Aspirin and alterations in DNA repair proteins in the SW480 colorectal cancer cell line

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Abstract. Regular aspirin intake is associated with a reduction in the incidence of colorectal cancer. Aspirin has been shown to be cytotoxic to colorectal cancer cells *in vitro*. The molecular basis for this cytotoxicity is controversial, with a number of competing hypotheses in circulation. One suggestion is that the protective effect is related to the induction of expression of the DNA mismatch repair (MMR) proteins hMLH1, hMSH2, hMSH6 and hPMS2 in DNA MMR proficient cells. We report that treatment of the DNA MMR competent/p53 mutant colorectal cancer cell line SW480 with 1 mM aspirin for 48 h caused changes in mRNA expression of several key genes involved in DNA damage signalling pathways, including a significant down-regulation in transcription of the genes *ATR*, *BRCA1* and *MAPK12*. Increases in the transcription of *XRCC3* and *GADD45α* genes are also reported. Regulation of these genes could potentially have profound effects on colorectal cancer cells and may play a role in the observed chemoprotective effect of aspirin *in vivo*. Although a correlation was not seen between transcript and protein levels of *ATR*, *BRCA1* and *GADD45α*, an increase in *XRCC3* encoded protein expression upon aspirin treatment in SW480 cells was observed by immunoblotting, immunofluorescence and immunohistochemical analysis. This is the first report of *XRCC3* gene transcription and encoded protein expression being susceptible to exposure to the non-steroidal anti-inflammatory drug, aspirin. Furthermore, this study indicates that alterations in gene transcription seen in microarray studies must be verified at the protein level.

Introduction

More than 940,000 colorectal cancer cases occur annually worldwide whilst approximately half a million patients die each year (1). There is abundant evidence that regular ingestion of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, can promote colorectal tumour regression and reduce the relative risk of developing colorectal cancer (2-6). There is also substantive evidence that aspirin is specifically cytotoxic against colorectal cancer cells cultured *in vitro* (7) and in animal models aspirin can inhibit cancers induced by carcinogens such as 1,2-dimethylhydrazine (8 and refs. therein).

The molecular basis for the anti-proliferative effect of NSAIDS, including aspirin, on colorectal cancer cells is controversial [reviewed in ref. (9)], with a range of theories in circulation such as; cyclooxygenase (COX) inhibition (7,10), NF-κB inhibition (11-13), NF-κB activation (14,15), down-regulation of Bcl-2 expression (16,17), thus making the cells less resistant to the initiation of apoptosis, and down-regulation of c-Myc, cyclin D1, cyclin A and proliferating cell nuclear antigen (PCNA) (18), the intrinsic antioxidant activity of aspirin preventing double stranded DNA breaks (19), aspirin induces translocation of Bax to mitochondria (20), NSAIDSs up-regulate 15-lipoxygenase-1 expression (21), inhibition of cytosolic phospholipase A_2 expression (22), depletion of intracellular polyamines (23), increased Rac 1 expression (24), increased NSAID-activated gene (NAG-1) protein (25 and refs. therein), selection for microsatellite stability (26), and induction of the DNA mismatch repair (MMR) proteins hMLH1, hMSH2, hMSH6 and hPMS2 in DNA MMR proficient cells, which ultimately facilitates programmed cell death (27). Identifying and understanding pathway modulation by aspirin is possibly key to understanding its chemopreventative effect on colorectal cancer.

Microarray analysis has been extensively utilised to examine alterations in gene transcription in response to NSAID exposure in a number of colon cancer cell lines with a subsequent large list of alterations in gene expression reported (24,28-31). This study aimed to investigate the relationship between aspirin and DNA repair genes, utilising a commercially available PCR array to analyse the expression of 84 different genes involved in DNA damage signalling pathways in the colorectal cancer cell line SW480 upon aspirin treatment. We report that *XRCC3* gene and protein expression is up-regulated in SW480 cells as a consequence of exposure to physiologically achievable levels of aspirin. Whilst *BRCA1* and *ATR* were noted to be significantly down-

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regulated at the mRNA level, there was no overt down-regulation of protein expression. We also show in concordance with previous reports (29,32) that *GADD45α* is induced at the mRNA level by cultured cells exposed to a NSAID.

Materials and methods

Cell culture and treatment. All materials were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK) unless otherwise stated. The human colon cancer cell line SW480 (MMR-proficient) was obtained from the European Collection of Cell Culture (ECACC, Health Protection Agency, Wiltshire, UK). Cells were cultured *in vitro* in 75 cm^2 culture flasks in L-15 Leibovitz medium supplemented with 10% Fetal Calf Serum (PAA Laboratories Ltd., Somerset, UK) and 1% (w/v) antibiotic solution [L-glutamine-penicillin-streptomycin solution (stabilised antibiotic solution)] and maintained at 37˚C.

For experimental treatments, aspirin was dissolved in 1 M Tris-HCl (pH 7.5) to a stock concentration of 0.5 M and pH adjusted to 7.2 with 4 M HCl [as described previously in (27)].

