Functional Characterization of Cyclooxygenase-2 Polymorphisms

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ABSTRACT

Cyclooxygenases (COX)-1 and -2 are the key enzymes in the conversion of arachidonic acid to prostaglandins. COX-2 appears to play an emerging role in inflammation and carcinogenesis. Nonsteroidal anti-inflammatory drugs (NSAIDs) are used for the treatment of numerous diseases and reduce the risk of developing colorectal cancer. Polymorphisms in the COX-2 gene could alter enzyme expression, function, and/or the response to NSAIDs. Therefore, they could modify individual risks for developing cancer and other diseases or the occurrence of side effects or sensitivity toward selective or nonselective COX inhibitors. We sequenced the COX-2 gene of 72 individuals and identified rare polymorphisms in the promoter and the coding region. A COX-2 molecular model was used to locate the coding region polymorphisms relative to functional sites in the protein, and the COX-2 V511A polymorphism was very near to the active site. This variant protein was expressed, and function was evaluated, but no difference was detected in metabolism of the COX-2 substrates, arachidonic acid, linoleic acid, and 2-arachidonyl glycerol, compared with the wild type. The $K_m$ values for arachidonic acid showed no differences between the COX-2 wild type and V511A mutant. Inhibition with selective or nonselective COX inhibitors was essentially the same for the two enzymes. The absence of functionally important polymorphisms in the COX-2 gene may suggest that there has been selective pressure against those single nucleotide polymorphisms because of the critical role of this enzyme in maintenance of homeostasis.

Cyclooxygenases (COX)-1 and -2 are the key enzymes in the conversion of arachidonic acid to prostaglandin (PG) H$_2$, the precursor of a diverse family of bioactive lipid mediators including PGs, thromboxane, and prostacyclin (Hamberg and Samuelsson, 1973). The human COX-1 gene, mapped to chromosome 9q32-q33.3, is about 22 kilobase pairs in size and contains 11 exons (Yokoyama and Tanabe, 1989; Kosaka et al., 1994). The human COX-2 gene, mapped to chromosome 1q25.2–q25.3, is about 8.3 kilobase pairs in size and contains 10 exons (Kosaka et al., 1994). COX-1 and -2 play key physiological roles in blood clotting, renal function, and maintenance of gastrointestinal integrity, and also participate in pathophysiological processes like inflammation, arthritis, and pain (reviewed in Dubois et al., 1999). COX-2 activity is primarily responsible for PG synthesis in the central nervous system and inflammatory cells. It is involved in pathophysiological responses including inflammation, arthritis, and pain (reviewed in Dubois et al., 1998). COX-2 also appears to play a role in tumor biology. High levels of COX-2 mRNA and protein were found in surgically resected colorectal adenocarcinomas (Eberhart et al., 1994; Kargman et al., 1995; Sano et al., 1995; Kutchera et al., 1996) and other epithelial tumors including gastric (Ristimaki et al., 1997), breast (Hwang et al., 1995; Kutchera et al., 1996) and other epithelial tumors including gastric (Ristimaki et al., 1997), breast (Hwang et al., 1995), and hepatocellular carcinomas (Shiota et al., 1998), lung (Wolff et al., 1998), esophageal (Zimmermann et al., 1999), and hepatocellular carcinomas (Shiota et al., 1999).

Nonsteroidal anti-inflammatory drugs (NSAIDs), like aspirin, which inhibit PG formation by inactivating COX (Vane and Botting, 1987), are used for the treatment of a wide variety of diseases. In the area of disease prevention, it has induced by a variety of mediators including cytokines, growth factors, tumor promoters (Kujubu et al., 1991; Harrison et al., 1994; Rimarachin et al., 1994), and UVB irradiation (Buckman et al., 1998). COX-2 activity is primarily responsible for PG synthesis in the central nervous system and inflammatory cells. It is involved in pathophysiological responses including inflammation, arthritis, and pain (reviewed in Dubois et al., 1998). COX-2 also appears to play a role in tumor biology. High levels of COX-2 mRNA and protein were found in surgically resected colorectal adenocarcinomas (Eberhart et al., 1994; Kargman et al., 1995; Sano et al., 1995; Kutchera et al., 1996) and other epithelial tumors including gastric (Ristimaki et al., 1997), breast (Hwang et al., 1998), lung (Wolff et al., 1998), esophageal (Zimmermann et al., 1999), and hepatocellular carcinomas (Shiota et al., 1999).

