DNA array analysis of the effects of aspirin on colon cancer cells: involvement of Rac1

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Aspirin and other non-steroidal anti-inflammatory drugs show efficacy in the prevention of colon cancer. The mechanism by which they do this is unclear. We used a commercially available DNA microarray to study changes in gene expression in 1176 cancer related genes in the HT29 colon cancer cell line induced by aspirin. Overall we find more genes that are significantly induced than are repressed. The pattern of gene expression changes is different at high concentrations of aspirin (5 mM) than at lower levels (500 and 50 μM). Genes involved in DNA damage signalling, nucleotide metabolism and the stress response are induced, and cell cycle related genes repressed. The small GTPase Rac1 is highly induced and this was confirmed by immunoblotting. We show using immunohistochemistry that Rac1 is expressed in mature colonocytes at the intercrypt table in human and mouse colon tissue. These results support the previous findings that aspirin has different actions at high concentrations than at low concentrations and further show the use of DNA array technology in the investigation of drug mechanisms of action. Furthermore, they point towards a role for Rac1 in the action of aspirin in colon cancer.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are effective in preventing colon cancer (1). Currently available compounds, however, have side-effect profiles that make them unsuitable for widespread use in the prevention of this commonly fatal disease. Understanding the mechanisms of action of NSAIDs in preventing colon cancer is central to the development of safer and more effective treatments for the chemoprevention of colon cancer. Despite much research effort there is still much controversy over the mechanisms involved in the chemopreventative actions of NSAIDs (2). Most research has focused on the first described molecular target of NSAIDs, prostaglandin synthetase or cyclooxygenase (COX). Two COX isofoms have been described. COX-1 is constitutively expressed in almost all tissues and may perform housekeeping functions. COX-2 is an immediate early gene, undergoing rapid transcriptional up-regulation in response to tissue injury. COX-2 is induced in colon cancer and may contribute to tumour growth by producing prostaglandins that inhibit apoptosis (3) and induce the formation of new blood vessels (4). Inhibiting COX-2 pharmacologically with COX-2 specific or non-specific COX inhibiting compounds reduces colon cancer growth both in animal models (5) and in an inherited form of colon cancer, familial adenomatous polyposis (6). Knocking out COX-2 genetically in mice also lowers their susceptibility to colon cancer (7).

However, there is increasing evidence that COX-independent actions of NSAIDs are important both for their anti-inflammatory and their chemopreventative actions. NSAID-related compounds with no COX-inhibitory activity retain their antitumour activity (8) and cancer cells lacking COX-2 enzyme are still sensitive to these compounds (9). At the same time new molecular targets for NSAIDs continue to be identified. Among these the inhibition of NF-κB (10) and PPARδ (11) provide alternative explanations for the tumour suppressive actions of NSAIDs. Many other possible COX-independent targets of NSAIDs in colon cancer have been suggested (reviewed in ref. 12). One of the criticisms of the COX-independent theories is that they require far higher drug concentrations than those needed to inhibit COX, concentrations that may not be achieved in vivo (2).

A relatively new method of drug target validation and the identification of alternative drug targets is use of DNA microarrays (13). Here we used a commercially available array to study the effects of aspirin on a large panel of cancer related genes. We hoped to find genes or groups of genes that were significantly up or down regulated by the treatment of colon cancer cell lines with aspirin, and thus to determine targets of aspirin relevant to neoplasia in colon cancer cells. We also addressed the question of drug concentration by performing parallel arrays where different concentrations of drug were used. Genes were classified according to function to look for patterns in the changes in gene expression induced by aspirin.

Materials and methods

Cell culture

The HT29 colon cancer cell line was obtained from the ATCC, and cultured in Dulbecco’s Modified Eagles Medium (Gibco, Paisley, Scotland) with 4.5 g/l glucose and l-glutamine. This was supplemented with penicillin (50 U/ml) and streptomycin (50 μg/ml) and, where serum was used, with 10% fetal calf serum (Gibco). Cells were grown in monolayers in a humidified atmosphere containing 5% CO2.

