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Evidence for colorectal cancer cell specificity of aspirin effects on NFκB signalling and apoptosis

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Epidemiological evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs) protect against colorectal cancer (CRC) to a greater degree than other non-gastrointestinal cancers, but the molecular basis for this difference is unknown. We previously reported that aspirin induces signal-specific IκB degradation followed by NFκB nuclear translocation in CRC cells, and that this mechanism contributes substantially to aspirin-induced apoptosis. Here, we explored the hypothesis that cell-type specific effects on NFκB signalling are responsible for the observed differences in protection by aspirin against CRC compared to breast and gynaecological cancers. We also assessed whether COX-2 expression, mutation status of adenomatous polyposis coli (APC), β-catenin, p53, or DNA mismatch repair (MMR) genes in CRC lines influenced aspirin-induced effects. We found that aspirin induced concentration-dependent IκB degradation, NFκB nuclear translocation and apoptosis in all CRC lines studied. However, there was no such effect on the other cancer cell types, indicating a considerable degree of cell-type specificity. The lack of effect on NFκB signalling, paralleled by absence of an apoptotic response to aspirin in non-CRC lines, strongly suggests a molecular rationale for the particular protective effect of NSAIDs against CRC. Effects on NFκB and apoptosis were observed irrespective of COX-2 expression, or mutation status in APC, β-catenin, p53 and DNA MMR genes, underscoring the generality of the aspirin effect on NFκB in CRC cells. These findings raise the possibility of cell-type specific targets for the development of novel chemopreventative agents.

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Keywords: IκB; NFκB; NSAIDs; chemoprevention; colorectal cancer

Colorectal cancer (CRC) is common in developed countries (Parkin et al, 1999) and is a major contributor to cancer-related morbidity and mortality. Chemoprevention is an inherently appealing approach to combat the disease, and non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with a substantial reduction in CRC incidence and mortality (Thun et al, 1993; Collet et al, 1999; Langman et al, 2000). Combined case–control data, including over 30 000 CRC cases, indicate a 45% reduction in the risk of developing CRC in subjects taking NSAIDs. Although there is evidence for a protective effect of NSAIDs against non-gastrointestinal cancers, the data are less convincing and the risk reduction much less. In breast cancer, reports show conflicting results and a recent meta-analysis revealed a risk reduction of only 13% in case–control studies (Khuder and Mutgi, 2001), considerably lesser than that in CRC. Similarly, in endometrial and ovarian cancer, the available evidence suggests that NSAIDs confer little, if any, protection (Cramer et al, 1998; Rosenberg et al, 2000; Fairfield et al, 2002; Meier et al, 2002). Collectively, published data suggest that there is considerable heterogeneity of NSAID anti-tumour effect between cancer types. The particular protective effect against CRC suggests the possibility that aspirin might target distinct molecular pathways in colonic epithelial cells. Elucidation of the molecular mechanism of this apparent differential sensitivity would lend further insight into both the mode of action of NSAIDs as well as identification of molecular markers of response.

The anti-tumour activity of NSAIDs has primarily been attributed to inhibition of the cyclooxygenase-2 enzyme (COX-2) and the resultant decrease in production of prostaglandins, as this remains the best-characterised effect (Vane, 1971). However, accumulating evidence from animal and cell culture experiments has shown that COX-2 inhibition is not the sole basis of NSAID anti-tumour activity (Alberis et al, 1995; Hanif et al, 1996; Elder et al, 1997; Piazza et al, 1997), suggesting that other targets are also involved. We previously reported that aspirin activates the NFκB signalling pathway and that this mechanism is of central importance to aspirin-mediated apoptosis in CRC cells (Stark et al, 2001). The NFκB transcription factor is normally sequestered in the cytoplasm by an inhibitor protein, IκBα. Following stimulation of the NFκB pathway, IκBα is phosphorylated, ubiquitinated and targeted for proteosomal degradation. Disassociation from IκBα results in translocation of NFκB to the nucleus, where it contributes to the co-ordinated transcription of genes involved in inflammation, cell proliferation and apoptosis (Pahl, 1999). Our previous work demonstrated that aspirin induces time- and dose-dependent signal-specific degradation of IκBα, nuclear translocation of NFκB and apoptosis in CRC cells. Time-course experiments indicated that IκBα degradation and NFκB nuclear
translocation preceded cell death, suggesting a causal relationship. This was confirmed in cells we engineered to continuously express a dominant-negative mutant IκBα (IκBαs32/36), which showed inhibition of both aspirin-induced NFκB nuclear translocation and apoptosis compared to their parental counterparts (Stark et al, 2001). This work alluded to the notion of specificity since the NFκB response was not observed in the control cell lines 293 HEK and A549, which were non-colorectal in origin.