Nonsteroidal anti-inflammatory drugs (NSAIDs), like aspirin, which inhibit PG formation by inactivating COX (Vane and Botting, 1987), are used for the treatment of a wide variety of diseases. In the area of disease prevention, it has

ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; NSAID, nonsteroidal anti-inflammatory drugs; 2-AG, 2-arachidonyl glycerol; DFU, 5,5-dimethyl-3-(3-flourophenyl)-4-(4-methylsulphonyl)phenyl-2(5H) furanone; PCR, polymerase chain reaction; TBS-T, Tris-buffered saline-Tween 20 0.05%; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; SNP, single nucleotide polymorphism; dbSNP, data base of single nucleotide polymorphisms.
been established that regular intake of NSAIDs reduces the risk of developing colorectal cancer (Giovannucci et al., 1994) and Alzheimer’s disease (reviewed in O’Banion, 1999). Besides the therapeutic effects, as many as 25% of individuals using NSAIDs experience some type of side effects, like gastrointestinal or renal dysfunctions (reviewed in Dubois et al., 1998). Whereas the therapeutic effects are attributed to COX-2 inhibition, the side effects are credited to the inhibition of COX-1 (Crockett, 1997; Needleman and Isakson, 1997). Therefore, specific COX-2 inhibitors, like celecoxib and rofecoxib, were developed that have an equivalent effect on pain relief and inflammation than nonselective NSAIDs but have reduced occurrence of side effects (Brooks and Day, 2000). Recent animal studies showed that selective COX-2 inhibitors seem to have antitumorigenic properties. In a rat tumor initiation/promotion model system, celecoxib significantly suppressed the incidence and multiplicity of adenocarcinomas of the colon (Reddy et al., 2000). Furthermore, celecoxib was found to be safe and effective for the prevention and regression of adenomas in a mouse model of adenomatous polyposis (Jaboy et al., 2000).

The present work is motivated by the possibility that genetic variation in the COX-2 gene could alter enzyme expression levels or biochemical function and consequently have an impact on prostaglandin biosynthesis. Therefore, polymorphisms might modify the individual risk of inflammatory disease, tumor incidence, or tumor malignancy. A second possibility is that COX-2 polymorphisms could change the response to NSAIDs resulting in decreased or increased sensitivity to selective or nonselective COX inhibitors. To investigate these hypotheses, we searched for polymorphisms by sequencing the COX-2 gene in 72 individuals of three different ethnic groups: Africans, Asians, and European/Caucasians. Selected polymorphisms in COX-2 were evaluated for functional impact using in vitro assays.

### Experimental Procedures

**Chemicals.** Arachidonic acid and 2-arachidonyl glycerol (2-AG) were purchased from Cayman Chemical (Ann Arbor, MI). Linsolitic acid was purchased from Nu-Chek Prep Inc. (Elysian, MN). Indomethacin was purchased from Sigma (St. Louis, MO). 5,5-Dimethyl-3-(3-flourophenoxy)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU) was kindly provided by Merck (Whitehouse Station, NJ); SC-58125 and celecoxib were kindly provided by Monsanto (St. Louis, MO). All additional chemicals utilized (unless otherwise noted) were purchased from Sigma-Aldrich (St. Louis, MO). All additional chemicals utilized (unless otherwise noted) were purchased from Sigma-Aldrich (St. Louis, MO). All additional chemicals utilized (unless otherwise noted) were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** The human COX-deficient colon carcinoma cell line HCT-116 was obtained from American Type Culture Collection (Manassas, VA). It was cultured in McCoy’s 5A medium supplemented with 10% fetal calf serum and gentamicin (1 mg/100 ml; Invitrogen, Carlsbad, CA). Cells were grown as a monolayer culture at 37°C in 5% CO₂.

The insect cell line S9 (from Spodoptera frugiperda) was obtained from BD PharMingen (San Diego, CA). It was grown in suspension culture in supplemented Grace’s insect medium (Invitrogen) containing 10% fetal calf serum, 1% Pluronic F-68 (Invitrogen) and antibiotics at 27°C.