DNA array

Confluent HT29 cells were treated by adding aspirin or vehicle (DMSO) for 24 h. At 24 h clear effects have been seen on the cell cycle while apoptosis is not yet found (14). Cells were then washed in ice-cold phosphate buffered saline (PBS) × 3. Total RNA was extracted using the ATLAS Pure Total RNA labelling system (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. Briefly, cells were lysed and total RNA extracted with three rounds of phenol:chloroform extraction. The resulting RNA was then treated with DNase and analysed on a denaturing agarose gel. Poly A+ Enrichment was performed using biotinylated oligo(dT) and avidin coated magnetic beads with a magnetic particle separator. Probes were made using the Atlas Human

Abbreviations: COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; PBS, phosphate buffered saline.
Cancer 1.2 array kit (Clontech) according to the manufacturer’s instructions. cDNA probes were transcribed using a mix of primers specific for each of the genes on the array and labelled with [α-32P]dATP. Probes were purified by removing unincorporated 32P-labelled nucleotides and small cDNA fragments using an extraction column. Nylon cDNA expression array membranes were pre-hybridized with sheared salmon testes DNA and then hybridized with the probes overnight. Membranes were then sealed in plastic wrap and exposed for 7 days to a phosphoimaging screen and then developed in a phosphoimager and analysed using DNA array analysis software. A complete list of the genes and gene categories can be found at http://atlasinfo.clontech.com/genelists/hucCa1.xls.

Signals were normalized between arrays using a correction factor calculated from the average expression of nine housekeeping genes. Spots were disregarded if neither treatment nor control levels were two times the average background level. Gene expression was considered significantly altered if the change was more than twice the standard deviation of the signal of the housekeeping genes. The change in gene expression is expressed as a multiple of the control (DMSO) value for that gene. The full array experiment from cell culture to phosphoimager was repeated twice for each condition and the change was more than twice the standard deviation of the signal of the housekeeping (DMSO) value for that gene. The full array experiment from cell culture to phosphoimager was repeated twice for each condition and the change was more than twice the standard deviation of the signal of the housekeeping genes. The change in gene expression is expressed as a multiple of the control (DMSO) value for that gene.

**Immunoblotting**

Treated cells were washed in ice cold PBS and scraped into 250 μl of lysis buffer (Cell Signalling, Beverly, MA) with the addition of 1 mM Pefabloc (Sigma, St Louis, MO). The lysates were sonicated and then centrifuged at 20,000 g for 10 min at 4°C and protein concentration measured with the BCA protein assay kit (Pierce Chemical, Rockford, IL). Sample buffer (125 mM Tris–HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1 mg bromphenol blue) was added so as to equalize protein concentrations. Seventy-five micrograms of protein per lane was loaded onto SDS–PAGE and blotted onto PVDF membrane (Millipore, Billerica, MA). The blots were blocked with 2% low fat milk powder in TBST (Tris-buffered saline with 1% Triton) for 1 h at room temperature and washed 3× 10 min in TBST before overnight incubation at 4°C with primary antibody in TBST with 2% milk. Blots were then washed 3× 10 min in TBST and incubated for 1 h at room temperature in 1/2000 horse radish peroxidase (HRP) conjugated secondary antibody in block buffer. After 30 min, slides were incubated for 5 min in LN 2-methyl acetamide (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected and quantified using a Lumi-Imager (Boehringer-Mannheim).

**Immunohistochemistry**

Sections (4 mm) were prepared from the formalin-fixed, paraffin-embedded tissue and mounted on slides coated with polylysine. Sections were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% H2O2 in PBS for 30 min and then washed in PBS. Antigen retrieval was performed by boiling slides for 10 min in 0.01 M sodium citrate pH 6.0. Non-specific binding sites were blocked with TEG (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.2% gelatin, 0.05% (v/v) Tween 20, pH 8.0) for 30 min, and then washed (3 × 5 min in PBS). Slides were incubated with the primary antibodies overnight at 4°C in PBS with 0.1% Triton and 1% bovine serum albumin. After washing (3 × 10 min in PBS), slides were then incubated with biotinylated secondary antibodies at room temperature for 1 h in PBS with 10% human serum. Slides were washed (3 × 5 min in PBS), incubated with streptavidin–biotin–horseradish peroxidase (Dako, Glostrup, Denmark) for 1 h, washed again (3 × 5 min in PBS), and peroxidase activity was detected with Fast DAB (Sigma). Finally, sections were counterstained with Mayer’s haematoxylin, dehydrated and mounted in ‘Entellan’ (Merck, Darmstadt, Germany) under cover slips.

**Antibodies**

Rac1 mouse monoclonal antibody IgG2b clone 23A8 was from Upstate (Lake Placid, NY) and was used for both immunoblotting and immunohistochemistry. Rac1 mouse monoclonal antibody IgG2b clone 102 was from Transduction Laboratories (Lexington, KY) and was used to confirm immunohistochemistry results (not shown). Cip1/WAF1 mouse monoclonal antibody IgG2a clone 104 was from Transduction Laboratories. HRP conjugated rabbit anti-mouse antibodies were from Cell Signalling, and biotinylated rabbit anti-mouse antibodies were from Dako.

