**p21<sup>WAF1/cip1</sup> Is an Important Determinant of Intestinal Cell Response to Sulindac in Vitro and in Vivo**

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**INTRODUCTION**

Sulindac, a nonsteroidal anti-inflammatory drug, inhibits intestinal tumorigenesis in humans and rodents. Sulindac induced complex alterations in gene expression, but only 0.1% of 8063 sequences assayed were altered similarly by the drug in rectal biopsies of patients treated for 1 month and during response of colorectal cells in culture. Among these changes was induction of the cyclin-dependent kinase inhibitor, p21<sup>WAF1/cip1</sup>. In Apc<sup>1638<sup>WAF1/cip1</sup></sup> mice, targeted inactivation of p21<sup>WAF1/cip1</sup> increased intestinal tumor formation in a gene-dose-dependent manner, but inactivation of p21 completely eliminated the ability of sulindac to both inhibit mitotic activity in the duodenal mucosa and to inhibit Apc-initiated tumor formation. Thus, p21 is essential for tumor inhibition by this drug. The array data can be accessed on the Internet at http://sequence.aecom.yu.edu/genome/.

**MATERIALS AND METHODS**

Ten biopsies of rectal mucosa 10–15 cm from the anal verge were taken from each of three patients at risk for colon neoplasia because they had previously had adenomatous polyps removed, and another 10 biopsies from each patient were taken after 1 month of oral treatment with 300 mg/day of sulindac. Patients were prepared for the procedures with a small tap water enema. Biopsies were immediately frozen in liquid nitrogen, and RNA was prepared using an RNeasy kit (Qiagen, Valencia, CA). The RNA was used to prepare probes that were labeled with Cy3 dUTP (before sulindac) or Cy5 dUTP (after sulindac; substituted nucleotides from Amersham Pharmacia Biotech), the two probes for each patient were combined and hybridized to a slide containing the PCR-amplified sequences of 8063 genes prepared by the Albert Einstein Microarray facility, as previously described (Ref. 12).4 Processing of the hybridized slides and data analysis were also done as described (12). In brief, signal/background ratio for each spot was calculated for each channel, a ratio of the ratios for each channel was derived, and values for this function of >1.25 or <0.8 were tentatively considered significant changes based on control experiments in which RNA samples from duplicate cell cultures showed that >98% of sequences fall within the 0.8–1.25 range (12). Data analysis was done in part using the Cluster and Treeview programs of Eisen (13). All microarray data as well as details of the AECOM Microarray Facility can be found on the Internet.4

For confirmation of altered p21 expression, SW620 cells were treated by changing the medium to fresh medium (control), or fresh medium containing 1.6 mM sulindac (Sigma Chemical Co., St. Louis, MO), and cells harvested for RNA extraction and Northern blot analysis of p21 expression exactly as described (12). The Apc<sup>p21</sup> mouse model has been reported (14). It was generated by mating Apc<sup>1638<sup>WAF1/cip1</sup></sup> mice (15) with p21<sup>−/−</sup> mice (16) to generate Apc<sup>−/−</sup>/H11021


tumor formation by sulindac. The function of the gene is essential for both the inhibition of mitotic activity in the mucosa as well as the inhibition of Apc-initiated tumor formation by sulindac.

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The abbreviations used are: APC, adenomatous polyposis coli; COX-2, cyclooxygenase-2; cdki, cyclin dependent kinase inhibitor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
p21<sup>−/−</sup> offspring (F1). These F1 mice were then mated to each other to generate F2 mice of appropriate genotype, that, after weaning, were randomized to either AIN-76A diet or AIN-76A containing 0.02% sulindac (Research Diets, New Brunswick, NJ). Animals were fed <i>ad libitum</i>, killed 36 weeks after being placed on diet, and tumors in the gastrointestinal tract scored as previously described (14). Sulindac in the diet had no effect on overall weight gain. This strategy of using littermates of different p21 genotype is used to minimize the chance that affects seen are attributable to loci unlinked to p21. Consistent with this, the group that was <i>Apc</i><sup>−/−</sup> and p21<sup>−/−</sup> developed tumors with the same frequency, intestinal distribution, and histopathology as has been reported a number of times for the parental <i>Apc1638N</i><sup>+/−</sup> strain that is on a homogeneous genetic background (e.g., 15, 17). Apoptotic and mitotic cells in the mucosa were scored as described previously (14).

