1.879	4.58
Carbonic anhydrase I	IncytePD:2101663	Hs.23118	1.865	5.75
Diacylglycerol kinase, β 90 kDa	IncytePD:1213571	Hs.158318	1.843	5.64
TATA box binding protein (TBP)-associated factor, RNA polymerase II, N, 68 kDa (*RNA-binding protein 56*)	IncytePD:1907369		1.825	4.66
Potassium inwardly-rectifying channel, subfamily J, member 2	IncytePD:630604	Hs.1547	1.815	5
Chemokine (C-X-C motif) ligand 6 (*granulocyte chemotactic protein 2*)	IncytePD:950310	Hs.164021	1.769	6.44
Homo sapiens–transcribed sequences	IncytePD:2289203	Hs.49132	1.764	3.98
Homo sapiens mRNA; cDNA DKFZp686H0155 (from clone DKFZp686H0155)	IncytePD:2906194	Hs.513302	1.747	5.46
Keratin, hair, acidic, 3B	IncytePD:3187903	Hs.32950	1.741	4.68
Down syndrome cell adhesion molecule	IncytePD:4774130	Hs.49002	1.739	4.53
Interleukin 9 receptor	IncytePD:2211721		1.731	4.82
Progestagen-associated endometrial protein (*placental protein 14, pregnancy-associated endometrial α-2-globulin, α uterine protein*)	IncytePD:1976179	Hs.82269	1.728	4.09
Agrin	IncytePD:4610962	Hs.273330	1.71	4.19
Homo sapiens mRNA; cDNA DKFZp686P24244 (from clone DKFZp686P24244)	IncytePD:1638327	Hs.119065	1.665	5.38
Prostaglandin I2 (prostacyclin) *receptor* (IP)	IncytePD:663798	Hs.458324	1.652	4.43
Retinoic acid receptor responder (tazarotene induced) 3	IncytePD:1927347	Hs.17466	1.647	4.29
Adlican	IncytePD:1402105	Hs.72157	1.618	4.45
KH-type splicing regulatory protein (FUSE binding protein 2)	IncytePD:3460518	Hs.91142	1.612	3.89
Small inducible cytokine subfamily C, member 1 (*lymphotactin*)	IncytePD:308896		1.6	5.03
Hypothetical protein LOC89958	IncytePD:2238576	Hs.19322	1.595	4.5
Ras homologue gene family, member F (in filopodia)	IncytePD:1905723	Hs.512618	1.594	5.98
Purinergic receptor P2Y, G-protein coupled, 1	IncytePD:1961218	Hs.2411	1.586	4.24
Copine VI (neuronal)	IncytePD:2284107	Hs.6132	1.585	4.3
Unc-5 homologue C (*Caenorhabditis elegans*)	IncytePD:3350064	Hs.125605	1.582	4.21
Spectrin, β, erythrocytic (includes spherocytosis, clinical type I)	IncytePD:2189383	Hs.438514	1.574	4.45
Hypothetical protein DKFZp667E0512	IncytePD:1214652	Hs.432998	1.563	4.97
Homo sapiens mRNA; cDNA DKFZp564B076 (from clone DKFZp564B076)	IncytePD:767447	Hs.21103	1.56	3.95
DNA segment on chromosome 6 (unique) 49 expressed sequence	IncytePD:2102881		1.536	4.63
Incyte EST	IncytePD:637290		1.529	3.91
Retinoic acid induced 3	IncytePD:899118	Hs.194691	1.503	3.89
Protein phosphatase 1, regulatory (inhibitor) subunit 16B	IncytePD:1678639	Hs.45719	1.501	4.36
Homo sapiens–transcribed sequences	IncytePD:2344817	Hs.444948	1.489	4.04
TUWD12	IncytePD:2815133	Hs.25130	1.482	4.17
Solute carrier family 38, member 4	IncytePD:778212	Hs.165655	1.472	6.44
Incyte EST	IncytePD:1607471		1.471	4.06
Src homology 2 domain-containing transforming protein D	IncytePD:1738134	Hs.7423	1.455	4.54
Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	IncytePD:3033662	Hs.290404	1.454	6.57
Glia maturation factor, γ	IncytePD:409089	Hs.5210	1.453	4.08
Inducible T-cell costimulator ligand	IncytePD:2758733	Hs.14155	1.447	4.32
Zinc finger protein 205	IncytePD:3315208	Hs.13128	1.434	5.45
Incyte EST	IncytePD:4291982		1.423	3.98
pancreatic polypeptide	IncytePD:254890	Hs.184604	1.41	4.2
cholinergic receptor, nicotinic, β polypeptide 3	IncytePD:4337986	Hs.96094	1.398	5.54
CLIP-170-related protein	IncytePD:2290302	Hs.7357	1.391	4.65
Zeta-chain (TCR) associated protein kinase 70 kDa	IncytePD:3287090	Hs.234569	1.386	3.85
Incyte EST	IncytePD:476348		1.38	4.75
ATP-binding cassette, subfamily C (CFTR/MRP), member 6	IncytePD:1830341		1.37	3.95
Granulysin	IncytePD:1878545	Hs.105806	1.365	4.62
Proteasome (prosome, macropain) subunit, β type, 9 (large multifunctional protease 2)	IncytePD:2018222	Hs.381081	1.35	4.15
Bone morphogenetic protein 7 (osteogenic protein 1)	IncytePD:2187660	Hs.170195	1.342	3.99
Potassium large conductance calcium-activated channel, subfamily M β member 3	IncytePD:2288482	Hs.120905	1.335	4.91
Thyroid hormone receptor interactor 8	IncytePD:4003794	Hs.442675	1.335	4.01
Prospero-related homeobox 1	IncytePD:1680706	Hs.159437	1.334	5.02
G protein pathway suppressor 2	IncytePD:606083	Hs.438219	1.325	5.71
RAB9A, member RAS oncogene family	IncytePD:1746329	Hs.444327	1.324	3.89
KIAA1145 protein	IncytePD:1477151	Hs.173392	1.319	3.78
Troponin C2, fast	IncytePD:1928824	Hs.182421	1.319	4.25
Homo sapiens–transcribed sequences	IncytePD:2959963	Hs.189955	1.319	4.77