**Sequence Analyses.** Genomic DNA was extracted from 72 human lymphoblastoid cell lines (Coriell Institute, Camden, NJ) obtained from healthy individuals with ancestries as follows: 24 African (16 African-Americans, 8 African Pygmies), 24 Asian (5 Indo-Pakistanis, 5 native Taiwanese, 5 mainland Chinese, 3 Cambodians, 3 Japanese, 3 Melanesians), and 24 European/Caucasian (9 European-Americans (Utah), 5 Druze (Lebanon), 5 Aygjei (eastern Europe), 5 from Moscow). This diverse sample of genomes was used to maximize discovery of polymorphisms (Cargill et al., 1999). Given the large degree of genetic diversity in human populations, difficulties in defining appropriate populations for sampling, and the small number of genomes sampled, the frequency data reported for any group (Table 1) must be considered an approximation. Sequence analyses were performed by the Lawrence Livermore National Laboratory under the supervision of Dr. H. Mohrenweiser under U.S. Interagency agreement (YI-ES-8054-05). The primer sequences and PCR conditions used for resequencing eight overlapping PCR products of the COX-2 gene can be found at the following website (http://manuel.niehs.nih.gov/egsnp/home.htm).

**Cloning, Site-Directed Mutagenesis, and Subcloning of COX-2 cDNA.** The full-length human COX-2 cDNA was cloned into pCDNA3.1 (Invitrogen) by reverse transcriptase-PCR as previously described (Hsi et al., 2000). The COX-2 V511A polymorphism was generated by in vitro mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The primer sequences containing the mutation were 5’-GGAGAATTCTGAGCTGGAGCACCATTCTCC-3’ for the forward and 5’-GGAGAATTTGCTCCGCTCTTCTACCATG-3’ for the reverse primer. Escherichia coli XL1 blue supercompetent cells were heat transformed with the mutagenesis reaction, plated out on LB agar plates containing 100 μg/ml ampicillin, and grown overnight. Ampicillin-resistant colonies were grown in LB-ampicillin broth overnight, and plasmid DNA was isolated with the QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA). The COX-2 insert containing the desired mutation with no secondary misincorporation was grown overnight in 250 ml of LB broth containing 100 μg/ml ampicillin and isolated with the QIAGEN Plasmid Maxi kit (QIAGEN) for transfection into HCT-116 cells. Wild type and V511A mutant COX-2 inserts were subcloned into baculovirus transfer vector pAcEG2 for expression in S9 cells.

### Table 1

Polymorphisms in the COX-2 gene (GenBank accession no. U04636)

<table>
<thead>
<tr>
<th>SNP Name</th>
<th>Location</th>
<th>Nucleotide Number (Accession no. U04636)</th>
<th>Polymorphism</th>
<th>Amino Acid Exchange</th>
<th>Amino Acid Position</th>
<th>Estimated Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-162G&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Promoter</td>
<td>671</td>
<td>C&gt;G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T10G&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-UTR</td>
<td>841</td>
<td>T&gt;G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R228H&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Exon 6</td>
<td>4383</td>
<td>G&gt;A</td>
<td>Arg→His</td>
<td>228</td>
<td>0.063 (3/48) 0 0</td>
</tr>
<tr>
<td>V511A&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Exon 10</td>
<td>6620</td>
<td>T&gt;C</td>
<td>Val→Ala</td>
<td>511</td>
<td>0.083 (4/48) 0.063 (3/48) 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> 5'-UTR, 5'-untranslated region.
<sup>b</sup> Only SNPs in the promoter region, the 5'-UTR, or polymorphisms causing AA exchanges in the protein shown.
<sup>c</sup> Number represents nucleotide position relative to the transcriptional start site.
<sup>d</sup> Number represents amino acid position.
Expression of Human COX-2 in HCT-116 and Sf9 Cells. HCT-116 cells were plated in 100-mm tissue culture plates at 3 × 10^6 cells/plate. After growing for 20 h, 10 μg of pcDNA3.1-COX-2 wild type and V511A were transfected with 50 μl of LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. After 5 h, serum-containing medium was added to the transfection supernatant. Cells were harvested 24 h after transfection in 1 ml of 100 mM Tris, pH 8.0, containing protease inhibitors (Sigma) and sonicated 2 × 20 s.