RESULTS

Microarray analysis of the expression of 8063 cDNA sequences was done using RNA isolated from rectal biopsies of three patients taken before and after 1 month of treatment with 300 mg sulindac/day. As we found for the effects of sulindac <i>in vitro</i> (12), there were extensive changes in gene expression seen in the biopsies from patients after 1 month of sulindac treatment (Fig. 1A). Moreover, there were large differences among the three patients in the extent of altered gene expression. Only 110 sequences (about 1%) exhibited altered expression in two of three patients, and only five sequences (0.06%) responded in all three patients. Thus, each patient showed altered expression of genes in the rectal mucosa after the period of sulindac treatment, but the response of each patient was different.

Each patient could be assayed only once using tissue taken before and after sulindac treatment. It is therefore possible that some of the heterogeneity results either from variability in the analysis or from cellular or biochemical heterogeneity in the biopsies, although each RNA preparation was from 10 pooled biopsies from each patient, both before and after treatment. To explore this issue, these data on the effects of sulindac <i>in vivo</i> were compared with the previous results we had obtained using a well-controlled system <i>in vitro</i>: the time course of response of SW620 colon carcinoma cells to sulindac (12). First, <i>in vitro</i>, sulindac generally reduced the expression of seven genes in the immunoglobulin gene family or that are characteristically expressed in lymphocytes; but these sequences were not expressed, or were unaffected, by sulindac <i>in vitro</i> (Fig. 1B). This most likely reflects the well-known activity of sulindac as an anti-inflammatory drug and, hence, an overall reduction in lymphocytes in the biopsies taken <i>in vivo</i> that does not take place in the homogeneous colon carcinoma cell line <i>in vitro</i>. This is evidence that overall, the microarray analysis accurately reflects the profile of gene expression in the tissue.

To focus on genes that were altered by sulindac, but which did not reflect heterogeneous or altered cell composition in the biopsies, we selected all sequences that were changed in expression by sulindac in at least two of three of the biopsies and that were altered similarly (induced or repressed) by the drug in the SW620 cells in culture (12). Only eight sequences of the >8000 screened (0.1%) satisfied these criteria (Fig. 1C). The identity of each was confirmed by sequence analysis.

One of the sequences that was induced both <i>in vitro</i> and <i>in vivo</i> was the cdki <i>p21<sup>WAF1/cip1</sup></i>. We have also found that sulindac induces <i>p21</i>
in HT29 colon carcinoma cells (data not shown), consistent with the work of others (18). For patients 1 and 3, the ratios of p21 expression in pooled biopsies taken after 1 month of sulindac treatment compared with expression in biopsies taken before treatment were elevated (red color; see “Materials and Methods”). There was no change in expression in patient 2 (ratio 0.94), although the Treeview program assigns a slight green color to the ratio < 1. For the effect of sulindac on SW620 cells in culture, we had the opportunity to confirm the microarray data by standard Northern blot analysis (Fig. 2). Quantitative analysis of the Northern blot data and comparison to the quantitative array data for p21, in both cases standardized for expression of GAPDH, showed that the increase in p21 expression is linear as a function of time over 48 h (P = 0.01 and 0.02 for array and Northern data, respectively). The kinetics of increase determined by the two methods were the same, although by Northern analysis, the overall increase in p21 mRNA was ~6-fold and only 3-fold by array analysis. Although we have found that array analysis generally underestimates the extent of altered gene expression relative to standard Northern analysis, these data again confirm the overall accuracy of the microarray data in reflecting the relative level of gene expression.