Cell viability. The effect of aspirin on cellular viability was measured by the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay (34). Cells were seeded into 96-well plates (10^4 cells per well). After 24 h of seeding, the cells were treated for 24, 48 and 72 h by adding various volumes of stock to obtain final concentrations of 0.5, 1, 3, 5 or 10 mM of aspirin. Control cells were treated with an equivalent volume of Tris-HCl (pH 7.2). After treatment, the cells were incubated with MTT (0.5 mg/ml) for 3 h at 37˚C. The insoluble formazan product was solubilised with DMSO. MTT conversion was determined by colourimetric analysis at 540 nm. Results were calculated from the average of three separate experiments with each experiment performed in quintuplicate and reported as the percentage of treated cells relative to the cells treated with medium alone.

RT² Profiler™ PCR Array. For PCR array analysis, the commercially available Human DNA Damage Signaling Pathways RT² Profiler™ Array (SuperArray Biosciences Corp., Frederick, MD) of 84 genes related to the DNA damage signalling pathway was utilised. Five reference genes (*B2M*, *HPRT1*, *RPL13A*, *GAPDH*, *ACTB*), RNA quality control, reverse transcription and PCR efficiency controls are included on the 96-well plate of the array.

At ~60% confluency, cells were treated with 1 mM aspirin and incubated at 37˚C for 48 h. Control cells were treated with an equivalent volume of Tris-HCl (pH 7.2). After 48 h flasks were washed with ice cold 1X PBS and total RNA was extracted using RNAqueous-4PCR kit for isolation of DNA-free RNA utilising DNase I treatment (Ambion, Austin, TX). Total RNA concentration was then quantified using a Pharmacia GeneQuant II spectrophotometer.

Total RNA (500 ng) was reverse transcribed using 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche, Hertfordshire, UK). Resulting cDNA was diluted 1 in 10 in nuclease-free water and added to SYBR Green Fluorescein Master Mix (PrimerDesign, Southampton, UK) which was subsequently aliquoted to each well of the PCR array for quantitative PCR. Thermal cycling and fluorescence detection were performed using a Biorad iCycler (Bio-Rad Laboratories, UK). The amplification process was as per the SA Biosciences protocol; briefly, the PCR conditions were 10 min denaturation at 95˚C followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C with melt curve analysis carried out with the following parameters: 1 min at 95˚C followed by 1 min at 55˚C, followed by 80 cycles of 10 sec at 55˚C with an increase in temperature after the second cycle by 0.5˚C. Five untreated samples and five treated samples were run on individual plates and data were analyzed with the Excel-based PCR Array Data Analysis Template downloaded from the SuperArray website (www.sabiosciences.com) and normalised to the expression level of housekeeping control genes provided on the arrays.

Immunoblot analysis. For protein extraction, cells (untreated or treated with 1 mM aspirin) were repeatedly washed with PBS, then for GADD45α and XRCC3 protein analysis, protein was extracted using protein extraction buffer (20 mM Tris/150 mM NaCl/1 mM EDTA/1% Triton X) on ice for 1 h, and centrifuged at 13000 g to remove cell fragments. Protein was quantified using Bio-Rad's DC Protein Assay as per manufacturer's instructions. Extracted cellular proteins were then dissolved in 2X Laemmli sample buffer, and analysed by discontinuous 12% SDS-PAGE, transferred to membrane and probed for protein expression. For XRCC3 and GADD45α gels, resolved proteins were transferred onto nitrocellulose membrane (Bio-Rad) at 100 v for 1 h by wet-transfer by standard protocol (Bio-Rad Laboratories). The nitrocellulose was blocked with 5% (w/v) non-fat milk/0.2% (v/v) Tween-20 in TBS for 60 min at room temperature and incubated with primary antibody overnight at 4˚C, prior to repeated rinsing with 0.2% (v/v) Tween-20 in TBS, then incubated with a secondary HRP conjugated antibody for 60 min at room temperature, repeatedly rinsed with 0.2% (v/v) Tween-20 in PBS and visualised using ECL reagent according to manufacturer's instructions with a STORM Phosphorimager (GE Healthcare UK Limited, Buckinghamshire, UK).

For BRCA1 and ATR protein analysis, samples were extracted using RIPA buffer [10% NP-40 (v/v)/10% Na-deoxycholate (v/v)/100 mM EDTA in Tris buffer pH 7.4 including Protease inhibitor cocktail tablet (Roche), Phosphatase inhibitor cocktail 1, Phosphatase inhibitor cocktail 2 and 200 mM PMSF]. Cell pellets were thawed on ice, re-suspended in 3 bed volumes of extraction buffer and incubated on ice for 20 min. Whole cell extracts were obtained by centrifuging samples at 13000 rcf at 4˚C for 15 min and samples were quantified using BCA Protein Assay kit (Pierce, Rockford, IL, USA). Protein samples were analysed using pre-cast 3-8% Tris-Acetate gels (Invitrogen, Paisley, UK). Resolved proteins were transferred onto PVDF membrane (which had been equilibrated in 100% methanol and then equilibrated in Transfer buffer for 15 min prior to transfer) at 350 mA for 1 h. Before blocking, PVDF membrane was re-hydrated in 100% methanol and washed briefly in PBS. The membrane was soaked in blocking buffer [4% (w/v) Marvel™ in PBS] for 1 h at room temperature and incubated with primary antibody (diluted in blocking buffer) overnight at 4˚C. The membrane was then washed 3 times in wash solution

