Differential Growth Inhibition by the Aspirin Metabolite Salicylate in Human Colorectal Tumor Cell Lines: Enhanced Apoptosis in Carcinoma and in Vitro-transformed Adenoma Relative to Adenoma Cell Lines

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Abstract

Regular aspirin intake may reduce the risk of colorectal cancer by 50%. However, the mechanism of this chemopreventive effect is not known. The effect of the aspirin metabolite salicylate on the growth of human colorectal tumor cell lines was determined. Salicylate showed dose-dependent inhibitory effects on all of the cell lines (IC50 1.65 ± 0.36 to 7.38 ± 1.08 mM), yet carcinoma and in vitro-transformed adenoma cell lines were more sensitive than adenoma cell lines. Salicylate caused all cell lines to accumulate in G0-G1 and induced apoptosis in carcinoma and in vitro-transformed adenoma cell lines but not in all adenoma cell lines. In those adenoma lines that did show salicylate-induced apoptosis, the extent was considerably less than that in the more transformed cell lines. The ability of salicylate to induce cell cycle arrest and apoptosis and, in particular, the increased sensitivity of cells at later stages of neoplastic progression may be mechanistically important in the chemopreventive action of aspirin toward colorectal cancer.

Introduction

Colorectal cancer remains one of the most common malignancies in the westernized world, and current treatment strategies have little effect on survival (1). However, NSAIDs3 such as aspirin and sulindac have recently shown considerable promise as agents effective in the chemoprevention of colorectal cancer. Data relating the effects of NSAIDs to human cancer are derived largely from epidemiological studies of aspirin use and the risk of colorectal cancer and clinical trials of sulindac for colonic polyposis. The epidemiological studies (reviewed in Refs. 1 and 2) indicate an approximate 50% reduction in the incidence of, or death from, colorectal cancer among regular users of aspirin. Results from clinical trials broadly support the chemopreventive action of NSAIDs apparent in the epidemiological studies. For example, sulindac causes regression of colorectal polyps in familial adenomatous polyposis (1, 2). Additionally, NSAIDs also have chemopreventive effects in rodent models of colorectal carcinogenesis, reducing the incidence and multiplicity of premalignant and malignant lesions (2, 3). Although these studies indicate a link between NSAID use and a reduced risk of colorectal cancer, the mechanism of chemoprevention remains unknown. It is probable that it results, at least in part, from the inhibitory effects of NSAIDs on prostaglandin biosynthesis (4). In this respect, it is notable that recent studies have shown prostaglandin E2 levels to be progressively increased from that in control mucosa to adenomatous polyps and then to adenocarcinomas (5). A reduction in prostaglandin levels resulting from NSAID treatment may have a number of potentially chemopreventive effects, reflecting the diversity of prostaglandin action in the intestine. For example, it may result in a decrease of mitogenic stimulation, restoration of the immune response, and a reduction in the formation of potential carcinogens (4, 6). However, two recent reports of studies using a single colorectal carcinoma cell line (7, 8) have suggested apoptosis as another mechanism for the chemopreventive effects of sulindac and its derivatives, which significantly does not necessarily involve the inhibition of prostaglandin biosynthesis (7). Although aspirin is the only NSAID to have been investigated widely in epidemiological studies and is substantially deacetylated to salicylate on the first pass through the portal circulation (9), there are no studies of the effects of salicylate on colonic epithelial cells. Also, it is likely that the relative sensitivity of cells at different stages of tumor progression is critical to the chemopreventive properties of NSAIDs, yet there are no reports of the effects of NSAIDs on premalignant colonic epithelial cells. Consequently, we chose to investigate the effect of salicylate on the proliferation and apoptosis of a range of colorectal tumor cell lines, including those representing early stages of tumor progression. The results of this study provide a new and significant insight into the effects of salicylate on human colorectal adenoma and carcinoma cell lines and a basis for the chemopreventive effect of aspirin.