(*Continued on the following page*)

Table 1. Genes differentially expressed ($P < 0.001$) in primary and follow-up biopsies of celecoxib-treated patients (Cont'd)

Genes*	Clone	UniGene cluster	Fold-difference, P/F†	t
Hepatitis B virus x associated protein	IncytePD:829521	Hs.20509	1.314	4.93
Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	IncytePD:513943	Hs.40968	1.303	4.38
N-myc downstream regulated gene 1	IncytePD:2055569	Hs.318567	1.296	4.01
Homo sapiens–transcribed sequence with strong similarity to protein sp:P43897 (H. sapiens) EFTS_HUMAN Elongation factor Ts, mitochondrial precursor (EF-Ts) (EF-TsMt)	IncytePD:2948353	Hs.340959	1.292	4.54
Homo sapiens mRNA; cDNA DKFZp667O0320 (from clone DKFZp667O0320)	IncytePD:1362601	Hs.137587	1.29	4.63
Platelet-derived growth factor α polypeptide	IncytePD:2158373	Hs.376032	1.274	3.75
Homo sapiens–transcribed sequences	IncytePD:694279	Hs.356812	1.259	3.77
Hermansky-Pudlak syndrome-6 protein	IncytePD:1738248	Hs.125133	1.245	3.85
Homo sapiens–transcribed sequence with moderate similarity to protein sp:P39192 (H. sapiens) ALU5_HUMAN Alu subfamily SC sequence contamination warning entry	IncytePD:1349433	Hs.121070	1.241	4.57
Homo sapiens cDNA: FLJ21513 fis, clone COL05778	IncytePD:232715	Hs.14555	1.22	4.67
Geranylgeranyl diphosphate synthase 1	IncytePD:221039	Hs.55498	1.217	3.91
Homo sapiens clone 24889 mRNA sequence	IncytePD:1984908	Hs.152335	1.21	3.88
Homo sapiens transcribed sequence with weak similarity to protein ref: NP_060265.1 (H. sapiens) hypothetical protein FLJ20378 [Homo sapiens]	IncytePD:2504786	Hs.513521	1.179	3.85
Homo sapiens transcribed sequence with weak similarity to protein ref: NP_079370.1 (H. sapiens) hypothetical protein FLJ22184 [Homo sapiens]	IncytePD:1451756	Hs.23272	0.862	−3.75
Zinc finger protein 263	IncytePD:2816931	Hs.124553	0.861	−3.76
BCL2/adenovirus E1B 19 kDa interacting protein 1	IncytePD:4069979	Hs.145726	0.86	−3.8
Vesicle-associated membrane protein 4	IncytePD:3038946	Hs.6651	0.854	−3.91
Zinc finger protein 512	IncytePD:2292949	Hs.294122	0.841	−3.98
Ubiquitin-activating enzyme E1C (UBA3 homologue, yeast)	IncytePD:3010845	Hs.154320	0.84	−3.78
Zinc finger protein 398	IncytePD:1382325	Hs.169452	0.839	−3.84
Chromosome 2 open reading frame 3	IncytePD:2779394	Hs.158969	0.839	−4.15
Nuclear cap binding protein 1, 80 kDa	IncytePD:999424		0.836	−3.84
2,3-bisphosphoglycerate mutase	IncytePD:2287957	Hs.198365	0.828	−5.02
Kinetochore associated 1	IncytePD:789903	Hs.300559	0.827	−4.09
Membrane component, chromosome 11, surface marker 1	IncytePD:827257	Hs.278672	0.826	−3.88
Syntaxin 6	IncytePD:2748585	Hs.157144	0.825	−5.37
Karyopherin α 1 (importin α 5)	IncytePD:465152	Hs.161008	0.824	−4
Hypothetical protein similar to RNA-binding protein lark	IncytePD:1793670	Hs.49994	0.821	−4.1
Eukaryotic translation initiation factor 4B	IncytePD:1663283	Hs.93379	0.821	−4.71
5-methyltetrahydrofolate-homocysteine methyltransferase reductase	IncytePD:2593323	Hs.153792	0.82	−4.44