Here, we focus on the important issue of the specificity of aspirin’s protective effects, as observed in epidemiological studies, and we set out to determine whether cell-type specific effects on the NFκB signalling pathway reflect the differential protective effects of aspirin in different cancer types. In particular, we wished to determine whether the lower protective effect observed for breast, ovarian and endometrial cancer can be explained by differing effects on the NFκB signalling pathway. We also investigated the generality of the NFκB response to aspirin in CRC by studying a panel of CRC cell lines with different genetic defects common in bowel malignancy. Here, we present evidence showing clear differences in NFκB response that parallel the epidemiological data, supporting the notion that the ability of aspirin to modulate the NFκB signalling pathway is a key determinant of the anti-tumour effect and that this is cell-type specific. Our findings provide further insight into the complex mechanisms by which NSAIDs exert an anti-tumour effect in CRC cells, and raise the possibility of cell-type specific molecular targets in CRC.

MATERIALS AND METHODS

Cell line culture and treatment

The CRC cell lines used were HRT-18, SW480, HT-29, DLD-1, LoVo and HCT116; breast cancer lines were T47-D, MCF-7 and MDA-MB-231; ovarian cancer line was A2780 and endometrial cancer line was HEC-1-A. All cancer cell lines are available from the American Type Culture Collection. The mutation status for the adenomatous polyposis coli (APC), p53, β-catenin and DNA mismatch repair (MMR) genes of the cell lines studied is shown in Table 1. Cell lines were grown as monolayers (37°C in 5% CO₂) in RPMI (HRT-18, DLD-1 and A2780), DMEM (HT-29, T47-D, MCF-7, MDA-MB-231, HEC-1-A), L-15 (SW480) and McCoy's 5A media (HCT116) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (media supplied by Gibco BRL, Paisley, UK). Cells were plated (1 × 10⁵ cells/ml) and grown until 60–70% confluent, prior to treatment with aspirin or carrier control at the same concentrations as the aspirin treatment. Aspirin (Sigma, St Louis, USA) was prepared as a 0.5 M stock solution in distilled water (final pH 7.0). Growth medium was replaced with the respective low serum (0.5% FCS) medium and solution in distilled water (final pH 7.0). Growth medium was added to the tube to collect any cells dislodged during washing. Cells were then washed with 2 ml of PBS, which was also added to the tube to collect any cells dislodged during washing. Cells were incubated with 1 ml of trypsin:versene (volume per volume) just until the cells detached and then resuspended in the conical tube containing the media with the floating and washed cells. Cells were counted using a haemocytometer and resuspended in cold 1 × binding buffer to approximately 1 × 10⁶ cells ml⁻¹. Media-binding reagent (10 μl) was added to 0.5 ml of the cell suspension, which was incubated with 1.25 μl of annexin V-FITC for 15 min at room temperature in the dark. Annexin V was then removed by centrifugation at 1000 g for 5 min and the cells were resuspended in 0.5 ml of cold 1 × binding buffer and placed on ice. The counting was done using a haemocytometer (two counting grids) in duplicate and this was carried out immediately following staining of cells, as apoptosis is an ongoing process and the FITC signal may be lost after an hour.

