p21\textsuperscript{WAF1/cip1} Is an Important Determinant of Intestinal Cell Response to Sulindac in Vitro and in Vivo

WanCai Yang, Anna Velcich, John Mariadason, Courtney Nicholas, Georgia Corner, Michele Houston, Winfried Edelmann, Raju Kucherlapati, Peter R. Holt, and Leonard H. Augenlicht

Department of Oncology [W. Y., A. V., J. M., C. N., G. C., M. H., L. H. A.], Department of Cell Biology [W. E., L. H. A.], and Department of Molecular Genetics [R. K.], Albert Einstein Cancer Center, Bronx, New York 10467, and Department of Medicine, St. Luke’s-Roosevelt Hospital Center and Columbia University, New York, New York 10025 [P. R. H.]

ABSTRACT

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\textsuperscript{WAF1/cip1}. In Apc\textsuperscript{1638+/−} mice, targeted inactivation of p21 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.

INTRODUCTION

Sulindac is representative of a class of nonsteroidal anti-inflammatory drugs that have significant activity in inhibiting colon tumor formation (1, 2). Sulindac has been shown to be effective in preventing intestinal tumors in familial adenomatosis polyposis patients that inherit a mutant allele of the APC\textsuperscript{3} gene (3, 4), in inhibiting tumor formation in a mouse model (Apc/Mm) in which an allele of the homologous mouse Apc gene was inactivated by a mutation (5), and in inhibiting chemically induced colon tumor formation in the rat (6).

Side effects of sulindac, which include gastrointestinal bleeding and ulceration, limits its clinical utility (7); but its clear efficacy in the inhibition of tumorigenesis makes it an important probe into the mechanisms by which tumors form and by which they may be inhibited. Thus, the effects of the compound in vivo and in vitro have been extensively studied. In particular, COX-2, which is inhibited by the binding of sulindac, is an important target of the drug (7, 8), consistent with the fact that the up-regulation of this enzyme has been linked to the development of colon tumors (9); and, in mice that have a targeted inactivation of the COX-2 gene, mutant Apc is much less effective in inducing tumor formation (10). However, the mechanisms by which the inhibition of COX-2 alters tumor development are not known, and other uncertainties regarding how nonsteroidal anti-inflammatory drugs in general inhibit tumor formation have recently been emphasized (11).

We have used microarray analysis of >8000 sequences to demonstrate that >500 genes (>$6\%) are altered in expression in the repro-
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 in vitro (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 in vivo were compared with the previous results we had obtained using a well-controlled system in vitro: the time course of response of SW620 colon carcinoma cells to sulindac (12). First, in vitro, 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 in vitro (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 in vivo that does not take place in the homogeneous colon carcinoma cell line in vitro. 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 in vitro and in vivo was the cdki p21^{WAF1/cip1}. We have also found that sulindac induces p21
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>, 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 a number of times for this Apc1638 model (14, 15, 17, 19). As in all mouse models that inherit a mutant Apc allele, the Apc-initiated tumors developed in the small intestine. On the control diet, the inactivation of p21 resulted in a gene-dosage-dependent increase in the incidence and frequency of small intestinal tumors/mouse (Table 1), consistent with our recent report (14). In the wild-type p21 mice, sulindac in the diet was highly effective in reducing tumor incidence in the small intestine from 89% to 53% and the tumor number/mouse by 56%, as shown by others in the ApcMin (5) and Apc1638N<sup>6</sup> mouse models. 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 in colon carcinoma cell lines in culture is associated with both cell cycle arrest and apoptosis (18). 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 colonic 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

Table 1. Incidence and frequency of tumors in the small intestine of Apc<sup>−/−</sup>, p21<sup>+/−</sup>, +/+ or −/− mice fed AIN-76A or AIN-76A plus sulindac diet for 36 weeks

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AIN-76A diet</th>
<th>AIN-76A + sulindac</th>
<th>Frequency&lt;sup&gt;a&lt;/sup&gt; (no. of tumors/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apc&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>89% (8/9)</td>
<td>53% (8/15)</td>
<td>1.67 ± 0.33</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>95% (18/19)</td>
<td>94% (15/16)</td>
<td>1.95 ± 0.31</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>100% (13/13)</td>
<td>100% (16/16)</td>
<td>2.62 ± 0.51&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are mean ± SE.  
<sup>b</sup> P < 0.01 in comparison to Apc<sup>−/−</sup>, p21<sup>+/−</sup> mice fed AIN-76A diet (by Student’s t test).  
<sup>c</sup> P < 0.001 in comparison to Apc<sup>+/−</sup>, p21<sup>+/+</sup> mice fed AIN-76A diet (by Student’s t test).  
<sup>d</sup> P < 0.05 in comparison to Apc<sup>+/−</sup>, p21<sup>+/−</sup> mice (by Student’s t test).

K. Yang, personal communication.

Fig. 2. Confirmation of altered p21 expression. A. standard Northern blot analysis of the expression of p21 and the control gene GAPDH was done with RNA isolated from cells treated for various periods of time with sulindac or with carrier. B. band intensities from A were quantified using a Phosphorimager (Storm System, Molecular Dynamics) and the altered expression of p21 at each time point corrected for the GAPDH signal is shown. Also shown is the altered expression of p21 as a function of time from the microarray data, corrected for the signal for GAPDH generated in the same way, which was on the slide containing the 8093 sequences. The graphs depicting p21 expression are derived from linear regression analysis of the data points.
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 p21⁻/− 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 p21/WAF1/Cip1 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 G1/S, 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, p27kip1, is haplo-insufficient for tumor suppression (27), and that p53, a principal 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 p21/WAF1/Cip1 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 nondoning 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⁺/−, p21⁻/− or p21⁻/− 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 intestinal tumor formation. Furthermore despite the fact that sulindac—a drug that induces long-term toxicity—has an important role in intestinal cell maturation and, hence, response to the drug. However, 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.

![Microarray data](image)

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Apoptotic index (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mitotic index (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-76A</td>
<td>AIN-76A + sulindac</td>
<td>AIN-76A</td>
</tr>
<tr>
<td>Apc⁻/⁻</td>
<td>p21⁺/−</td>
<td>0.71 ± 0.17</td>
</tr>
<tr>
<td>Apc⁻/⁻</td>
<td>p21⁻/−</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>Apc⁻/⁻</td>
<td>p21⁻/⁻</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>AIN-76A + sulindac</td>
<td>AIN-76A</td>
</tr>
<tr>
<td>Apc⁻/⁻</td>
<td>p21⁺/−</td>
<td>1.38 ± 0.16</td>
</tr>
<tr>
<td>Apc⁻/⁻</td>
<td>p21⁻/−</td>
<td>2.16 ± 0.21</td>
</tr>
<tr>
<td>Apc⁻/⁻</td>
<td>p21⁻/⁻</td>
<td>2.86 ± 0.18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± SE.  
<sup>b</sup> P < 0.05 in comparison to the Apc⁻/⁻, p21⁺/− mice fed AIN-76A diet (by Student’s t test).  
<sup>c</sup> P < 0.05 in comparison to the Apc⁻/⁻, p21⁻/− mice (by Student’s t test).  
<sup>d</sup> P < 0.01 in comparison to the Apc⁻/⁻, p21⁻/− mice (by Student’s t test).  
<sup>e</sup> P < 0.001 in comparison to the Apc⁻/⁻, p21⁻/− mice (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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REFERENCES


$p21^{WAF1/cip1}$ Is an Important Determinant of Intestinal Cell Response to Sulindac \textit{in Vitro} and \textit{in Vivo}

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