[0.1% (v/v) Tween-20 in PBS] for 5 min each time and incubated with secondary antibody [diluted in 5% (w/v) Marvel™ /0.1% (v/v) Tween-20 in TBS] for 1 h at room temperature. The membrane was again washed 3 times using wash solution for 5 min each time and then incubated with Western Lightning™ Plus-ECL oxidising reagent plus, enhanced Luminol reagent plus (Perkin-Elmer Life Sciences). The membrane was then analysed using Intelligent Dark box, LAS-3000 (Fujifilm) and Image Reader, LAS-3000 with an exposure time of 20 min.

Samples were quantified to ensure equal concentrations of samples were loaded for comparison studies (20 μg total protein). To illustrate equal band loading samples were probed for ß-tubulin protein expression using a monoclonal anti-ß-tubulin antibody (Sigma) or monoclonal anti-α-tubulin antibody (Abcam, Cambridge, UK).

The primary antibodies used for immunoblotting analysis were anti-XRCC3 antibody (1:5000 dilution; Abcam), anti-BRCA1 antibody (1:1000; Cell Signalling Technology), anti-ATR antibody (1:100; Cell Signalling Technology) and anti-GADD45α antibody (1:250; Santa Cruz Biotechnology).

Immunofluorescence analysis. For immunofluorescence analysis, briefly, cells were fixed using a methanol/acetone mixture, washed and blocked with 5% BSA (w/v)/0.2% (v/v) Tween-20 in PBS then repeatedly washed with 0.2% (v/v) Tween-20 in PBS, and incubated with primary antibody at room temperature for 60 min. Cells were again washed with 0.2% (v/v) Tween-20 in PBS prior to incubation with the secondary antibody and incubated for 60 min at room temperature. Cells were washed with 0.2% (v/v) Tween-20 in PBS, then transferred to a glass slide whereupon they were analysed using a Carl Zeiss LSM 510 Meta confocal microscope. Primary antibodies used were the same as for immunoblotting analysis. Secondary antibodies used were donkey anti-rabbit IgG-FITC and donkey anti-mouse IgG-Texas Red (both Santa Cruz Biotechnology).

Immunohistochemical analysis and analytical digital photomicroscopy (ADP). For immunohistochemical analysis, briefly, cells were fixed using a methanol/acetone mixture, washed and blocked with 5% BSA (w/v)/0.2% (v/v) Tween-20 in PBS then repeatedly washed with 0.2% (v/v) Tween-20 in PBS, and incubated with anti-XRCC3 antibody (1:5000 dilution, Abcam), at room temperature for 60 min. Cells were again washed with 0.2% (v/v) Tween-20 in PBS prior to incubation with the anti-rabbit-HRP conjugated secondary antibody (1:1000 dilution, Oncogene Research Products) and incubated for 60 min at room temperature. Cells were washed with 0.2% (v/v) Tween-20 in PBS, then incubated with 1-Step™ TMB substrate (Pierce) for 4 min before the reaction was stopped with ultra-pure water. The coverslips were then mounted onto slides with mounting medium (Dako, CA, USA). Images were obtained using a Nikon Eclipse TS100 inverted microscope and Nikon DS-Fi1 camera head and subsequently analysed using analytical digital photomicroscopy (ADP) as outlined by Biocolor Ltd., UK (www.biocolor.co.uk). Briefly, the digitally acquired images were posterized using Adobe® Photoshop® software: blue pixels (as the substrate conversion resulted in a subtle blue stain) were counted in each image and recorded to give semi-quantitative values for comparison analysis. All photographs were taken at a constant magnification and all fields of view were of similar confluence. Three photographs were taken of each slide to obtain an average pixel value.

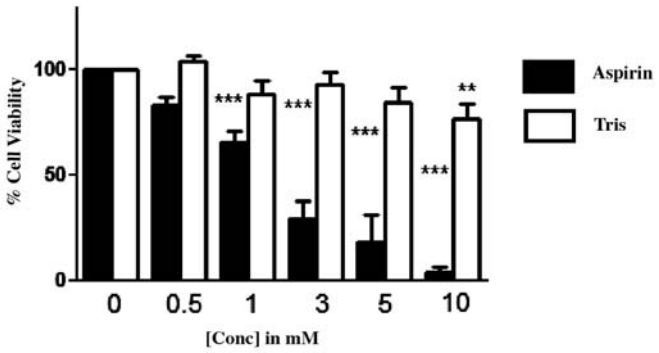

Figure 1. Cell viability analysis of SW480 cells when treated with aspirin for 48 h. Forty-eight hour incubation with 1 mM aspirin caused significant decreases in SW480 cell viability as assessed by MTT assay. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software. All graphs are displayed as means ± SEM from 3 experiments performed in quintuplicate. $^{**}P<0.05$ and $^{***}P<0.005$ (One-way ANOVA with Tukey's multiple comparison test). In control experiments, cells were treated with equivalent levels of Tris buffer to determine toxicity associated with carrier molecule used.