**Results**

**More genes are induced by aspirin treatment than repressed**

Of the 1176 genes whose expression was analysed, 149 were significantly up-regulated and 51 significantly down-regulated when average gene expression was analysed over the three doses of aspirin used. Induction or repression is expressed as a multiple of the DMSO control value for that gene. For example, the expression of prostate differentiation factor after aspirin treatment using three different concentrations is on average nearly four times higher than the control value. The 10 most up regulated and 10 most down regulated genes using 5 mM aspirin are shown in Tables I and II.

To look at the effect of aspirin dosage on gene expression we compared the results obtained for each gene in each condition to obtain a correlation coefficient for the various comparisons. We found that increasing the aspirin dose by a factor of 10 from 0.05 to 0.5 mM leads to very little difference in gene expression, a further factor of 10 increase, from 0.5 to 5 mM, results in a very different gene expression pattern. This is interesting as it agrees with data regarding the minimum effective dose required to influence many of the new non-COX targets of NSAIDs. These all require aspirin doses in the 1–10 mM range (12). Aspirin mediated effects such as induction of apoptosis (14,15) and inhibition of angiogenesis (16,17) also need doses in this millimolar range, doses that are at least 100-fold higher than those needed to inhibit prosta-glandin synthesis (18). Our results confirm that aspirin has different actions in the millimolar range than at doses below this. The differences seen involve large numbers of genes and hence our representation of this using the correlation coefficient
In the two dot plot graphs we have plotted the mean fold induction and repression values for two different aspirin concentrations against each other. Thus, expression levels of each gene obtained using one concentration of aspirin divided by the DMSO controls were plotted against the expression levels obtained with a different aspirin concentration divided by the DMSO controls. The greater the similarity between the gene expression patterns the more linear the dot plot graph. In Table III we show a small selection of the genes that are exclusively altered by high dose (5 mM) aspirin treatment.

Table III. A selection of the genes highly affected using 5 mM aspirin treatment, which are not affected using the other two aspirin concentrations

<table>
<thead>
<tr>
<th>GenBank acc. no.</th>
<th>Gene name</th>
<th>↑ or ↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>X54941</td>
<td>CDC28 protein kinase 1</td>
<td>↑</td>
</tr>
<tr>
<td>J04111</td>
<td>v-jun avian sarcoma virus 17 oncogene homologue</td>
<td>↑</td>
</tr>
<tr>
<td>M11810</td>
<td>2′,5′-Oligoadenylate synthetase 1</td>
<td>↑</td>
</tr>
<tr>
<td>U48296</td>
<td>Protein tyrosine phosphatase type IVA, member 1</td>
<td>↑</td>
</tr>
<tr>
<td>U09579</td>
<td>Cyclin-dependent kinase inhibitor 1A (CDKN1A); p21 Cip1/WAF1</td>
<td>↓</td>
</tr>
<tr>
<td>M32800</td>
<td>HLA-G histocompatibility antigen, class I, G</td>
<td>↓</td>
</tr>
<tr>
<td>U90313</td>
<td>Glutathione S-transferase like; glutathione transferase omega</td>
<td>↓</td>
</tr>
<tr>
<td>M19645</td>
<td>Heat shock 70 kDa protein 5</td>
<td>↓</td>
</tr>
<tr>
<td>M58603</td>
<td>Nuclear factor-κB (p105)</td>
<td>↓</td>
</tr>
</tbody>
</table>

All genes within the top 20 most up-regulated or top 20 most down-regulated genes using 5 mM aspirin, but unaffected by the other concentrations, are listed.

Aspirin represses genes involved with the cell cycle

We further analysed whether there was a pattern to the significantly down-regulated genes. Fifty-one of these were categorized and categories significantly over-represented amongst the down-regulated genes are displayed in Table IV. Interestingly both of these are cell cycle related. The effects of aspirin and other NSAIDs on various cell cycle proteins are well documented (21,22).

This also fits well with some of the up-regulated genes such as p21 Cip1/WAF1, a cyclin-dependant kinase inhibitor that induces cell cycle arrest. This appears high in the list of most up-regulated genes by 5 mM aspirin. This we checked by immunoblotting HT29 cells treated with aspirin as shown in Figure 2. This confirms that cell cycle inhibition is one of the major effects of aspirin in colon cancer cells, and also confirms the reliability of the array results.