The gene expression data therefore provided presumptive evidence that p21 may be important in the response of colonic cells to sulindac. To pursue this more definitively, we generated mice that were Apc<sup>+/−</sup>, to initiate tumor formation, and also either p21<sup>+/+</sup>,<sup>−/+</sup>,<sup>−/−</sup> or <sup>−/−</sup>. Littermates of different genotypes were then randomized to a control AIN76A diet, or the diet was supplemented with 0.02% sulindac, for 36 weeks. The Apc<sup>+/−</sup> mice that were wild type for p21 and fed the control diet for 36 weeks developed an average of 1.67 tumors/mouse, consistent with previous reports (14). There was no apparent difference in the ratio of adenomas to adenocarcinomas before and after sulindac treatment, suggesting that the drug was equally effective on tumors at both stages. However, homozygous inactivation of p21 completely eliminated the ability of sulindac to reduce the incidence of mice with small intestinal tumors or the number of these tumors (Table 1). Interestingly, loss of a single p21 allele was equally effective in eliminating the tumor inhibition elicited by sulindac. Thus, p21 is essential for tumor repression by sulindac.

The sulindac induction of p21 expression, cell lines in culture is associated with both cell cycle arrest and apoptosis (15). Moreover, we have demonstrated previously that the inactivation of p21 in mice is associated with defects in three major pathways of cell maturation in the intestinal mucosa: cell cycle arrest, lineage-specific differentiation, and apoptosis (14). Therefore, it is unclear which of these cell maturation pathways in which p21 plays a role is most important in the tumor inhibition elicited by sulindac. We addressed this by, first, determining the expression of p21 in a number of cell systems in which the inhibition of cell cycling is dissociated from either a differentiation response or the stimulation of apoptosis. Thus, both cycle arrest in G<sub>0</sub>-G<sub>1</sub> and stimulation of apoptosis is seen in SW620 treated with the short-chain fatty acid butyrate (20, 21), but there is no induction of differentiation markers in this cell line in response to sulindac. Furthermore, in Caco-2 cells (22, 23) and clone 16E and clone 19A cells (24), growth arrest and differentiation triggered by contact inhibition do not lead to apoptosis. In each case, p21 expression was induced as part of the genetic reprogramming that characterizes the maturation of the cells (Fig. 3). Thus, these data suggest that the primary pathway regulated by p21 in colon cells is a G<sub>0</sub>-G<sub>1</sub> cell cycle arrest. This is consistent with the fact that p21 is expressed in the intestinal tract coincident with the exit of cells from the proliferative compartment as they migrate up the crypt toward the intestinal lumen (25).

These results led us to hypothesize that sulindac would have a marked effect on the inhibition of cell proliferation in the intestinal mucosa, but not on apoptosis. The data in Table 2 demonstrated that this is correct. With loss of either a single or both p21 alleles, the mice showed significantly increased mitosis and decreased apoptosis in the duodenal mucosa, similar to results we have reported previously (14). In the Apc<sup>+/−</sup>, p21<sup>+/+</sup> mice, where sulindac was effective in reducing tumor formation (Table 1), the drug significantly decreased mitotic
activity, but did not alter apoptosis, in the mucosa (Table 2). This effect of sulindac on decreasing mitotic activity was eliminated in the Apc+/−, p21+/− or −/− mice (Table 2).

DISCUSSION

Microarray analysis of gene expression in colon carcinoma cells in culture treated with sulindac, and of biopsies of patients given sulindac daily for 1 month, identified p21WF16/πp1 as one of only eight sequences that were altered similarly by sulindac both in vitro and in vivo. This was presumptive evidence that p21 was functionally important in tumor inhibition by sulindac. Use of a mouse model that combined a targeted inactivation of Apc, to initiate tumor formation, and inactivation of p21 was then definitive in showing that p21 was necessary for inhibition of Apc-initiated tumor formation by this drug.