Materials and Methods

Cell Lines and Culture Conditions. The human colorectal tumor cell lines used in this study are detailed in Table 1 (10–16). In addition to tumorigenic carcinoma cell lines, they include non-tumorigenic adenoma cell lines and derivatives of these that have been transformed in vitro either to tumorigenicity or to anchorage independence. Unless otherwise stated, they were grown on tissue culture plastic in DMEM (Life Technologies, Inc.) with 20% fetal bovine serum (batch selected) as described previously (11), except that the DMEM was not supplemented with hydrocortisone sodium succinate. The inclusion of hydrocortisone may have interfered with the mobilization of arachidonic acid. This is the substrate for cyclooxygenase, both isoforms of which are inhibited by NSAIDs. The non-trypsinizable adenoma cell line PC/AA was routinely maintained in the presence of Swiss 3T3 feeder cells (10), but was grown on plastic in the absence of feeder cells for these studies.

Treatment with Sodium Salicylate. Sodium salicylate was prepared as a 50 mM stock solution in water (tissue culture tested; Life Technologies, Inc.) with the final pH adjusted to 7.2. Water alone was added to control cultures. Except in the case of the PC/AA line, exponentially growing cells were trypsinized prior to seeding at a density so that there were 2–3 × 106 cells/T25 flask at the time of treatment (3–5 days after seeding). The non-trypsinizable PC/AA adenoma cells were passaged using dispase (10) and seeded for experiment in DMEM in the absence of 3T3 feeder cells; again, at a density so that there were 2–3 × 106 cells/T25 flask at the time of treatment. Cells were treated in triplicate with 1–5 mM sodium salicylate (Sigma, Poole, United Kingdom) for 4 days, and then the attached cells (those remaining adhered to the tissue culture flask) and floating cells (those having detached from the tissue culture flask) were counted separately and stained with acridine orange for analysis by fluorescence microscopy (see below). IC50 values were determined from dose-response curves of the salicylate concentration versus attached cell yield.

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2 To whom requests for reprints should be addressed.

3 The abbreviation used is: NSAID, nonsteroidal anti-inflammatory drug.
Salicylate Differentially Inhibits the Growth of Colorectal Adenoma and Carcinoma Cell Lines. The effect of salicylate on the growth of colorectal adenoma, in vitro-transformed adenoma, and carcinoma cell lines (Table 1) was determined after 4 days of treatment. In all cases the adenoma cell lines were less sensitive to the growth inhibitory properties of salicylate (as determined by the IC50 values) than the carcinoma cell lines (lines 7 and 8) and the adenoma AA/C1 (P = 0.012) and RR/C1 (P = 0.0003) cell lines were relatively resistant to the induction of cell death by salicylate (Fig. 2). The response was not dose dependent in any case, and the maximum induction of cell death ranged from 1.25 ± 0.13-fold to 1.9 ± 0.09-fold consistency the control value. The maximum induction of cell death was highly significant for the more transformed cell lines (P = 0.0003, 0.0015, 0.0005, and 0.0001, respectively, for lines 5-8) but less so for the adenoma AA/C1 (P = 0.012) and RR/C1 (P = 0.005) cell lines, reflecting the extents of cell death shown in Fig. 2. The induction of cell death was not significant (P > 0.05) for the PC/AA and RG/C2 adenoma lines.

To determine whether the increase in floating cells was due to the induction of apoptosis rather than just necrosis, the floating cells were analyzed using fluorescence microscopy following staining with acridine orange. This showed that at the highest salicylate concentration (5 mM) the proportion of floating cells that were apoptotic was equal

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**Table 1 Human colorectal tumor cell lines used**