Rac1 is induced by aspirin treatment

The gene most highly induced by aspirin treatment is prostate differentiation factor (PDF) a gene otherwise known as millimolar doses of aspirin to effect these changes, p21 and heat shock protein are reviewed by Tegeder et al. The effects of aspirin on NF-κB are also reviewed and largely ascribed to inhibition of IκB kinase. This also requires millimolar concentrations of aspirin. Here we show reduced transcription of p105, which could provide a further explanation. NF-κB is relevant to colorectal cancer as we (19) and others have shown (20).

Aspirin induces genes involved with DNA damage, nucleotide metabolism and stress response

To analyse whether there was a pattern to the up-regulated genes we analysed them by functional category to see if any categories were significantly over represented. For example, of the 1176 genes, seven code for ribosomal proteins. Six of these genes are among the 149 genes significantly up-regulated by aspirin. If 149 genes were selected at random from 1176 then on average they would contain 0.88 genes from the ribosomal protein gene category. As shown in Table III, genes involved with DNA damage signalling, stress response and nucleotide metabolism were among the most significantly affected categories.

Fig. 1. The list of values obtained for gene expression under the various treatments were compared to give a correlation coefficient between −1 and 1, representing the degree to which gene expression between two treatments is linearly related. While there was a good correlation in gene expression pattern between 0.05 and 0.5 mM aspirin treatments, the gene expression pattern obtained with 5 mM aspirin was very different to that of 0.5 mM aspirin treatment.
suggests that Rac1 is involved in differentiation of the colonic epithelium. Thus, Rac1 expression increases as the colonocytes differentiate and is highest in mature colonocytes about to undergo apoptosis. This would support evidence that macrophage inhibitory cytokine or NSAID activated gene (NAG), which as the latter name suggests, has already been described as being up regulated by aspirin treatment (23). Another interesting gene that is highly induced by aspirin is Rac1, a molecule involved with differentiation of intestinal epithelial cells (24). This up regulation was also shown by immunoblotting aspirin-treated HT29 cells. Rac1 expression was induced in a time- and concentration-dependant manner as shown in Figure 3.

**Rac1 is expressed most highly by mature colonocytes in human and mouse colon**

One possible way to determine the function of Rac1 in the colon is to study its expression pattern by immunohistochemistry. As shown in Figure 4, Rac1 is expressed most highly at the intercrypt table in both mouse and human colonic epithelium. Thus, Rac1 expression increases as the colonocytes differentiate and is highest in mature colonocytes about to undergo apoptosis. This would support in vivo evidence that suggests that Rac1 is involved in differentiation of the colonic epithelium.

**Discussion**

The exciting prospect of being able to prevent colon cancer with NSAIDs has led to intense interest in their working mechanism in preventing colon cancer. Since the discovery that NSAIDs inhibit COX (25), most of the work has concentrated on this as the working mechanism. However, in the last few years a number of new molecular targets of NSAIDs have been reported and several of these have been suggested to provide alternative working mechanisms for NSAIDs in colon cancer (12). One of the criticisms of many of these COX-independent mechanisms is that they require far higher doses of NSAIDs than required for COX inhibition, concentrations that may be difficult to achieve in vivo (2). In this study we set out to determine the changes in gene expression induced by aspirin at three different concentrations in the colon cancer cell line HT29 using a commercially available DNA microarray consisting of 1176 genes selected for their known or suspected roles in cancer. We analysed both the average expression obtained with the three aspirin doses and also the differences seen between the different doses.

Trials that address the issue of aspirin dosage and chemoprevention of colorectal cancer seem to indicate that aspirin at low dose (81 mg/day) is as effective as higher dosages (325 mg/day) (26). Epidemiological studies also show a statistically significant reduced risk for the development of colorectal cancer in those taking approximately 75 mg aspirin/day (27). It seems that even a low number of aspirin tablets (16 × 325 mg) per month taken regularly over 10 years reduces the risk of colorectal cancer development (1). Clinical evidence from the use of aspirin in other diseases suggests that for use as an anti-inflammatory drug far higher doses are required than when used for pain relief (28,29). Ultimately in vitro concentrations of aspirin are difficult to equate to in vivo concentrations. While plasma levels of up to 1 mM have been obtained in subjects taking 900 mg of aspirin/day (30), this may under-estimate the concentration to which the intestinal mucosa is exposed as the drug is taken orally.