The mouse data do not define the level of p21 necessary for the response to sulindac, but from the consistency with which sulindac and other agents that arrest colon cells in G0-G1 induce response to sulindac, but from the consistency with which sulindac was functionally important in tumor inhibition by sulindac. Use of a mouse model that combined a targeted inactivation of Apc, to initiate tumor formation, and inactivation of p21 was then definitive in showing that p21 was necessary for inhibition of Apc-initiated tumor formation by this drug.

The mouse data do not define the level of p21 necessary for the response to sulindac, but from the consistency with which sulindac and other agents that arrest colon cells in G0-G1 induce p21 (e.g., 26), we believe that induction above a threshold level is required. In this regard, it was of interest that inactivation of a single allele of p21 was sufficient to eliminate the tumor inhibitory effects of sulindac. This is reminiscent of the fact that another cdki, p27kip, is haplo-insufficient for tumor suppression (27), and that p53, a principle regulator of p21 expression, also shows gene-dosage effects (28). Therefore, the level of p21 in tumor promotion and inhibition appears to be critical, and the data suggest that in the heterozygous mice (i.e., p21+/−), sulindac is not able to induce expression to levels sufficiently high to inhibit tumor formation. However, because p21 expression is restricted to cells that have exited the proliferative compartment (25), this will have to be studied by methods that are highly quantitative for p21 level and activity on a single-cell basis.

The identification of the cdki p21WF16/πp1 as an essential element in the response to sulindac is consistent with earlier reports that showed the induction of p21 in cells in culture after sulindac treatment (18, 29). The role of p21 that we have revealed in both tumor formation (14) and inhibition is significant in regards to the fact that p21 expression is restricted to nondividing cells in the normal crypt (25). p21 therefore plays an important role in intestinal cell maturation and homeostasis. This is consistent with the fact that the essential pathway in tumor initiation by Apc is elevation in β-catenin-Tcf complex formation and activity (30–32). c-myc is a transcriptional target of this signaling pathway (33), which in turn elevates cdk4 expression (34), that can be inhibited by p21. Thus, in addition to the reported role of sulindac on tumor promotion (1, 2), the effects of sulindac on p21 expression in the flat mucosa of patients (i.e., Fig. 1) and on the inhibition of mitosis in the small intestine of the Apc1638 mouse (Table 2) indicates that sulindac may also alter early events in intestinal tumor formation. Furthermore despite the fact that p21 can play roles in apoptosis (14, 35) and in cell differentiation (14), the data we have presented here suggest that p21 expression is most closely linked to its regulation of the cell cycle of intestinal cells (36–39). Moreover, there was no obvious size reduction in the tumors that were present in the Apc+/−, p21+/−, +/−, or −/− mice fed the sulindac diet compared with those fed control diet, again suggesting that a major effect of sulindac may be on early stages of tumor formation. However, whether p21 plays a role exclusively in tumor initiation or promotion, or in a combination of both early and later events, the inhibition of tumor formation in the Apc/p21 mouse model demonstrates the importance of p21 in Apc-initiated tumorigenesis and its inhibition.

The three patients investigated showed markedly different alterations in gene expression after sulindac treatment. There are at least two sources of this variability. First, in this initial study, we have no information regarding pharmacokinetics or drug disposition, nor have we factored in confounding variables such as diet and/or genetic background, both of which can profoundly influence the physiology of the gastrointestinal tract and, hence, response to the drug. However, we also believe that heterogeneous changes, whether direct or indirect effects of sulindac, are what would be expected of a pharmacological agent that induces long-term toxicity.

Regardless of the source of the heterogeneity, comparisons of the data bases led to identification of a key gene in the response. This approach of comparing in vivo and in vitro gene expression profiles first was used by us in comparing transformation of colonic epithelial cells in vivo with their response to inducers of cell maturation in vitro (40), which identified the role of mitochondrial gene expression (20, 41, 42) and then mitochondrial function (43, 44) in these two complementary processes. Therefore, as a general approach, the generation and comparison of appropriate in vivo and in vitro data bases may be highly effective in identifying key target genes from complex microarray data bases.