Statistical analysis. Graphical representations of changes in cell viability were calculated using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA). For each set of experiments, where 'n', is stated; 'n' refers to individual experiments, each run in quintuplicate. All graphs are displayed as means ± SEM.

Results

Microarray analyses of colorectal cells exposed to non-steroidal anti-inflammatory drugs (such as aspirin) consistently point to an altered and increased expression of the *GADD45α* gene. With respect to alterations to expression of other DNA repair genes, Hardwick *et al* reported that 5 mM aspirin can induce a 2.5-fold induction of *DNA-damage-inducible transcript 3* (24), whilst Iizaka *et al* reported that *uracil DNA glycosylase* and *XRCC5* were down-regulated by sulindac in SW480 and SW948 colorectal cancer cells (29). In light of the likely long-term nature of aspirin treatment as a chemoprotective agent, we reasoned that it would be informative with respect to understanding the effect of the use of aspirin on colorectal cell genome stability to re-examine DNA repair gene expression, using a dedicated PCR array, and exposing cells to 'physiological' levels (1 mM) of aspirin. We utilised the well-studied SW480 colorectal cell line, which is DNA mismatch repair competent but is compromised for the *APC* 'gatekeeper' and *p53* 'guardian' genes and does not express cyclooxygenase-2 (COX-2) (35), as a reasonable *in vitro* surrogate for colorectal cancer.

Aspirin reduces cell viability at physiological levels. The effect of clinically relevant concentrations of aspirin on the SW480 cell line are shown in Fig. 1 as assessed in the MTT

Table I. Comparison of the transcriptional level of 84 genes important for DNA damage signalling pathways analysed by RT² Profiler™ PCR Array.

Accession no.	Gene name	T-test P-value	Fold difference (treated/untreated)
Hs. 431048	*ABL1*	0.14	3.03
Hs.601206	*ANKRD17*	0.16	0.30
Hs.73722	*APEX1*	0.42	0.73
Hs.367437	*ATM*	0.46	0.59
Hs.271791	***ATR***	**0.001**	**0.24**
Hs.533526	*ATRX*	0.83	1.29
Hs.194143	***BRCA1***	**0.04**	**0.15**
Hs.519162	*BTG2*	0.46	1.84
Hs.292524	*CCNH*	0.08	0.09
Hs.184298	*CDK7*	0.34	0.63
Hs.24529	*CHEK1*	0.19	0.52
Hs.291363	*CHEK2*	0.40	0.57
Hs.135471	*CIB1*	0.54	1.28
Hs.249149	*CIDEA*	0.48	0.48
Hs.151573	*CRY1*	0.10	0.42
Hs.290758	*DDB1*	0.37	0.72
Hs.505777	*DDIT3*	0.71	0.83
Hs.339396	*DMC1*	0.74	0.81
Hs.435981	*ERCC1*	0.74	0.82
Hs.487294	*ERCC2*	0.21	1.79
Hs.498248	*EXO1*	0.34	0.70
Hs.591084	*FANCG*	0.26	6.45
Hs.409065	*FEN1*	0.08	0.48
Hs.292493	*G22P1*	0.41	0.40
Hs.80409	***GADD45α***	**0.16**	**14.78**
Hs.9701	*GADD45γ*	0.77	0.75
Hs.545196	*GML*	0.17	0.19
Hs.577202	*GTF2H1*	0.84	1.09
Hs.191356	*GTF2H2*	0.62	0.67
Hs.386189	*GTSE1*	0.83	1.22
Hs.152983	*HUS1*	0.63	0.64
Hs.503048	*IGHMBP2*	0.23	0.45
Hs.17253	*IHPK3*	0.62	0.38
Hs.61188	*KUB3*	0.60	0.79
Hs.1770	*LIG1*	0.17	0.55
Hs.463978	*MAP2K6*	0.98	0.99
Hs.432642	***MAPK12***	**0.05**	**0.41**
Hs.35947	*MBD4*	0.18	0.52
Hs.195364	*MLH1*	0.56	0.59
Hs.436650	*MLH3*	0.53	0.81
Hs.509523	*MNAT1*	0.61	0.58
Hs.459596	*MPG*	0.47	0.46
Hs.192649	*MRE11A*	0.97	1.02
Hs.597656	*MSH2*	0.23	0.65
Hs.280987	*MSH3*	0.71	0.77
Hs.271353	*MUTYH*	0.84	1.22
Hs.396494	*N4BP2*	0.71	0.63
Hs.492208	*NBS1*	0.22	8.14
Hs.66196	*NTHL1*	0.39	1.77
Hs.380271	*OGG1*	0.63	0.79
Hs.20930	*PCBP4*	0.11	0.21
Hs.147433	*PCNA*	0.28	0.64
Hs.424932	*PDCD8*	0.71	0.80
Hs.111749	*PMS1*	0.49	1.58
Hs.632637	*PMS2*	0.11	0.66
Hs.225784	*PMS2L3*	0.69	0.86
Hs.78016	*PNKP*	0.60	1.97
Hs.631593	*PPP1R15A*	0.33	0.40
Hs.491682	*PRKDC*	0.86	1.16
Hs.531879	*RAD1*	0.86	1.10
Hs.16184	*RAD17*	0.65	1.30
Hs.375684	*RAD18*	0.87	1.21
Hs.81848	*RAD21*	0.27	0.65
Hs.128904	*RAD50*	0.16	9.79
Hs.631709	*RAD51*	0.83	1.42
Hs.172587	*RAD51L1*	0.07	0.47
Hs.240457	*RAD9A*	0.11	0.28
Hs.546282	*RBBP8*	0.94	1.03
Hs.443077	*REV1L*	0.84	0.84
Hs.461925	*RPA1*	0.89	0.95
Hs.408846	*SEMA4A*	0.18	9.55
Hs.591336	*SESN1*	0.46	2.00
Hs.211602	*SMC1L1*	0.22	0.55
Hs.81424	*SUMO1*	0.91	0.92
Hs.408312	*TP53*	0.17	0.57
Hs.192132	*TP73*	0.65	0.43
Hs.344812	*TREX1*	0.82	0.74
Hs.191334	*UNG*	0.63	0.87
Hs.591907	*XPA*	0.85	1.09
Hs.475538	*XPC*	0.86	0.94
Hs.98493	*XRCC1*	0.30	0.61
Hs.647093	*XRCC2*	0.78	1.42
Hs.592325	***XRCC3***	**0.05**	**7.37**
Hs.444451	*ZAK*	0.32	2.46