Western blotting

Cells were washed with PBS, centrifuged (1200 r.p.m., 10 min) and cell pellets resuspended in lysis buffer (50 mM NaCl, 10 mM HEPES, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% Triton X-100) containing complete Protease Inhibitor Cocktail and 100 mM Pefabloc (Roche Diagnostics, Manheim, Germany). The cell suspension was centrifuged (6000 r.p.m., 15 min, 4°C) and the supernatant containing cytoplasmic proteins aliquotted. Protein content was measured by the method of Bradford (BioRad, Hercules, California, USA). Cytoplasmic proteins (30 μg) were separated on a 10% SDS–PAGE gel, transferred to a polyvinylidene difluoride membrane (BioRad) and blocked in 4% non-fat dry milk solution with 0.3% Tween20 (Sigma). Membranes were probed with a sheep polyclonal IκBα antibody (a gift from Professor R Hay, University of St Andrews, UK), rabbit polyclonal p65 antibody (Santa Cruz, California, USA) or mouse monoclonal COX-2 antibody (Cayman Chemicals, Michigan, USA). COX-2 electrophoresis standard (Cayman Chemicals or mouse monoclonal COX-2 antibody (Cayman Chemicals, Michigan, USA). Cytoplasmic proteins (30 μg) were separated on a 10% SDS–PAGE gel, transferred to a polyvinylidene difluoride membrane (BioRad) and blocked in 4% non-fat dry milk solution with 0.3% Tween20 (Sigma). Membranes were probed with a sheep polyclonal IκBα antibody (a gift from Professor R Hay, University of St Andrews, UK), rabbit polyclonal p65 antibody (Santa Cruz, California, USA) or mouse monoclonal COX-2 antibody (Cayman Chemicals, Michigan, USA). COX-2 electrophoresis standard (Cayman Chemicals) was used to indicate the correct COX-2 band. Monoclonal antibody to Cu/Zn SOD (The Binding Site, Birmingham, UK) and to actin (Santa Cruz) was used as a control for protein loading. Antigen–antibody complexes were visualised with chemiluminescence (Amersham ECL Reagents, UK).

Immunofluorescence analysis

Cells grown to 60–70% confluence on glass coverslips were treated with carrier or 10 μM aspirin for 24 h (in the respective 0.5% FCS medium). After treatment, cells were washed with PBS, fixed with acetone: methanol (volume per volume) (−20°C, 10 min) and blocked in 10% pre-immune donkey serum (Sigma) for 1 h. Rabbit polyclonal antibody to NFκB p65 (Santa Cruz) was applied for 1 h, followed by incubation with FITC-conjugated donkey anti-rabbit IgG for 1 h. The nuclei were stained with DAPI and the coverslips mounted with Vectorshield (Vector Laboratories, Burlingame, California, USA).

| Table 1 | Mutation status of cancer cell lines studied |
|---------|-------------------------------------------|
| Cell line | APC | β-catenin | p53 | MMR |
| HRT-18 | Mutant | Wild type | Mutant | Deficient |
| SW480 | Mutant | Wild type | Mutant | Proficient |
| HT-29 | Mutant | Wild type | Mutant | Proficient |
| DLD-1 | Mutant | Wild type | Mutant | Deficient |
| LoVo | Mutant | Wild type | Wild type | Deficient |
| HCT-116 | Wild type | Mutant | Wild type | Deficient |
| MCF-7 | Wild type | Wild type | Wild type | Proficient |
| MDA-MB231 | Wild type | Wild type | Mutant | Not known |
| T47D | Wild type | Wild type | Mutant | Proficient |
| A2780 | Wild type | Wild type | Wild type | Proficient |
| HEC-1-A | Not known | Not known | Not known | Deficient |

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RESULTS

Colorectal cancer cells are more susceptible to aspirin-induced apoptosis than non-CRC cells

We studied the effect of aspirin on the growth of a panel of CRC cell lines (HRT-18, SW480, HT-29, DLD-1, LoVo and HCT116) in comparison to cell lines derived from other cancer types: breast (MCF-7, MDA-MB-231, T47D), ovarian (A2780) and endometrial (HEC-1-A). The non-CRC cell lines were chosen based on epidemiological data, where there is some evidence to suggest a protective effect in breast cancer and less so in ovarian and endometrial cancer.

In triplicate dose–response experiments, cell lines were treated for 24 h with aspirin at concentrations of 1, 3, 5 and 10 mM and viable cell number determined by haemocytometric counts. We found a concentration-dependent decrease in viable cell number in each of the six CRC cell lines studied (Figure 1A). In contrast, there was no demonstrable effect of aspirin on the viability of the non-CRC cell lines MCF-7, MDA-MB-231, A2780 and HEC-1-A (Figure 1A). Interestingly, the T47D breast cancer cells did exhibit a dose-dependent reduction in viability, although this effect was not so pronounced as that seen in CRC cells at low aspirin concentrations. The IC50 values were calculated from the growth curves of the aspirin-treated CRC cell lines only, as there was no consistent reduction in cell viability in the non-CRC cell lines (Table 2). The mean IC50 value for the CRC cell lines was 2.38 mM and the greatest incremental reduction in viability in these cells was observed between 0 and 1 mM concentrations, which is comparable to serum concentrations attainable in humans (Pachman et al, 1979).