Poly(A) binding protein interacting protein 1	IncytePD:2360751	Hs.374614	0.815	−3.86
Ash2 (absent, small, or homeotic)-like (Drosophila)	IncytePD:2635641	Hs.6856	0.814	−3.82
Tousled-like kinase 1	IncytePD:664380	Hs.369280	0.813	−3.79
Zinc finger protein 262	IncytePD:3409136	Hs.150390	0.81	−3.94
Cerebral cavernous malformations 1	IncytePD:2779795	Hs.438833	0.81	−3.86
Bone morphogenetic protein receptor, type II (serine/threonine kinase)	IncytePD:2967280	Hs.53250	0.807	−4.76
Hypothetical protein FLJ22002	IncytePD:2547084	Hs.461485	0.804	−3.84
Solute carrier family 33 (acetyl-CoA transporter), member 1	IncytePD:2842553	Hs.285176	0.801	−4.27
Hypothetical protein MGC23401	IncytePD:2884394	Hs.435048	0.797	−4.17
MADS box transcription enhancer factor 2, polypeptide D (myocyte enhancer factor 2D)	IncytePD:2722916	Hs.77955	0.796	−4.19
Myotubularin related protein 4	IncytePD:2890336	Hs.141727	0.796	−4.43
Zinc finger protein 451	IncytePD:1812489	Hs.188662	0.796	−3.83
Osmosis responsive factor	IncytePD:2078614	Hs.280811	0.794	−3.84
Incyte EST	IncytePD:2477854		0.793	−4.2
KIAA0725 protein	IncytePD:1824577	Hs.434966	0.793	−5.54
Laminin, α 2 (merosin, congenital muscular dystrophy)	IncytePD:2187108	Hs.445120	0.792	−4.01
TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150 kDa	IncytePD:998069	Hs.122752	0.79	−4.73
Minichromosome maintenance deficient (Saccarhomyces cerevisiae) 4	IncytePD:103669		0.788	−3.81
Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), α 1	IncytePD:1794012	Hs.128312	0.785	−5.45
KIAA0643 protein	IncytePD:1981939	Hs.155995	0.784	−5.83
Cohen syndrome 1	IncytePD:2201626	Hs.185585	0.783	−3.79
DKFZP434D193 protein	IncytePD:1425925	Hs.19150	0.782	−3.75
Transcriptional adaptor 2 (ADA2 homologue, yeast)-like	IncytePD:2045628	Hs.125156	0.781	−3.88
Hypothetical protein FLJ12085	IncytePD:3737086	Hs.310422	0.78	−4.97
Vacuolar protein sorting 45A (yeast)	IncytePD:2821078	Hs.134231	0.78	−5.04
Replication factor C (activator 1) 5, 36.5 kDa	IncytePD:3590056	Hs.443227	0.777	−4.25
Solute carrier family 30 (zinc transporter), member 4	IncytePD:3350921	Hs.112282	0.773	−4.64
Zinc finger protein 450	IncytePD:2808265	Hs.409876	0.771	−3.92
Dmx-like 1	IncytePD:3272587	Hs.181042	0.769	−4.53
Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	IncytePD:2374366		0.768	−3.8
Phosphoinositide-3-kinase, catalytic, β polypeptide	IncytePD:267803	Hs.239818	0.767	−3.76
Homo sapiens, clone IMAGE:5268696, mRNA	IncytePD:2396760	Hs.90286	0.763	−3.89
3-hydroxyisobutyryl-Coenzyme A hydrolase	IncytePD:3254666	Hs.236642	0.761	−5.34
KIAA0373 gene product	IncytePD:2069349	Hs.150444	0.76	−4.32
Acyl-Coenzyme A binding domain containing 3	IncytePD:2071833	Hs.6831	0.758	−3.89
Itchy homologue E3 ubiquitin protein ligase (mouse)	IncytePD:2383269	Hs.314676	0.755	−4.05

(*Continued on the following page*)

Table 1. Genes differentially expressed ($P < 0.001$) in primary and follow-up biopsies of celecoxib-treated patients (Cont'd)