<table>
<thead>
<tr>
<th>Cell linea (passage no.)</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoma cell linesb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:PC/AA (18-20)</td>
<td>Adenoma derived from familial adenomous polyposis patient, nonclonogenic</td>
<td>10</td>
</tr>
<tr>
<td>2:AA/C1 (75-82)</td>
<td>Clonogenic (passaged with trypsin) variant of PC/AA</td>
<td>11</td>
</tr>
<tr>
<td>3:RR/C1 (32-38)</td>
<td>Sporadic adenoma-derived, clonogenic</td>
<td>12</td>
</tr>
<tr>
<td>4:RG/C2 (44-49)</td>
<td>Sporadic adenoma-derived, clonogenic</td>
<td>13</td>
</tr>
<tr>
<td>In vitro transformants of adenoma cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:AA/C1/SB10 (173-181)</td>
<td>Chemical carcinogen transformed variant of AA/C1, tumorigenic</td>
<td>11</td>
</tr>
<tr>
<td>6:RG/C2/GV (127-134)</td>
<td>Chemical carcinogen and radiation transformed variant of RG/C2</td>
<td>14</td>
</tr>
<tr>
<td>Carcinoma cell linesc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7:HT29 (284-290)</td>
<td>Anchorage independent but nontumorigenic</td>
<td>15</td>
</tr>
<tr>
<td>8:SK/S (42-48)</td>
<td>Sporadic carcinoma</td>
<td>16</td>
</tr>
</tbody>
</table>

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*a The numbering of the cell lines corresponds to that used in the figures and text.

*b The adenoma cell lines (lines 1–4) are nontumorigenic in athymic nude mice. The carcinoma cell lines (lines 7 and 8) are tumorigenic in athymic nude mice.

*c RG/C2/GV was derived from RG/C2 by treatment with 1 µg/ml N-methyl-N'-nitro-N-nitrosoguanidine, selection for growth in soft agar followed by irradiation with five fractions of 1.5 Gy each at 6-week intervals.

**Measurement of Apoptosis.** As we and others have described previously, the level of apoptosis in cultured epithelial cell lines can be assessed by measuring the proportion of the total cell population that has detached from the cell monolayer and is floating in the medium and determining the fraction of these floating cells that are apoptotic (16–18). Following salicylate treatment, the proportion of the total cell population that was floating was determined for each salicylate dose. To determine whether the induction of floating cells was due to apoptosis, the proportion of the attached and floating cell populations that were apoptotic was established by staining with acridine orange (5 µg/ml in PBS) and analysis by fluorescence microscopy as described previously (16, 19). Apoptotic cells were identified by their characteristically condensed chromatin stained by the acridine orange. Necrotic cells were identified as cells with poorly staining “hollow” nuclei. Acridine orange-stained cells were photographed using Fujichrome Provia (ISO 400) film. Statistical analysis was carried out using Student’s unpaired t test; P < 0.05 was considered to be statistically significant.

**Analysis of Cell Cycle Distribution.** For flow cytometric analysis, cells were treated with 5 mM salicylate for 24 to 72 h. Samples of attached cells were fixed with 70% ethanol and stained with 20 µg/ml propidium iodide (Sigma) before being analyzed for red (FL2) fluorescence on a FACScan (Becton Dickinson), as detailed previously (19). The cell cycle distribution was calculated from the resultant DNA histograms using Cell FIT software, based on a rectangular S-phase model.