We next wished to establish whether the reduction in viable cell number that we observed in the CRC cell lines was due to induction of apoptosis. Annexin-V binding of phosphatidylserine residues externalised during apoptosis was used to determine the proportion of cells undergoing programmed cell death in response to increasing concentrations of aspirin. We found that aspirin treatment induced a concentration-dependent increase in apoptosis in all six of the CRC cell lines studied, confirming that induction of apoptosis is responsible for the observed reduction in cell viability (Figure 1B). There was no dose-dependent increase in apoptosis in the non-CRC cell lines following aspirin treatment, which was consistent with the lack of effect on cell viability (Figure 1A).

Since these findings suggested a cell-type specific NFκB response to aspirin, we next determined whether the disparate IκBα response was accompanied by a differential effect on NFκB nuclear translocation in the CRC compared to the non-CRC cell lines. Immunofluorescence analysis showed that p65, the transcriptionally active subunit of NFκB, was primarily located in the cytoplasm in untreated cells as expected (Figure 2C, D, first panel). Following aspirin treatment, there was nuclear accumulation of p65 in all of the CRC cell lines (Figure 2C, second panel). However, in keeping with our observation that there was no IκBα degradation in the non-CRC cells, aspirin treatment did not induce nuclear translocation of p65 in any of these cell lines (Figure 2D, second panel). These data establish that the disparity in viability following exposure to aspirin in CRC cell lines compared to lines derived from other cancer types is associated with markedly differing responses of the NFκB pathway to aspirin. This work suggests that the effect of aspirin on NFκB signalling may be implicated in the differential sensitivity of cancer types to aspirin-induced apoptosis.

 Basal IκBα and p65 protein levels and aspirin-induced apoptosis in CRC cell lines

High basal NFκB activity and aberrant IκBα expression have been observed in a number of cancers including CRC (Rayet and Gelinas, 1999). In view of our findings of a cell-type specific NFκB and death response to aspirin, we considered whether the basal levels of IκBα and p65 might determine increased sensitivity to apoptosis, and so could be potential molecular markers of response. We used immunoblot analysis of cytoplasmic extracts to examine basal levels of IκBα and p65 in both the CRC and non-CRC cell lines (Figure 3). There was no difference in expression of IκBα or p65 or their relative levels (analysed by densitometry, data not shown) between colorectal and non-CRC cells that could account for increased sensitivity to apoptosis. These results indicate that sensitivity to aspirin-induced apoptosis is not related to the cytoplasmic pool of either protein available for stimulation.

 Basal COX-2 protein levels do not determine the NFκB response to aspirin

Increased COX-2 expression has been observed both in premalignant colonic lesions and CRCs (Eberhart et al, 1994), and COX-2 inhibition has been shown to play a role in aspirin-mediated cell death (Boobool et al, 1996). Hence, we considered whether COX-2 expression might explain the heterogeneity of the aspirin response between the CRC and non-CRC cell lines. Immunoblot analysis of
cytoplasmic proteins demonstrated considerable variation in basal levels of COX-2 between the CRC cell lines (Figure 4). The CRC cell lines SW480 and HCT116 do not express COX-2, whereas HT-29 and LoVo do express COX-2, and yet all underwent apoptosis following aspirin treatment. In the non-CRC panel, the MCF-7 cell line does not express COX-2 but the MDA-MB-231 cell line does.
express COX-2, but neither undergoes aspirin-induced apoptosis. Similarly, there was variability of COX-2 levels between the CRC lines and the non-CRC lines (Figure 4). Thus, we found no association between basal levels of COX-2 expression and sensitivity to aspirin-induced apoptosis, providing further support for the notion that COX-independent mechanisms play an important role in the anti-tumour effect of NSAIDs.

**DISCUSSION**

The work presented here demonstrates a striking difference in the response to aspirin between CRC cell lines and lines derived from other cancer types, with respect to both cell viability and NFκB signalling. We show that aspirin-induced apoptosis, associated with IκBα degradation and NFκB nuclear translocation, was restricted to CRC cells. This relationship between aspirin-induced apoptosis and the effect on NFκB signalling suggests a molecular rationale for the particular sensitivity of CRC to NSAIDs compared to other cancers. These findings also extend our previous observations on the importance of the NFκB pathway as a key NSAID target.