Genes*	Clone	UniGene cluster	Fold-difference, P/F†	t
Translin	IncytePD:986855	Hs.75066	0.755	−3.8
Golgi autoantigen, golgin subfamily b, macrogolgin (with transmembrane signal), 1	IncytePD:1506093	Hs.445083	0.754	−4.77
Protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	IncytePD:2726895	Hs.83572	0.752	−4.05
Eukaryotic translation initiation factor 2, subunit 1 α, 35 kDa	IncytePD:1224219	Hs.151777	0.751	−3.82
Optic atrophy 1 (autosomal dominant)	IncytePD:3140082	Hs.131273	0.751	−4.12
Ubiquitination factor E4A (UFD2 homologue, yeast)	IncytePD:4215584	Hs.75275	0.749	−3.79
Nucleoporin 153 kDa	IncytePD:341994	Hs.146449	0.748	−4.6
Adenosine deaminase, tRNA-specific 1	IncytePD:3836721	Hs.188661	0.744	−4.12
Myosin, light polypeptide 1, alkali; skeletal, fast	IncytePD:973629	Hs.187338	0.743	−3.99
Thyroid autoantigen 70 kDa (Ku antigen)	IncytePD:3773032	Hs.169744	0.742	−4.02
Origin recognition complex, subunit 3-like (yeast)	IncytePD:822017	Hs.312419	0.741	−3.84
CGI-85 protein	IncytePD:2603232	Hs.442630	0.74	−4.08
Phosphatidylinositol-4-phosphate 5-kinase, type II, β	IncytePD:1315666	Hs.291070	0.738	−5.06
Alkylglycerone phosphate synthase	IncytePD:334227	Hs.407933	0.734	−4.54
Transmembrane 9 superfamily protein member 4	IncytePD:2372824	Hs.79305	0.73	−4.56
Homo sapiens transcribed sequence with moderate similarity to protein ref: NP_004563.1 (H. sapiens) plakophilin 2 [Homo sapiens]	IncytePD:1873338	Hs.436379	0.726	−4.21
DKFZP434C212 protein	IncytePD:1282176	Hs.287266	0.724	−3.98
Hypothetical protein BC005868	IncytePD:2046989	Hs.184846	0.722	−4.77
Zinc finger and BTB domain containing 5	IncytePD:736418	Hs.3682	0.718	−4.47
Soc-2 suppressor of clear homologue (Caenorhabditis elegans)	IncytePD:3597760	Hs.104315	0.717	−4.59
Adaptor-related protein complex 3, mu 2 subunit	IncytePD:2135517	Hs.77770	0.715	−4.69
Ubiquitin-conjugating enzyme E2H (UBC8 homologue, yeast)	IncytePD:2275196	Hs.372758	0.715	−3.96
v-yes-1 Yamaguchi sarcoma viral oncogene homologue 1	IncytePD:1887736	Hs.194148	0.703	−4.61
RAP1, GTPase activating protein 1	IncytePD:1439934	Hs.433797	0.698	−3.82
Zinc finger protein 175	IncytePD:1306335	Hs.119014	0.695	−4.33
Implantation-associated protein	IncytePD:876466	Hs.323562	0.694	−3.75
Homo sapiens−transcribed sequences	IncytePD:3220149	Hs.30385	0.69	−4.53
Nicastrin	IncytePD:2471087	Hs.4788	0.686	−3.89
Hypothetical protein MGC3067	IncytePD:1879888	Hs.241576	0.68	−4.75
Coatomer protein complex, subunit α	IncytePD:3296228	Hs.477132	0.672	−4.27
U2-associated SR140 protein	IncytePD:3179051	Hs.370907	0.67	−4.7
Chromosome 20 open reading frame 194	IncytePD:2956129	Hs.119021	0.667	−3.83
Catenin (cadherin-associated protein), delta 1	IncytePD:1976602	Hs.166011	0.665	−4.87
KIAA1210 protein	IncytePD:3355572	Hs.97594	0.655	−3.93
Phosphoserine aminotransferase 1	IncytePD:2825369	Hs.286049	0.619	−4.54
Nuclear receptor interacting protein 1	IncytePD:2366468	Hs.155017	0.605	−4.15
Basonuclin	IncytePD:3564654	Hs.64025	0.588	−3.84
Myosin, heavy polypeptide 11, smooth muscle	IncytePD:1866751	Hs.78344	0.579	−4.32
Synaptonemal complex protein 2	IncytePD:1692253	Hs.202676	0.568	−4.74

*Genes are sorted by *P/F* ratio.
†*P/F*, geometric mean of ratios of gene expression in primary to follow-up biopsies.

each sample and each gene. Serial dilutions of a mixture of colon total RNAs from several other colon biopsies were used to calculate PCR efficiency for a gene in the range of 10 to 100 ng of total RNA per reaction. Gene expression was normalized to the amount of ribosomal 18S RNA in a sample, for which reverse transcription-PCR reactions were done on the same 96-well plate in separate wells, together with serial dilutions of colon total RNA to calculate PCR efficiency for ribosomal 18S RNA. Data were analyzed with Q-Gene software (56).

Results

Twenty-five pairs of pretreatment and posttreatment descending colon biopsies from patients exposed to celecoxib (from 14 patients receiving 200 mg bid and 11 patients receiving 400 mg bid of celecoxib) and 14 pairs of biopsies from patients treated with placebo were analyzed on a total of 88 arrays (with some samples analyzed in duplicate). Upon statistical analysis, data on duplicate arrays were averaged and treated as one entry in the analysis. Mean Pearson correlation coefficient (r) for gene expression log ratios between duplicate arrays was 0.84 (95% confidence interval, 0.78-0.91) for 10 different aRNA pairs (for two duplicate arrays of the same printing set hybridized with the same aRNA, $r = 0.95$).