Data was analyzed with the Excel-based PCR Array Data Analysis Template downloaded from the SuperArray website (www.sabiosciences.com) and normalised to the expression level of reference control genes provided on the arrays. Genes in bold are those which showed a significant fold difference in gene expression ($P<0.05$). *GADD45α* is also in bold.

viability assay. As noted by a number of other researchers (7,31), we find that aspirin can exhibit a dose-dependent inhibition of proliferation of colorectal cell lines. We have also shown that low levels of aspirin are more toxic to colorectal cancer cells than other cancer types such as glioma (U373MG) and breast cancer (MCF-7, MDA-231-MB) cells (data not shown). We were particularly interested in the effect of aspirin at relatively low levels (~1 mM) in comparison to other studies where microarray analysis has been utilised [see for example (30,31)], because we wished to ensure that the results obtained were as physiologically

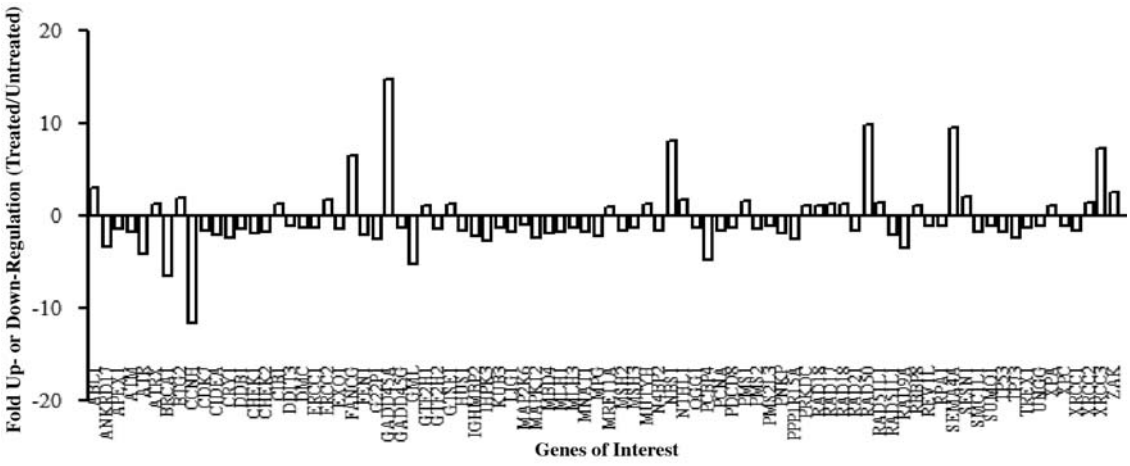

Figure 2. Graphical representation of Table I. Fold (up or down) alteration of gene transcription after treatment with 1 mM aspirin for 48 h compared to untreated samples employing the RT^2 Profiler™ PCR Array. Twenty seven out of 84 genes were up-regulated whilst 57 were down-regulated.