Sf9 cells were plated in 60-mm tissue culture dishes at 2 × 10^6 cells/dish. They were cotransfected with 0.5 μg of Baculogold linearized baculovirus DNA (BD Pharmingen) and 5 μg of pAcSG2 containing the human COX-2 inserts according to the manufacturer’s instructions. From the transfection supernatants, plaque assays were performed to identify recombinant viral clones. Therefore, a wide range of viral dilutions was infected, and plaques were visualized on agarose overlays. Five days later, a second agarose overlay was applied containing 0.1 mg/ml neutral red. On the 6th day, clear plaques could easily be visualized against a red cell background. Clear plaques were picked without further plaque purification and amplified once before expression was assessed by Western blot analysis on whole cell lysates. Recombinant clones were amplified in Sf9 monolayer cultures at a multiplicity of infection <1, and viral titers were estimated by endpoint dilution assay (Crosen and Gruenwald, 1999). For large scale COX-2 expression, 3 liters of Sf9 cells at 1.5 × 10^9 cells/ml were infected with viral stock at a multiplicity of infection of 20 and harvested at a cell viability of 50 to 70%.

High-Pressure Liquid Chromatography. COX-2 wild type and V511A mutant catalytic activity in transiently transfected HCT-116 cells was performed using [3H]arachidonic acid as a substrate as described previously (Hsi et al., 2000).

Western Blot. For Western blot analysis, lysed HCT-116 cells or baculovirus expressed, solubilized COX-2 enzyme was normalized for protein (BCA protein assay; Pierce Chemical, Rockford, IL), bands were visualized by exposure to Hyperfilm-MP (Amersham Pharmacia Biotech), and bands were visualized.

COX-2 Enzyme Preparation. Infected Sf9 cells were centrifuged for 10 min at 4°C at 2000 rpm in a Sorvall centrifuge (Newton, CT). Cell pellets were washed with cold phosphate-buffered saline and centrifuged again with the same conditions as described above. After removing the supernatant, cell pellets were frozen at −80°C for cell lysis and afterward thawed on ice in a 80 mM Tris, 2 mM EDTA buffer, pH 7.2, containing 0.1 mM diethyldithiocarbamate and protease inhibitors (Sigma-Aldrich). Cell lysate was ultracentrifuged 45 min at 4°C at 40,000 rpm and the pellet was homogenized in 200 mM Tris, 0.1 mM EDTA, pH 8.0, containing 0.4% CHAPS. Homogenate was stirred for 1 h at 4°C bringing the CHAPS concentration to 1% (w/v) and subsequently ultracentrifuged 45 min at 4°C at 40,000 rpm. The supernatant contained solubilized COX-2 enzyme and was stored in aliquots at −80°C (J. J. Prusakiewicz, personal communication).

COX-2 Enzymatic Assay. COX-2 activity was determined by measuring oxygen consumption at 37°C in an oxygraph chamber with an oxygen electrode. The reaction buffer consisted of 100 mM Tris, pH 8.0, with 500 mM phenol. For estimating the K_m for arachidonic acid, the solubilized COX-2 wild type (600 μg of total protein) and V511A mutant (740 μg of total protein) enzymes were reconstituted for 1 min with 10 μM hematin on ice. Subsequently, they were incubated for 1 min at 37°C in reaction buffer before being challenged with final concentrations of 3, 10, 30, 100, and 200 μM arachidonic acid. Oxygen consumption was monitored for 180 min. Comparisons of substrate oxygenation of arachidonic acid, linoleic acid, and 2-AG were performed under the same experimental conditions as described above. Six hundred micrograms of total protein for each genotype was used for the reactions and incubated with 100 μM each substrate.

For determining the IC_{50} values for the COX inhibitors, indomethacin, DFU, SC-58125, and celecoxib, the experimental conditions were as described above. After reconstitution with hematin, the wild type and V511A mutant enzymes were incubated for 2 min at 37°C in reaction buffer with appropriate concentrations of inhibitors before being challenged with a final concentration of 200 μM arachidonic acid.

Statistical Methods. To evaluate whether the differences in the IC_{50} values of the wild type and the V511A mutant COX-2 for the COX inhibitors (indomethacin, DFU, SC-58125, and celecoxib) were statistically different from each other, we used the following models to fit the data.

\[
\text{indomethacin and celecoxib: } \log(y) = \alpha + \beta x + \gamma x^2 + \epsilon \quad (1)
\]

\[
\text{SC-58125: } \log(y) = \alpha + \beta \sqrt{x} + \gamma x + \epsilon \quad (2)
\]

\[
\text{DFU: } \log(y) = \alpha + \beta \sqrt{x} + \epsilon \quad (3)
\]

In each of the above models, \( \epsilon \) is the random error associated with the model. We assume that \( \epsilon \) is normally distributed with constant variance across all observations. This assumption appears to be valid for the given data.