Paired t test identified 173 genes that showed statistically significant (at a univariate $P < 0.001$) differences in expression in pretreatment and posttreatment biopsies from 25 patients (58 arrays) who received celecoxib (Table 1). Only 14 genes were differentially expressed (at $P < 0.001$) in pretreatment and posttreatment biopsies of 14 patients (30 arrays) who received placebo (Supplementary Table S1). The probability of getting 173 and 14 differentially expressed genes out of 9,128 by chance (multivariate class label permutation Ps) is 0.001 and 0.094, respectively. The magnitude of gene expression differences is rather small: maximal fold differences are 1.76 for 92 overexpressed genes and 2.10 for 81 genes underexpressed in posttreatment biopsies of celecoxib-treated patients. For five selected genes, array data showing overexpression after celecoxib treatment were confirmed by real-time reverse transcription-PCR (Supplementary Tables S2 and S3).

Twenty-three of 25 pairs of samples (92%) from celecoxib-treated patients were classified correctly in a leave-one-out cross-validation using the CCP and SVMP. Random permutation of class label showed that the probability of getting as small a cross-validated misclassification rate (8%) as obtained with the true class label by chance (CCP/SVMP permutation Ps) is 0.001 for both classifiers. In contrast, less than half of the samples from placebo-treated patients were correctly classified in cross-validation with CCP (43%, permutation $P = 0.589$) or SVMP (21%, $P = 0.682$).

Thus, the patterns of gene expression in pretreatment and posttreatment biopsies from celecoxib-treated patients are different, but no such differences, exceeding those expected

by chance, are seen in the placebo-treated patients. Celecoxib-treated colonic mucosa biopsies can be correctly identified by the pattern of gene expression, again in contrast to biopsies from patients who received placebo.

One of the known confounding variables that can affect the results of array gene expression analysis is the use of different printing sets of arrays (i.e., arrays which were printed on different dates with the same cDNA clones). We identified (using F test) genes that showed statistically significant variation in log ratios between printing sets (6,130 and 6,831 genes at $P < 0.001$ and $P < 0.005$, respectively), excluded them, and repeated the analysis for the remaining 2,841 or 2,140 genes. Class comparison and class prediction analysis confirm the existence of a distinguishing gene expression signature in colonic mucosa of celecoxib-treated patients, which is robust and can withstand withdrawal of two thirds of the genes present on the array. Using SVMP classifier, up to 96% of pretreatment and posttreatment biopsies from celecoxib-treated patients can be classified correctly in a leave-one-out cross-validation (with one misclassified pair of biopsies out of 25 pairs) based on the expression of 2,841 genes.

Another confounding issue in the analysis is the possibility that the observed differences between the celecoxib- and placebo-treated groups may be a function of the difference in population size. To rule out this possibility, we randomly selected 250 sets of 14 patients from the 25 celecoxib-treated patients. Class comparison and class prediction were done based on the expression of 2,841 genes. Analysis of the distribution of the number of misclassified samples for the 250 sets of 14 patients showed that the probability of getting by chance a misclassification rate such as that for placebo-treated patients is <0.0002. This indicates that the difference in outcome of our analysis of placebo-treated patients compared with celecoxib-treated patients cannot be dismissed as a result of a smaller number of samples in the placebo group.

Among patients who received celecoxib, 14 were given the 200 mg dose, and 11 were given the 400 mg dose. Most (130 genes or 75%) of the 173 genes whose differences in expression are significant at $P < 0.001$ showed greater fold differences in expression between pretreatment and posttreatment biopsies for patients treated with 400 mg of celecoxib compared with patients who received 200 mg of celecoxib. The Wilcoxon matched pairs signed rank t test shows that the median fold differences in expression of 173 genes for the group of patients treated with 400 mg (1.46) and 200 mg (1.31) of celecoxib are statistically significant ($P < 0.0001$). These dose-dependent changes in expression are more characteristic for genes overexpressed in posttreatment biopsies (Fig. 1) than for underexpressed genes (Fig. 2): 91% (84 of 92) of overexpressed genes display a dose response effect compared with only 57% (46 of 81) of underexpressed genes.

SAM is less restrictive in defining the significance of gene expression changes, although as shown in an experiment (49) and in simulation of microarray hybridization experiments (57), it can still generate reliable lists of genes that show reproducible changes in expression between compared samples. When gene expression patterns in pretreatment and posttreatment colonic biopsies of patients treated with celecoxib were compared, 1,584 genes were found to be significant by SAM with a median false discovery rate of 9.9% and Δ = 0.63 (for log 2 of relative difference). Of 1,584 genes, 756 were overexpressed, and 828 genes were underexpressed in posttreatment biopsies (Supplementary Table S4). All 173 genes that showed differences in expression significant at univariate $P < 0.001$ were present in the list of 1,584 genes, and differences in expression of 1,454 of 1,584 genes were significant at $P < 0.05$. A dose effect is still present: 996 genes (63%) display greater fold differences in gene expression in patients treated with 400 mg of celecoxib compared with patients treated with 200 mg of celecoxib. Differences in the median fold difference are significant by Wilcoxon matched pairs signed rank t test for the whole set of 1,584 genes (1.30 and 1.24 for patients who received 400 and 200 mg of celecoxib, respectively; $P < 0.0001$), and in a separate comparison for 756 genes overexpressed in posttreatment biopsies (median fold differences = 1.31 and 1.19; $P < 0.0001$) but not for 828 genes underexpressed in posttreatment biopsies.