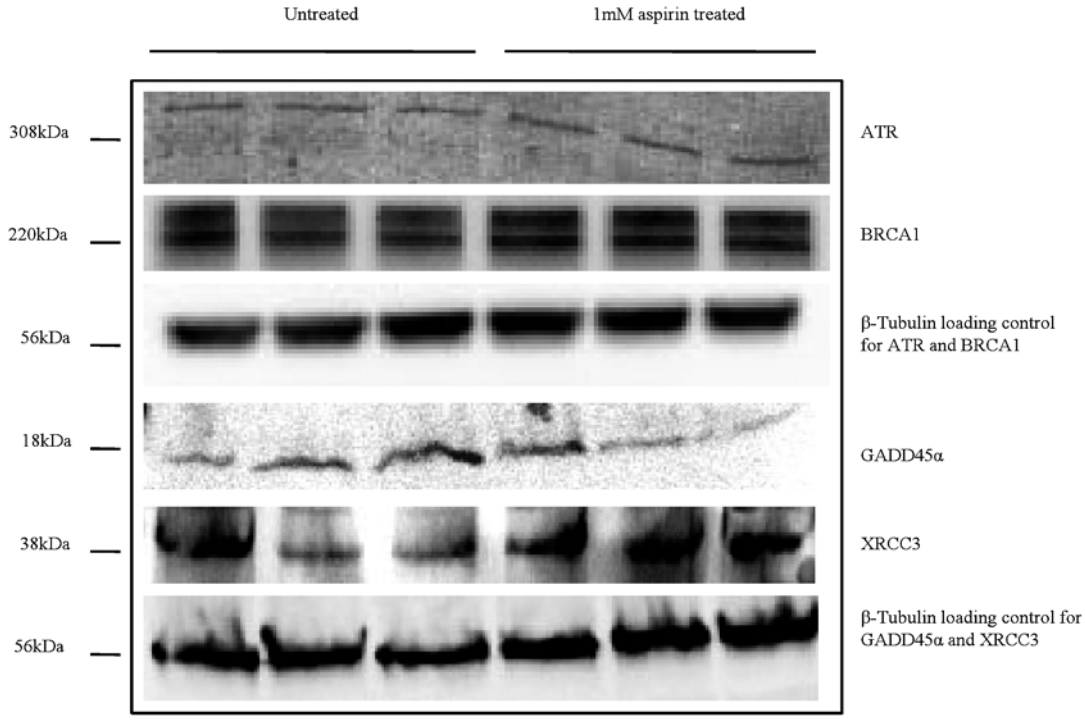

Figure 3. Immunoblot analysis demonstrating changes in XRCC3 but not ATR, BRCA1 or GADD45α protein expression upon 1 mM aspirin treatment over a 48-h period compared with untreated samples in SW480 cells. Immunoblot analysis was used to examine alterations in expression of repair proteins in SW480 cells exposed to aspirin previously highlighted by micorarray analysis. ß-tubulin was used as a loading control (n=3 for each treatment). Most notably, little alteration in protein expression was seen for GADD45α but XRCC3 expression was substantially altered.

relevant as possible. Salicylate levels of 1 mM have been reported to be achievable in serum in human subjects given analgesic doses of aspirin (14,15,36). We selected a 48 h incubation period with aspirin for the following reasons: cell viability results (Fig. 1) indicate that 48 h treatments with 1 mM aspirin cause a significant decrease in cell viability of SW480 cells [24 h treatment did not cause a significant decrease in cell viability (data not shown)]. Stark *et al* reported that significant nuclear translocation of NF-κB was possible in SW480 cells at 48 h incubation at a concentration of 1 mM (14). Fig. 1 also shows that the diluent (Tris-HCl buffer) had no significant effect on cell viability at low concentrations.

Aspirin affects several key genes involved in DNA damage signalling pathways. DNA mismatch repair proteins have been shown to be affected by aspirin treatment (14) and may play a part in the mechanism of action of aspirin. To investigate whether aspirin affected expression of genes involved in DNA damage signalling we utilised a commercially available PCR array to assess the expression of 84 different genes involved in DNA damage signalling pathways. Genes featured on the array included those associated with the ATR/ATM signalling network and transcriptional targets of DNA damage response.