The formula for the IC_{50} values depends upon the model used to fit the data.

For model (1):

\[
\text{IC}_{50} = \frac{-\beta - \sqrt{\beta^2 - 4\gamma \ln(2)}}{2\gamma}.
\]

For model (2):

\[
\text{IC}_{50} = \frac{\left(\frac{\beta + \sqrt{\beta^2 - 4\gamma \ln(2)}}{2\gamma}\right)^2}{\beta}.
\]

For model (3):

\[
\text{IC}_{50} = \frac{(\log(2))^2}{\beta}.
\]

Results

Sequencing of the Human COX-2 Gene. The COX-2 gene was sequenced in 72 individuals (144 chromosomes), and 20 differences from the published sequence (GenBank accession number U04636) were detected. The full set of polymorphisms can be accessed at the National Institute of Environmental Health Sciences website listed under Experimental Procedures. The majority of polymorphisms identi-
identified were intronic or synonymous changes where functional effects are not likely. Table 1 displays four polymorphisms with potential impact on the COX-2 phenotype. Two polymorphisms in the promoter region, C-162G and T10G, were not located in any known transcriptional regulatory elements but could potentially impact transcription. Preliminary expression studies of these variants using luciferase reporter plasmids in the presence and absence of known COX-2 inducers were unpromising (data not shown).

As a first step toward evaluation of polymorphisms within the coding region, we located the two polymorphisms leading to amino acid changes in the molecular model of the COX-2 protein. The model was developed by Dr. R. J. Bienstock, National Institute of Environmental Health Sciences (unpublished communication), based on the published structure of the murine COX-2 protein (Kurumbail et al., 1996; Fig. 1). The R228H polymorphism is positioned on the surface of the protein without being close to any functionally significant site. While this change could, in principle, affect contact with other proteins, we did not investigate this further. However, since the V511A polymorphism lies very close to the active site of COX-2, we choose to carry out further analyses focusing on whether this variant protein was functionally different from the wild-type protein.

**COX-2 Wild Type and V511A Expression in HCT-116 Cells.** To evaluate the function of COX-2 wild type and

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**Fig. 1.** Three-dimensional model of the murine COX-2 enzyme. The R228H and V511A polymorphisms are marked. V509I and R513H, which contribute to inhibition behavior and substrate specificity in vitro, are also highlighted.
V511A variant proteins, we expressed both alleles in COX-deficient HCT-116 cells. Figure 2A shows the Western blot of cell lysates from transiently transfected HCT-116 cells. High-pressure liquid chromatography analyses of arachidonic acid metabolism show that both proteins produced similar amounts of primary metabolite, PGE2, and other minor metabolites (Fig. 2B). Thus, it appears that the V511A polymorphism does not result in an inactive COX-2 enzyme or change the metabolite profile.

**Baculovirus Expression of COX-2 Wild Type and V511A Polymorphism.** The expression in HCT-116 cells did not permit complete analysis of potential differences in activity between wild type and V511A COX-2. Therefore, the enzymes were expressed in the baculovirus expression system that yields high levels of recombinant protein. A Coomassie Blue staining of the S9 solubilized protein fraction shows that the main protein of this fraction was COX-2 (Fig. 3A). Therefore, analysis proceeded with no further enzyme purification.

Densitometry on the Western blot of the large scale COX-2 enzyme preparation indicated a small difference in COX-2 expression for the wild type and the V511A mutant enzyme (Fig. 3B). Comparison with the COX-2 standard allowed for an estimation of 53 ng of COX-2 wild type and 64 ng of COX-2

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**Fig. 2.** A. Western blot from cell lysates of HCT-116 cells transiently transfected with vector, COX-2 wild type (wt), or COX-2 V511A mutant (V511A). Ten nanograms of COX-2 standard, 30 μg of total protein/lane. B, high-pressure liquid chromatography analyses of arachidonic acid metabolism in the same cell lysates used for the Western blot in A.
V511A mutant within 100 ng of total solubilized protein. The enzymatic assays were normalized to O$_2$ uptake and COX-2 protein expression.