**Results**

Salicylate Differentially Inhibits the Growth of Colorectal Adenoma and Carcinoma Cell Lines. The effect of salicylate on the growth of colorectal adenoma, in vitro-transformed adenoma, and carcinoma cell lines (Table 1) was determined after 4 days of treatment. In the range of 1–5 mM, salicylate showed dose-dependent inhibitory effects on the attached cell yield of all cell lines tested (Fig. 1, A–C). The dose-response curves of each cell line were used to determine the specific IC50 value for the reduction of cell numbers by salicylate (Fig. 1D). There was considerable variation in the sensitivities of the cell lines to the growth inhibitory effects of salicylate with IC50 values (±SD) ranging from 1.65 ± 0.36 to 7.38 ± 1.08 mM (Fig. 1D). However, in all cases the adenoma cell lines were less sensitive than the carcinoma and in vitro-transformed adenoma cell lines to the growth inhibitory effects of salicylate. Only in a comparison of the IC50 value of the RG/C2 adenoma line and that of the HT29 carcinoma line was this difference found not to be significant (P = 0.1079; Fig. 1D, lines 4 and 7). The differential effect was apparent not only between unrelated cell lines at different stages of neoplastic transformation but also between adenoma lines and their in vitro-transformed derivatives. Progression of the parental PC/AA adenoma cell line (the least sensitive of the cell lines to salicylate) through the clonogenic variant AA/C1 and to the tumorigenic line AA/C1/SB10 (Table 1; Ref. 11) resulted in the reduction of the IC50 value for salicylate from 7.38 ± 1.08 mM to 4.69 ± 0.45 mM to 2.77 ± 0.56 mM, respectively (Fig. 1D, lines 1, 2, and 5). Thus, conversion of a non-tumorigenic adenoma cell line to a tumorigenic adenocarcinoma resulted in an increased sensitivity to the growth inhibitory properties of salicylate. Similarly, progression of the RG/C2 adenoma cell line (IC50 3.89 ± 0.15 mM) to an anchorage-independent line with increased colony-forming efficiency on plastic was associated with a reduction of the IC50 value for salicylate to 2.57 ± 0.22 mM (Fig. 1D, lines 4 and 6).

To examine a possible mechanism by which salicylate had an antiproliferative effect, the cell cycle phase distribution of cell lines 1–8 (Table 1) was compared for control and 5 mM salicylate-treated cultures (data not shown). In all of the cell lines tested, salicylate treatment caused an accumulation of cells in G0-G1 and a decrease in the proportion of cells in the S-phase. Notably, the cell lines that were more sensitive to the growth inhibitory properties of salicylate (as determined by the IC50 values) did not necessarily exhibit an increased cell cycle arrest when compared with less sensitive cell lines.

Salicylate Induces Apoptosis in Colorectal Carcinoma and in *In Vitro*-transformed Adenoma Cell Lines but Significantly Less So in Adenoma Cell Lines. Having established that salicylate was growth inhibitory to the colorectal tumor cell lines, we investigated whether salicylate induced apoptosis. We have shown previously that in the routine culture of colorectal epithelial tumor cells, the majority of cells that spontaneously detach from the tissue culture flask and float in the medium are apoptotic (16). Fig. 2 illustrates the effect of salicylate treatment on the proportion of the total cell population (attached and floating) that is floating in the medium. In the carcinoma and transformed adenoma cell lines (lines 5–8; Table 1), salicylate was found to increase, in a dose-dependent manner, the proportion of cells floating. The maximum extent of the induction of cell death in these cell lines ranged from 3.7 ± 0.37-fold to 14.6 ± 1.56-fold the control value. In contrast, adenoma cell lines (lines 1–4; Table 1) were relatively resistant to the induction of cell death by salicylate (Fig. 2). The response was not dose dependent in every case, and the maximum induction of cell death ranged from 1.25 ± 0.13-fold to 1.9 ± 0.09-fold consistency the control value. The maximum induction of cell death was highly significant for the more transformed cell lines (P = 0.0003, 0.0015, 0.0005, and 0.0001, respectively, for lines 5–8) but less so for the adenoma AA/C1 (P = 0.012) and RR/C1 (P = 0.005) cell lines, reflecting the extents of cell death shown in Fig. 2. The induction of cell death was not significant (P > 0.05) for the PC/AA and RG/C2 adenoma lines.