PCR array analysis (Table I and Fig. 1) demonstrates that 48-h incubation of SW480 cells with 1 mM aspirin caused

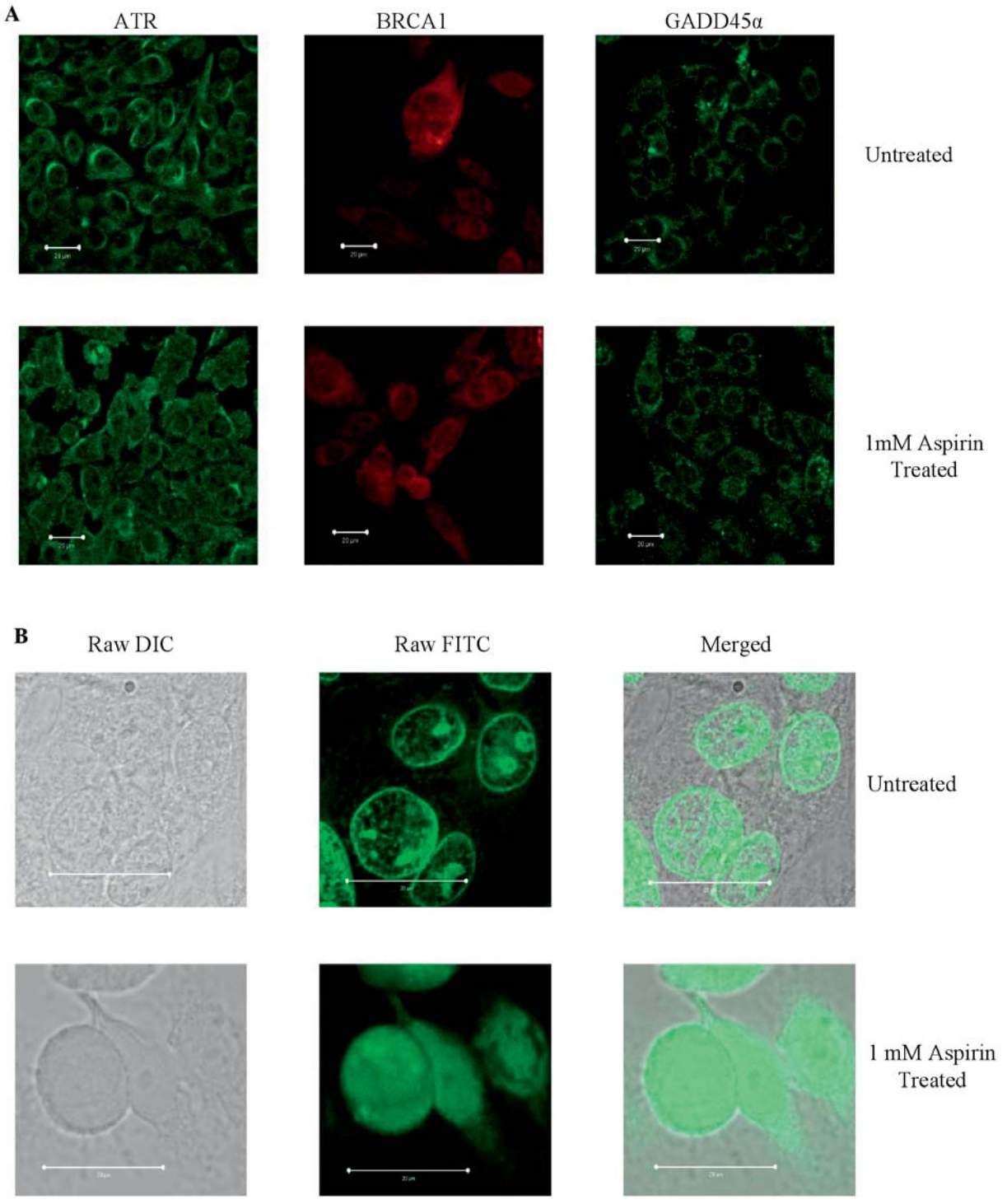

Figure 4. Immunofluorescence analysis of repair protein expression in SW480 cells treated for 48 h with 1 mM aspirin. Scale bar, 20 μm. (A) Representative FITC or Texas-Red images from three independent experiments are shown. Immunofluorescence analysis of SW480 cells probed with rabbit anti-ATR and donkey anti-rabbit FITC demonstrated no change in expression of ATR protein upon treatment with 1 mM aspirin over a 48-h period compared with untreated samples. Immunofluorescence analysis of SW480 cells probed with mouse anti-BRCA1 and donkey anti-mouse Texas-Red demonstrates no change in expression of BRCA1 protein upon treatment with 1 mM aspirin over a 48-h period compared with untreated samples. Immunofluorescence analysis of SW480 cells probed with rabbit anti-GADD45α and donkey anti-rabbit FITC demonstrated no change in expression of GADD45α protein upon treatment with 1 mM aspirin over a 48-h period compared with untreated samples. (B) Immunofluorescence analysis of SW480 cells probed with rabbit anti-XRCC3 and donkey anti-rabbit FITC. For clarity, the Raw differential interference contrast microscopic (DIC) and Raw FITC (FITC channel only) image is shown alongside the merged image. In contrast to ATR, BRCA1 and GADD45α an increase in expression of XRCC3 protein upon treatment with 1 mM aspirin over a 48-h period compared with untreated samples was consistently noted.

marked decreases in mRNA expression of several key genes involved in DNA damage signalling pathways including significant decreases in *BRCA1* (fold change = 0.15; P=0.04), *ATR* (fold change = 0.24; P=0.001) and *MAPK12* (fold change = 0.41; P=0.05) and a significant increase in *XRCC3* (fold change = 7.37; P=0.05). Whilst not statistically signi-