Substrate Spectrum and Enzymatic Activity of COX-2 Wild Type and V511A Mutant. An oxygraph was used to evaluate the rate of metabolism for the three known COX-2 substrates, arachidonic acid, linoleic acid, and the recently discovered 2-AG (Kozak et al., 2000). We showed that 1 μg each of the wild type and the mutant enzyme metabolized 100 μM each substrate at a comparable rate and extent (Fig. 4). These data agree with previous results that arachidonic acid and 2-AG are comparable substrates (Kozak et al., 2000) and that linoleic acid is a poorer substrate for COX-2 (Laneuville et al., 1995). No differences in COX-2 activity between the wild type and V511A proteins could be detected during the incubations (180 min), indicating similar stability for both proteins.

A detailed analysis of the $K_m$ values for the main COX-2 substrate, arachidonic acid, was performed. The estimated $K_m$ values for COX-2 wild type were 9 μM and the mutant enzyme 10 μM. These show that the activity of the two enzymes for the substrate arachidonic acid is the same. These $K_m$ values are in the same range as reported by others (Kozak et al., 2000).

Inhibition of COX-2. The sensitivity of wild type and V511A mutant COX-2 to inhibition by nonselective and selective COX-2 inhibitors was examined. We selected indomethacin as the prototype for nonselective COX inhibitors. For selective COX-2 inhibitors, we chose celecoxib, a commercially available drug, and SC-58125, a derivative of celecoxib. In a reported study, the selectivity of SC-58125 for COX-2 versus COX-1 could be reversed by introducing the V509I mutation into the COX-2 enzyme (Gierse et al., 1996). The other therapeutically used drug, rofecoxib, was not available to us. Therefore, we used its analog, DFU. Figure 5, A to D, shows the inhibition curves for the COX-2 wild type and V511A mutant. Each data point represents three individual measurements. From these data, the IC$_{50}$ values for the different genotypes and inhibitors were calculated (Table 2). The IC$_{50}$ values determined for the COX-2 wild type and V511A mutant with respect to the four drugs did not differ significantly from each other.

Discussion

The possibility that single nucleotide polymorphisms (SNPs) may be useful in identifying candidate disease genes and individuals at risk of disease has led to extensive projects aimed at discovery and organization of SNPs into databases. As of February 2001, ~2 million candidate SNPs were available in the public dbSNP databases (Sachidanandam et al., 2001) suggesting that if the promise of polymorphism analysis is realized, we are entering into an "era of personalized medicine" (Kwok and Gu, 1999). Far less has been done to functionally characterize polymorphisms in coding regions. The present study aimed to find and phenotypically characterize COX-2 polymorphisms that might be associated with disease or with individual responses to drug therapies.

Three observed SNPs, C-162G, T10G, and V511A, were also identified in other SNP discovery projects (P. J. Oefner, unpublished data; A. Halushka et al., 1999) and are present in the National Center for Biotechnology Information dbSNP database (http://www.ncbi.nlm.nih.gov/ SNPs/). The estimated allele frequencies for the C-162G, T10G, and V511A variant alleles (Table 1) were similar to the other projects (P. J. Oefner, personal communication; Halushka et al., 1999). Multiple reports of the same variant provide validation for detection of SNPs. The COX-2 R228H polymorphism has not been reported by any other group but was observed in one Asian chromosome in our population. Although the allele frequency estimates displayed in Table 1 are very approximate, each of these variants are relatively uncommon.

The inhibition of COX-2 is one likely mechanism by which NSAIDs are associated with a reduced incidence of and mortality from sporadic adenoma and colon cancer in epidemiologic and rodent studies (reviewed in Janne and Mayer, 2000). Altered function of COX-2 was hypothesized to change the number of colonic polyps in patients with an inherited predisposition to colon cancer, and two COX-2 gene sequencing studies in patients with adenomatous polyposis have been reported. Among patients with attenuated adenomatous polyposis coli, a silent G/C polymorphism in exon 3 was reported at a frequency of 0.098 (nucleotide number 2191 of GenBank accession number U04636), but no association with polyp number in this disease group was observed (Spirio et al., 1998). This polymorphism was also observed at an allele.
frequency of 0.269 in a group of familial adenomatous polyposis patients from Switzerland. No association was found between the polymorphism and development of extracolonic manifestations of the disease or total colonic polyp number (Humar et al., 2000). We found this mutant allele with a frequency of 0.104 in the Caucasian part of our population (data not shown). Functional characterization for this variant was not pursued. Other SNPs in COX-2 were reported by Humar et al. (2000), but there has been no suggestion that these SNPs play a role in the inherited predisposition to colon cancer. Possible associations between COX-2 polymorphisms and other diseases have not been investigated.