Epidemiological evidence indicates that NSAIDs impart greater protection against CRC than other cancer types, but the molecular basis for this effect is not known. Several previous reports, including our own, have shown that aspirin induces apoptosis in CRC cells (Hanif et al, 1996; Elder et al, 2001; Planchon et al, 1995). The graphs represent three independent experiments and the bars on the graphs are standard error bars.

| Cell line | IC50 (mM) |
|-----------|-----------|
| HRT-18    | 3.12 ± 0.69  |
| SW480     | 1.48 ± 0.12  |
| HT-29     | 1.98 ± 0.68  |
| DLD-1     | 2.92 ± 0.58  |
| LoVo      | 2.07 ± 0.25  |
| HCT-116   | 2.71 ± 0.46  |

**Table 2** IC50 values for colorectal cancer cell lines

The observed differences in sensitivity to aspirin-induced apoptosis between CRC cell lines and non-CRC cell lines (Figure 4) provide further support for a causal role of the NFκB pathway in the differential response to aspirin between CRC cells and other cancer types. These findings also extend our previous observations on the importance of the NFκB pathway as a key NSAID target.

Epidemiological evidence indicates that NSAIDs impart greater protection against CRC than other cancer types, but the molecular basis for this effect is not known. Several previous reports, including our own, have shown that aspirin induces apoptosis in CRC cells (Hanif et al, 1996; Todd et al, 1997; Planchon et al, 1995). The graphs represent three independent experiments and the bars on the graphs are standard error bars.

**Figure 1** Differential effect of aspirin on cell viability and apoptosis in CRC and non-CRC cell lines. Aspirin treatment (0–10 mM) for 24 h induces a concentration-dependent decrease in viable cell number (determined by haemocytometric counts) in all CRC cell lines, but there is no consistent change in the non-colorectal cancer cell lines (A). Annexin V binding assay is used to determine whether all CRC cell lines undergo apoptosis after aspirin treatment (0–5 mM) for 24 h, but there was no change in apoptosis in the non-CRC cell lines (B). Annexin V binding assay is used to determine whether the non-CRC cell lines are less susceptible to aspirin-induced apoptosis compared to the CRC cell line SW480 following treatment for 72 h with aspirin (0–3 mM) (C). The graphs represent three independent experiments and the bars on the graphs are standard error bars.
Figure 2  Aspirin-induced IxBz degradation and p65 nuclear translocation is restricted to CRC lines. Western blot analysis shows that aspirin treatment (0–10 mM) for 24 h induces IxBz degradation in a concentration-dependent manner in the CRC cell lines (A), but not in the non-CRC cell lines (B). Following aspirin treatment, cytoplasmic extracts were made from untreated and treated cells and probed with sheep polyclonal IxBz antibody. The western blot shown is representative of at least three independent experiments, and Cu/Zn SOD was used as a control for protein loading. Micrographs (×63) of immunocytochemically stained cells show that aspirin treatment (10 mM) for 24 h induces nuclear accumulation of p65 in the CRC cell lines (C), but not in the non-CRC cell lines (D).
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Aspirin concentrations used here are relevant to pharmacological levels in clinical practice (1–3 mIu) (Insel, 1996). Nonetheless, comparisons between cell culture concentrations and plasma levels are somewhat artificial, because of the inability to accurately mimic in vivo metabolism and tissue concentration of the agent in epithelial or tumour cells. Decreased basal levels of apoptosis and hyperproliferative mucosa have been observed in patients with adenomas, suggesting the existence of a ‘field effect’ in the colonic mucosa (Antl et al., 2001). Although we observed proportionally less apoptosis at lower concentrations of aspirin, there is evidence that low levels of apoptosis translate into significant tumour regression over time in cell kinetics studies (Pritchard and Watson, 1996). It remains to be determined whether aspirin redresses the balance by inducing apoptosis de novo in newly transformed colorectal epithelial cells destined to become malignant clones. There is evidence of NFκB involvement in colonic crypt differentiation and cell turnover in mouse colon, where NFκB activity is greater in proliferating cells at the base of crypts compared to mature cells at the surface (Inan et al., 2000). Thus, it is also possible that the drug corrects deranged mechanisms that permit escape from normal cellular turnover and apoptosis.

In summary, the data presented here demonstrate that there are substantial differences in the anti-tumour effects of aspirin and modulation of NFκB signalling between cancer cells of different tissue origin. The effect of aspirin on NFκB signalling and apoptosis does not appear to be related to expression levels of COX-2 or mutation status of APC, β-catenin, p53 and DNA MMR genes. This is important when considering translating these findings to clinical studies aimed at defining the NFκB response to aspirin in human colonic epithelium and tumours. The molecular basis of NSAID anti-tumour activity is complex, and our findings provide further evidence that the effects of aspirin on NFκB signalling have particular relevance to CRC chemoprevention.

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