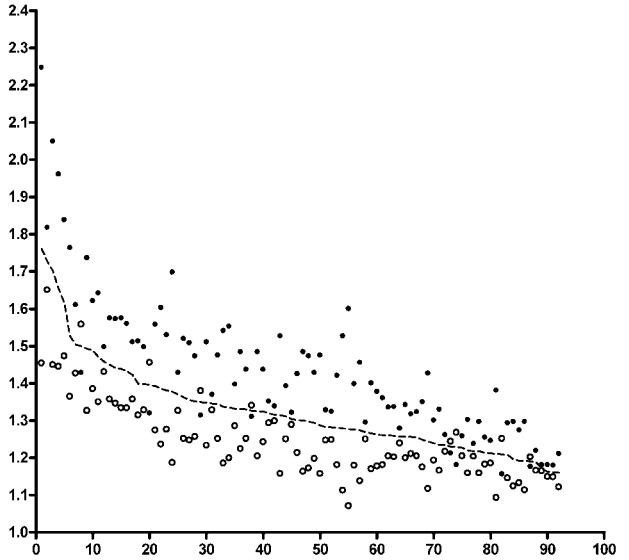

Figure 1. Comparison of the fold differences in expression for 92 genes overexpressed after treatment of patients with celecoxib. *Abscissa,* gene number; *ordinate,* fold difference in gene expression in posttreatment relative to pretreatment biopsies. Ninety-two genes were sorted by mean fold difference in expression (*dash line*). ○, fold difference in patients treated with 200 mg of celecoxib; ●, fold difference in patients treated with 400 mg of celecoxib.

The 1,584 cDNA clones for which changes in posttreatment biopsies of celecoxib-treated patients are identified as significant by SAM are mapped to 1,448 unique UniGene Cluster ID, among which 696 (48%) are overexpressed, and 752 (52%) are underexpressed after celecoxib treatment. Analysis of the distribution of these genes across Gene Ontology categories indicates that immune response, including immune cell activation and inflammation (as part of ''response to wounding'' category; EASE score for the category ''inflammatory response'' by itself is 0.065), response to stress, cell adhesion, and regulation of apoptosis are the main biological processes affected by changes in gene expression in colonic mucosa of patients treated with celecoxib (Supplementary Table S5). Genes controlling cell communication and cell-to-cell signaling are also overrepresented among genes affected by celecoxib, and changes in their activity are likely responsible for shaping the list of most affected molecular functions, which include receptor binding and cytokine and signal transducer activity (Supplementary Table S6). There is also an enrichment in the number of genes whose expression has been changed by celecoxib that encode proteins residing in the plasma membrane and proteins that form the extracellular matrix (Supplementary Table S7).

Discussion

The data show that treatment of patients with celecoxib at recommended clinical doses (200 and 400 mg p.o. bid), in contrast to treatment with placebo, leads to changes in expression of a substantial number of genes in healthy colon.

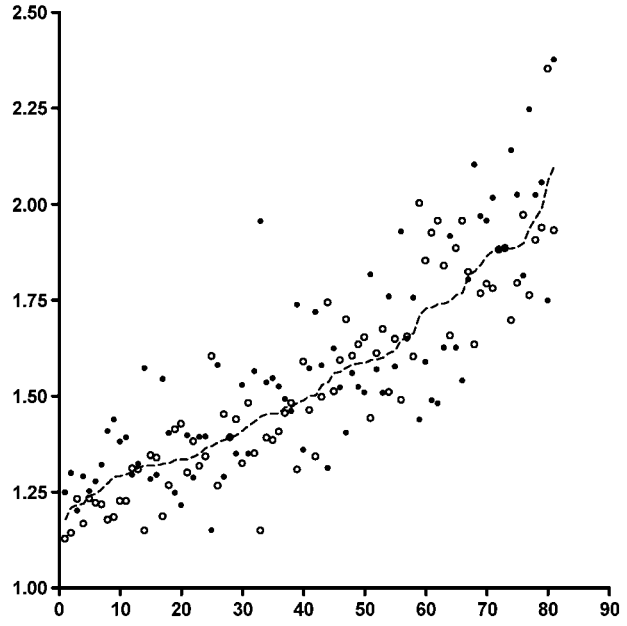

Figure 2. Comparison of the fold differences in expression for 81 genes underexpressed after treatment of patients with celecoxib. *Abscissa,* gene number; *ordinate,* fold difference in gene expression in pretreatment relative to posttreatment biopsies. Eighty-one genes was sorted by mean fold difference in expression (*dash line*). ○, fold difference in patients treated with 200 mg of celecoxib; ●, fold difference in patients treated with 400 mg of celecoxib.