Besides the discovery of SNPs in the COX-2 gene, various studies on site-directed mutagenesis of COX-2 have been published to elucidate the function and inhibition of the enzyme. Gierse et al. (1996) aligned human COX-1 with the structure of human COX-2 to evaluate specific residues for their contribution to the selective inhibition of compounds. A number of amino acid differences at the mouth of the cyclooxygenase substrate channel and in the active site were

![Fig. 4. Comparison of metabolism of COX-2 wild type (wt) and COX-2 V511A mutant (V511A) for 100 μM each of the substrates arachidonic acid (AA), linoleic acid (LA), and 2-AG measured by oxygen consumption per minute per microgram of COX-2 with an oxygen electrode as described under Experimental Procedures.]

![Fig. 5. Inhibition of arachidonic acid metabolism of COX-2 wild type (wt) and V511A mutant (V511A) by indomethacin (A), DFU (B), SC-58125 (C), and celecoxib (D). Values determined by measuring oxygen consumption per minute per microgram of total protein with an oxygen electrode as described under Experimental Procedures and shown as percentage of control (% control). Each data point represents the mean of three independent measurements.]

revealed. The corresponding amino acid to isoleucine at position 523 in COX-1 is a valine at position 509 in COX-2, located in the active site of the enzyme. The single amino acid exchange V509I confers selectivity of COX-2-specific inhibitors in the class of SC-58125, whereas conventional NSAIDs such as indomethacin showed no change in selectivity (Gierse et al., 1996). The V511A polymorphism observed and tested in this study is located two amino acids away from the important 509 site (Fig. 1). We compared the inhibition of the COX-2 V511A protein with the COX-2 wild-type protein by NSAIDs and observed no effect on COX-2 inhibition by indomethacin, SC-58125, DUF, or celecoxib. The reason may be in the orientation of the amino acid 511. Compared with the 509 site, the 511 residue points away from the active site and, therefore, probably does not contribute to the active site. Another location that is positioned very closely to the V511A site is position 513, which is part of the COX-2 side pocket. This side pocket is the binding site for the sulfonamide or sulfone groups of the COX-2-selective inhibitors, celecoxib, and rofecoxib. The triple mutant COX-2 V523I/R513H/V434I, which represents the major side pocket differences between COX-1 and -2, showed another function of this site as it promotes efficient metabolism of the newly discovered COX-2 substrate, the endocannabinoid 2-AG (Kozak et al., 2000). The investigation of the metabolism of 2-AG compared with the known COX-2 substrates arachidonic and linoleic acid for the COX-2 wild type and V511A polymorphism demonstrated that there was no difference in O$_2$ uptake between the two. The COX-2 V511A polymorphism plays no important role in the formation of the COX-2-specific side pocket and, therefore, does not participate in COX-2-specific inhibition or metabolism of 2-AG.

In conclusion, the COX-2 V511A polymorphism investigated here did not have any impact on COX-2 activity, COX-2 inhibition by COX-2-specific inhibitors, or substrate preference in these systems. Therefore, it is unlikely that it has any impact on modification of the risk of cancer or other diseases. Neither hypothesis, that polymorphism in the COX-2 gene could contribute to individual susceptibility to cancer nor that COX-2 polymorphisms may have an impact on individual reaction to NSAIDs, appears to be supported. The absence of functionally important polymorphisms in the COX-2 gene may suggest that there has been selective pressure against those SNPs because of the high importance of this enzyme in maintenance of body homeostasis. We suggest that SNPs in other genes of the prostaglandin synthesis pathway, like phospholipases, specific prostaglandin synthases/isoenzymes, or possibly drug-metabolizing enzymes may be responsible for the interindividual differences in susceptibility to cancer and reactions toward NSAIDs. More studies are necessary to elucidate the association between the cancer preventive properties or side effects of NSAID use and individual genotypes.

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References


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