There are seven additional sequences that are altered in expression by sulindac both in vivo and in vitro (Fig. 1C). Among these, decorin is particularly intriguing because it is expressed in colon tumors (45) and modulation of its expression in colon carcinoma cells can influence cell growth (46) linked to alterations in p21 expression (47, 48). Alteration in ladinin expression, a basement membrane protein that may be involved in the stability of epithelial-mesenchyme interactions, is also of interest (49). Additional data on the functional relevance of these genes in tumor formation and inhibition will be necessary, however, before the significance of each of these observations is understood. It is also important to note that the 8063 sequences screened may represent less than one-quarter of the number of human genes (the complete data bases are available on the Internet). There-

<table>
<thead>
<tr>
<th>Time</th>
<th>SW620 cells treated with Sulindac</th>
<th>--5, 2, 12, 16, 24, 48 hours</th>
<th>SW620 cells treated with Butyrate</th>
<th>--5, 2, 12, 16, 24, 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HT29 Clone 16E cells--day 3, 5, 7, 10, 15, 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT29 Clone 19A cells--day 3, 5, 7, 10, 15, 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caco-2 cells--day 2, 5, 14, 21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Alteration of p21 expression in in vitro models of colonic cell maturation. The data for SW620 cells treated with sulindac or butyrate have been previously released and are a comparison of RNA from cells at each time point for which the media was changed with or without the inducing agent. The data for the HT29 clone 16E and clone 19A cells, representing the mucin secreting or absorptive cell pathways, respectively (24, 52), were generated by comparing RNA from day-3 undifferentiated cells to RNA from a similar day-3 culture, or to cells grown for up to day 20, during which time they exhibit cell cycle arrest and lineage-specific differentiation. Similarly, the data for Caco2 cells was generated by comparing RNA from day-2 undifferentiated cells to RNA from each of the other time points up to day 21. Microarray data were generated and analyzed as described previously (12).

Table 2 Apoptosis and mitosis in the duodenum of Apc+/−, p21+/−, +/− or −/− mice fed AIN-76A or AIN-76A plus sulindac diet for 36 weeks

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Apoptotic index (%)</th>
<th>Mitotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AIN-76A</td>
<td>AIN-76A + sulindac</td>
</tr>
<tr>
<td>Apc+/−, p21+/−</td>
<td>0.71 ± 0.17</td>
<td>0.99 ± 0.16</td>
</tr>
<tr>
<td>Apc+/−, p21+/−</td>
<td>0.38 ± 0.06</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td>Apc+/−, p21+/−</td>
<td>0.28 ± 0.08</td>
<td>0.32 ± 0.09</td>
</tr>
</tbody>
</table>

a Values are means ± SE.

b *P < 0.05 in comparison to the Apc+/−, p21+/− mice fed AIN-76A diet (by Student’s t test).
c *P < 0.05 in comparison to the Apc+/−, p21+/− mice fed AIN-76A diet (by Student’s t test).
d *P < 0.01 in comparison to the Apc+/−, p21+/− mice fed AIN-76A diet (by Student’s t test).
e *P < 0.001 in comparison to the Apc+/−, p21+/− mice fed AIN-76A diet (by Student’s t test).
fore, there may be many more genes that are involved in the response to sulindac in vivo and in vitro. These could be genes in pathways already identified in the response of cells in culture to sulindac, such as COX-2 (7, 8), and other genes involved in arachidonic acid and ceramide synthesis (50), additional genes involved in cell-cycle regulation or in apoptotic (51) or signaling (12, 26) pathways. How these are integrated with p21 function in inhibiting tumor formation is a critical question, and it may be that determinants of efficacy of sulindac in tumor inhibition, which function upstream and downstream of this central regulator of cell cycling, are highly complex, especially when projected against a background of genetic and dietary heterogeneity.

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11. Acknowledgments

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References


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