It is generally accepted that NSAIDs and, particularly, COX-2-specific inhibitors prevent colon tumorigenesis by inhibiting tumor cell proliferation and tumor-related angiogenesis, reducing carcinogen activation and inflammation, enhancing immune surveillance, and promoting apoptosis in tumor cells through COX-dependent and COX-independent mechanisms (1, 58-61). However, the relative importance of each of these effects still remains to be established and, in addition, because most of the data supporting these conclusions were obtained in experiments using tumor cells, it is not known which, if any, effects occur in normal colonic mucosa of healthy patients.

The normal colorectal mucosa has three main elements: a single layer of surface epithelial cells forming crypts (composed of absorptive cells, goblet cells, undifferentiated precursor cells, specialized enteroendocrine cells, and rare Paneth cells, which are supported by a thin basement membrane of collagen and other proteins), lamina propria (that extends between crypts and contains many cell types: fibroblasts, lymphocytes, plasma cells, eosinophils, macrophages, and mast cells), and muscularis mucosa. The colonic epithelium is an actively proliferating and dynamic system in which cells originating from stem cells on the bottom of crypts migrate toward the intercrypt table while undergoing differentiation before being shed into the lumen. Standard colon pinch biopsies contain mostly crypt cells (80%), some lamina propria cells, and some colon-associated lymphoid cells. Thus, the observed changes in gene expression after celecoxib treatment may originate from changes in different cells, reflecting changes in a particular cell characteristic, or in the balance of proliferation and differentiation processes in colonic mucosa.

In this study, we only considered a subset of the total set of human genome transcripts. It is possible that a study of a larger number of transcripts might reveal genes more significantly up-regulated and down-regulated. However, even with this limited set, some interesting relationships emerge:

Immune Response, Inflammation, and Angiogenesis. Among the 103 genes belonging to the Gene Ontology category ''immune response'' and affected by celecoxib, 22 are overexpressed, and 81 are underexpressed in posttreatment biopsies of celecoxib-treated patients (Supplementary Table S8), indicating that immune reactions may be somewhat suppressed in these patients. The disparity in the number of overexpressed and underexpressed genes is even more pronounced for genes controlling the cell defense response (Supplementary Table S9). Of 30 genes affected by celecoxib that belong to Gene Ontology category ''inflammatory response,'' 21 are underexpressed (Supplementary Table S10), again indicating that the inflammatory reaction, especially in the early steps, could be muted in the colonic mucosa of celecoxib-treated patients. Among genes affected by celecoxib treatment, five encode proteins controlling early steps of the inflammation reaction: *CD40* (*TNFSF5*), *LCK* and *zap70* protein kinases, *IL2*, and *IL4*, with all five being underexpressed (Supplementary Tables S4 and S10). Angiogenesis occurs during wound healing and in tumorigenesis, and COX-2-stimulated tumor angiogenesis can influence tumor development not only in late but also in the very early stages (62), whereas suppression of angiogenesis is considered an important component of the cancer-preventing effect of COX-2 inhibitors (reviewed in ref. 63). Nine genes belonging to the ''angiogenesis'' Gene Ontology category are affected by celecoxib, with five being overexpressed, and four being underexpressed (Supplementary Table S11). The hierarchical structure of the Gene Ontology categories does not reflect, naturally, the complexity of regulation of biological processes. For example, an important role in angiogenesis belongs to cell adhesion proteins, particularly to integrins (64, 65). Integrins α_1, α_2, and β_3 are overexpressed in tumor-associated angiogenesis, known to be suppressed by NSAID, and all of them are underexpressed after celecoxib treatment (Supplementary Table S10).

Cell Adhesion. Cell-cell and cell-extracellular matrix adhesion play important roles in the maintenance of mechanical and functional integrity of colonic mucosa and in control of cell polarity, proliferation, and differentiation (66), and proteins implicated in cell adhesion are frequently targeted in colon tumorigenesis (67). Of 77 genes that encode proteins in the ''cell adhesion'' Gene Ontology category, 29 are overexpressed, and 48 are underexpressed after celecoxib treatment (Supplementary Table S13). Forty of these genes (16 overexpressed and 24 underexpressed after celecoxib treatment) encode proteins that target focal contacts, cellular structures linking intracellular actin cytoskeleton to extracellular matrix at points of cell contacts with substratum (Supplementary Table S1).

Analysis of possible effects of changes in expression of these 40 genes done with the PathwayAssist 2.5/MedScan program (Ariadne Genomics, Inc., Rockville, MD) and then manually reviewed indicates that most of the genes overexpressed after celecoxib treatment encode proteins that inhibit formation of focal contacts, whereas genes underexpressed by celecoxib encode mainly proteins that stimulate formation of focal contacts. Cell signaling, including signaling involved in reorganization of cell cytoskeleton and focal contacts, is mainly controlled by a number of different intracellular protein tyrosine kinases and usually does not require changes in gene expression and may supersede such changes (68). Nevertheless, if celecoxib-induced changes in gene expression are translated into corresponding changes in protein quantities and activities, the cumulative effect of celecoxib treatment is most likely the inhibition of cell-cell and cell-extracellular matrix adhesion in colonic mucosa. The observation that subapoptotic doses of sulindac sulfide cause loss of focal adhesion contacts and reorganization of the actin cytoskeleton in human colon carcinoma cells (69) supports this possibility.