To look for general patterns within the complex changes induced in this large number of genes, we have grouped genes into functional groups and looked to see whether the affected genes fell into these groups more frequently than would be expected by chance. We also compared the pattern of gene expression found at each of the three aspirin concentrations to see to what extent they overlapped. Finally, we selected one of the most highly up-regulated genes and investigated this in more depth.

The most highly up-regulated gene has been identified previously by subtractive hybridization as the gene most highly up-regulated by NSAIDs. PDF, otherwise known as NAG or macrophage inhibitory cytokine, is highly up regulated by NSAID treatment. It is a member of the TGF-beta family and has proapoptotic properties (23,31). Our finding that this is the most highly up-regulated gene confirms the reliability of our

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**Table IV. A list of the gene categories significantly over represented among the genes induced by aspirin treatment**

<table>
<thead>
<tr>
<th>Gene category</th>
<th>Up regulated</th>
<th>Total</th>
<th>Expected P value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear proteins</td>
<td>61</td>
<td>283</td>
<td>35.6</td>
<td>0.00000016</td>
</tr>
<tr>
<td>Ribosomal proteins</td>
<td>6</td>
<td>7</td>
<td>0.88</td>
<td>0.00025</td>
</tr>
<tr>
<td>Stress response proteins</td>
<td>11</td>
<td>31</td>
<td>3.9</td>
<td>0.0014</td>
</tr>
<tr>
<td>DNA damage signalling/repair proteins</td>
<td>13</td>
<td>40</td>
<td>5.0</td>
<td>0.0016</td>
</tr>
<tr>
<td>mRNA processing/turnover/transport</td>
<td>7</td>
<td>15</td>
<td>1.89</td>
<td>0.0024</td>
</tr>
<tr>
<td>Transcription activators and repressors</td>
<td>20</td>
<td>94</td>
<td>11.8</td>
<td>0.0071</td>
</tr>
<tr>
<td>Nucleotide metabolism</td>
<td>10</td>
<td>35</td>
<td>4.4</td>
<td>0.0084</td>
</tr>
<tr>
<td>Others involved in protein turnover</td>
<td>4</td>
<td>9</td>
<td>1.13</td>
<td>0.022</td>
</tr>
<tr>
<td>DNA replication</td>
<td>6</td>
<td>21</td>
<td>2.64</td>
<td>0.033</td>
</tr>
<tr>
<td>Factors of translation</td>
<td>3</td>
<td>6</td>
<td>0.76</td>
<td>0.035</td>
</tr>
<tr>
<td>Chromatin proteins</td>
<td>3</td>
<td>6</td>
<td>0.76</td>
<td>0.035</td>
</tr>
</tbody>
</table>

For each gene category the columns show the number of up-regulated genes that belonged to the category, the number of genes in the whole array belonging to the category, the number of genes in the category that would be expected to appear by chance among the up-regulated genes, and the probability of obtaining the number of genes observed. (Using the binomial distribution function.)
array technique. We further checked the reliability of our results by analysing protein levels by the immunoblotting of a number of highly affected genes. One interesting gene never found at the villus tip and this we show is also the site of highest Rac1 expression in humans and mice. This provides supporting evidence that Rac1 may be involved with colono-

### Table V.

<table>
<thead>
<tr>
<th>Gene category</th>
<th>Down regulated</th>
<th>Total</th>
<th>Expected</th>
<th>P value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclins</td>
<td>3</td>
<td>8</td>
<td>0.34</td>
<td>0.0047</td>
<td>0–1.48</td>
</tr>
<tr>
<td>Other cell cycle proteins</td>
<td>4</td>
<td>33</td>
<td>1.4</td>
<td>0.038</td>
<td>0–3.69</td>
</tr>
</tbody>
</table>

For each gene category the columns show the number of down-regulated genes that belonged to the category, the number of genes in the whole array belonging to the category, the number of genes in the category that would be expected to appear by chance among the down-regulated genes, and the probability of obtaining the number of genes observed. (Using the binomial distribution function.)

least partly due to promotion of differentiation (39), and that this may be due to its effects on RAC1.

In conclusion, we find evidence that aspirin at 5 mM concentration has very different effects on cells than at two lower concentrations, 50 and 500 μM, a finding that supports clinical evidence that aspirin at low and high dose has different effects. We find that aspirin predominantly induces genes involved with DNA damage signalling, nucleotide metabolism and the stress response. On a single gene level a member of the TGF-beta family is the most highly induced gene and Rac1 is also highly induced. Rac1 plays an important role in cell–cell adhesion and its induction by aspirin may therefore underlie the beneficial effects of aspirin in colon cancer progression.

### References


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