To determine whether the increase in floating cells was due to the induction of apoptosis rather than just necrosis, the floating cells were analyzed using fluorescence microscopy following staining with acridine orange. This showed that at the highest salicylate concentration (5 mM) the proportion of floating cells that were apoptotic was equal...
EFFECTS OF SALICYLATE ON COLORECTAL TUMOR CELLS

to control values (86–95% dependent on cell line). Hence, the increase in the proportion of cells floating in salicylate-treated cultures was due largely to the induction of apoptosis and not simply a result of increased necrosis. Only in the case of S/KS, the most sensitive cell line, was the proportion of floating cells (in 5 mM treated cultures) that were apoptotic slightly reduced compared with control levels (see legend to Fig. 2). At lower doses the apoptotic fraction of the floating cells at different stages of neoplastic progression. The IC50 values are the salicylate treatment with the aspirin metabolite salicylate on the proliferation of colorectal tumor cells in vivo following 4 days of treatment with the aspirin metabolite salicylate. The data indicate the level of floating cells, as a proportion of the total cell number (attached and floating), following salicylate (1–5 mM) treatment in relation to the control (spontaneous) level (assigned as 1) for the respective cell line. The control (spontaneous) level (mean percentage ± SD) of floating cells for each cell line was as follows: Adenomas (A): 1, PC/AA (2.66 ± 0.67); 2, AA/C1 (0.92 ± 0.11); 3, RR/C1 (6.48 ± 1.81); 4, RG/C2 (6.14 ± 1.61). In vitro-transformed adenomas (B): 5, AA/C1/SB10 (1.00 ± 0.02); 6, RG/C2/GV (3.50 ± 0.41). Carcinomas (C): 7, HT29 (4.23 ± 1.05); 8, S/KS (4.87 ± 1.14). The morphology of the (acridine orange-stained) floating cells from control and treated cultures was examined at the end of each experiment to determine whether the increase in floating cells with salicylate treatment was due to apoptosis. For each cell line (apart from S/KS when treated with 5 mM salicylate), the proportion of floating cells that were apoptotic in salicylate-treated cultures was similar to that of control cultures, where this proportion was 86–95% dependent on the cell line. For S/KS (the cell line most sensitive to the growth inhibitory properties of salicylate), the proportion of floating cells that were apoptotic decreased from 94.3% ± 1.2 in control cultures to 82.5% ± 2.1 in cultures treated with 5 mM salicylate, with a corresponding increase in cells appearing to be necrotic. Details of the cell lines are presented in Table 1. Each experiment was carried out with triplicate determinations for each salicylate concentration (0–5 mM). The data presented are the means of the three experiments. Bars, SD.

Discussion

Epidemiological and clinical studies have left little doubt that aspirin and related compounds have considerable potential as chemopreventive agents for colorectal cancer. It is therefore important to understand their effect on colorectal tumor cells. This study has shown that the aspirin metabolite salicylate inhibits growth and induces apoptosis in colorectal tumor cells. We have also found salicylate to cause cell cycle arrest in these cells. Recently, it has been shown that sulindac and its derivatives have similar effects on HT29 colon carcinoma cells (7, 8). The response of colorectal carcinoma cells to salicylate, sulindac, and its derivatives may in part explain the chemopreventive nature of these compounds in colorectal cancer. However, a critical issue in cancer chemoprevention or chemotherapy is the relative sensitivity of cells at different stages of neoplastic progression. Thus, the importance of the present study lies not only in the use of the aspirin metabolite salicylate but also of premalignant colorectal tumor cells in addition to carcinoma cells. Colorectal carcinoma and in vitro-transformed adenoma cells were found to be more sensitive than colorectal adenoma cells to growth inhibition and, particularly, to the induction of apoptosis by salicylate. Indeed, salicy-
prostaglandin biosynthesis, may be involved in the cellular response to NSAIDs. This has been proposed for sulindac sulfone which induces apoptosis in the colon carcinoma cell line HT29 but does not inhibit prostaglandin biosynthesis (7).

Although the molecular mechanisms remain unclear, our study demonstrates cellular responses to salicylate that may contribute to the chemopreventive effects of regular aspirin intake toward colorectal cancer. Of particular importance is the demonstration that in vitro-transformed adenoma and carcinoma cell lines are more sensitive than adenoma cell lines to growth inhibition and the induction of apoptosis by salicylate. Such differential effects will be central to the development of NSAID chemoprevention strategies.

References

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