Transforming Growth Factor-β Signaling Pathway. The genes for both type I and type II transforming growth factor-β

(TGF-β) receptors and bone morphogenetic protein receptors are overexpressed and so is the gene for SMAD4, the common mediator of TGF-β receptor signaling, whereas the gene for SMAD7, an antagonist of TGF-β/bone morphogenetic protein receptor type I signaling, is underexpressed after celecoxib treatment (Supplementary Table S14). Increased activity of the TGF-β receptor signaling pathway may be responsible for the observed overexpression of the gene for SRY-interacting protein (SIP1), a transcription factor known to be induced by TGF-β signaling (70). It is interesting that rat epithelial cells genetically engineered to overexpress COX-2 have reduced TGF-β receptor type II level, which can be increased by treatment of the cells with sulindac sulfide (71).

Obviously, observed changes in gene expression are mediated by changes in the activity of transcription factors, with many genes encoding transcription factors also being affected by celecoxib treatment.

Cell Proliferation and Apoptosis. There are 139 genes in the Gene Ontology category ''cell proliferation,'' which are affected by celecoxib treatment (Supplementary Table S15), although the enrichment of affected genes of this category is not significant according to EASE analysis (EASE score 0.45). Among them, 80 are overexpressed, and 59 are underexpressed in posttreatment biopsies of celecoxib-treated patients, reflecting modulation of the balance between cell proliferation and differentiation rather then stimulation of cell proliferation. Consideration of genes implicated in cell death and apoptosis regulation leads to the same conclusion: 25 of the genes encoding apoptosis-inducing and antiapoptotic proteins are induced, and 35 are underexpressed after celecoxib treatment (Supplementary Table S16). It is interesting that genes encoding proteins that are involved in induction of apoptosis by extracellular signals (*DAP3, DPF2*, and *MAP3K5*) are induced by celecoxib, whereas genes encoding proteins implicated in induction of apoptosis via death domain receptors [*BID, FADD*, and *TNFRSF10A* (*DR4*)] are suppressed by celecoxib.

The results show that celecoxib, at clinically approved doses, and in contrast to placebo, induces changes in expression of a substantial number of genes. The targeted biological processes fit reasonably to the current models of cancer preventive action of NSAIDs. In fact, a comparison of gene expression changes induced by celecoxib in normal colonic mucosa with changes in gene expression observed in tumor cells in culture treated with NSAID reveals similarly affected genes. Among 10 known genes whose expression was changed after treatment of human rectal carcinoma cells with NSAIDs (72), two that were induced by the COX-2 inhibitor NS-398 are found in the list of 1,448 genes affected by celecoxib, and both are overexpressed in posttreatment biopsies of celecoxib-treated patients. Three genes induced by celecoxib treatment are present in both our list of 1,448 genes and the list of 31 known (with UniGene ID) genes induced by treatment of human colorectal cell lines with sulindac sulfide (73). Even when the observed gene expression changes were compared with changes induced by celecoxib treatment of a human mammary epithelial cell line (obviously with significant tissue-specific differences in gene expression compared with colonic mucosa cells; ref. 74), there were still 31 similarly reacting genes (21 induced and 10 suppressed). In experiments with cultured tumor cells, the conditions of treatment with NSAIDs are usually selected to achieve significant if not complete cell growth arrest and eventually apoptotic cell death. Gene expression changes observed under such conditions define cancer therapeutic effects of NSAIDs rather than possible cancer preventive action.

We have recently completed a study of patterns of gene expression in human colonic aberrant crypt foci (ACFs). ACFs are considered to be an early preneoplastic lesion in the colon (75), and the ability to decrease the frequency of ACF after carcinogen treatment is used routinely in experimental models of colon cancer to estimate the cancer preventive effect of different agents (76). It is particularly well established that NSAIDs (77-79) and celecoxib (80-83) decrease the frequency of carcinogen-induced ACFs in the colon in animal models. It has also been shown that the number of ACF in patients who received sulindac sulfide is decreased (84). It seems that we can distinguish ACFs from normal colonic mucosa by the pattern of gene expression. Among the 90 genes for which changes in expression in ACF compared with normal colonic mucosa were identified by SAM (false discovery rate = 9.9%, $\Delta = 0.547$) as significant, 22 genes are present in the list of 1,584 genes whose expression was affected by celecoxib treatment. However, for 21 of 22 genes, the observed changes in expression after celecoxib treatment and in ACF compared with normal mucosa were in the opposite direction: genes that were overexpressed in ACF were underexpressed after celecoxib treatment and vice versa (Table 6 in the accompanying article). It is possible that the cancer preventive effects of celecoxib are directly reflected by the alterations in the expression of a subset of genes required in early stages of cellular transformation.

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