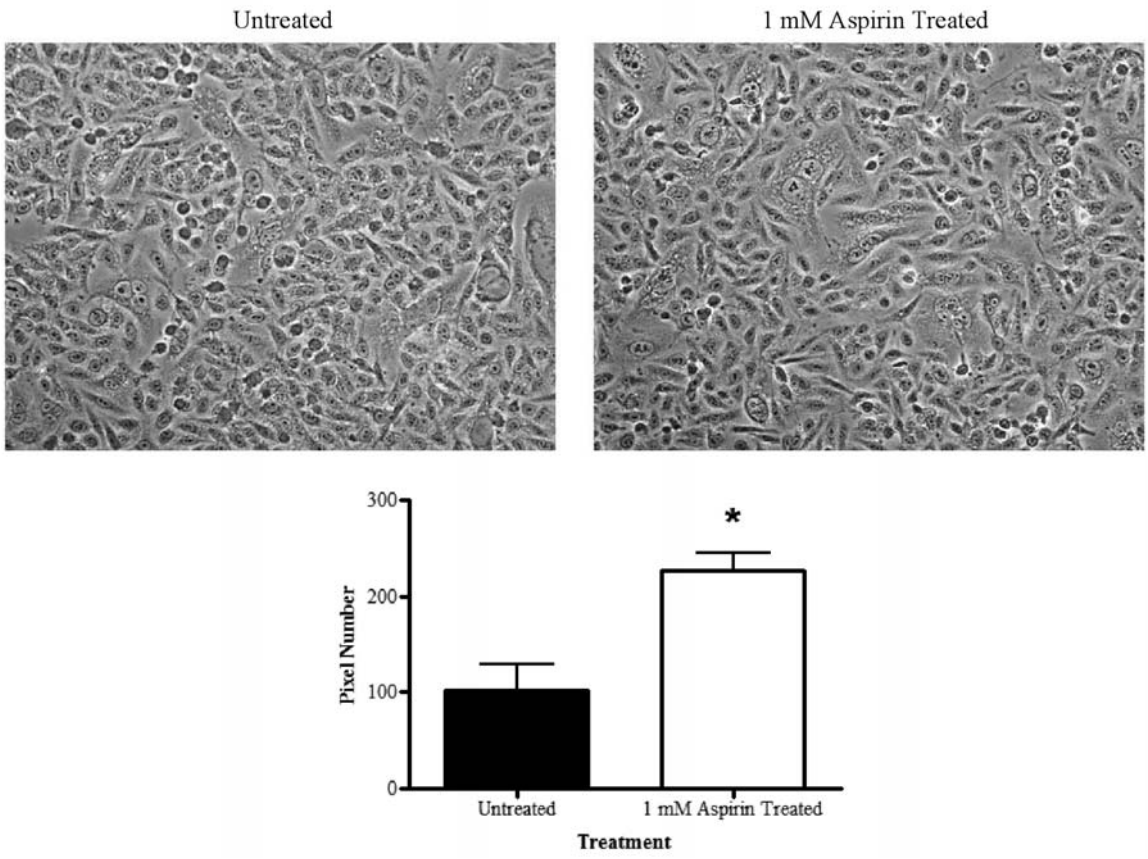

Figure 5. Analytical digital microscopic analysis of XRCC3 expression in SW480 cells exposed to aspirin. Digital images of SW480 cells untreated or treated with 1 mM aspirin for 48 h were taken at x200 magnification. To quantify the subtle alterations in substrate development, pixel values were quantified using analytical digital photomicroscopy. Statistical analysis of pixel number consistently indicated a significant increase (*P>0.05, using unpaired t-test) in aspirin treated cells compared to untreated cells, showing an increase in XRCC3 protein expression upon aspirin treatment. Controls with secondary antibody only and primary antibody only were also carried out and pixel number analysis demonstrated values lower than those of experimental slides indicating specificity of the reaction and that the antibodies on their own did not cause any significant change to pixel number (data not shown).

ficant, an increase in mRNA expression of *GADD45α* is of key interest due to the large fold difference of expression seen between treated and control samples (fold changes = 14.8; P=0.16).

Aspirin increases XRCC3 protein expression but does not affect BRCA1, ATR or GADD45α protein expression. We investigated expression of *BRCA1*, *ATR*, *XRCC3* and *GADD45α* encoded proteins by immunofluorescence and immunoblotting to determine correlation with changes of mRNA expression. These proteins were chosen for analysis based on P-value significance (P<0.05) and difference in expression level between control and treated samples (>4-fold). Whilst the P-value for *GADD45α* is considered non-significant (>0.05), the large fold change and previous experimental research indicating an up-regulation of *GADD45α* (29,31) warranted further investigation of this protein.

A strong correlation was noted for *XRCC3* gene and protein expression by immunoblotting (Fig. 3) and immunofluorescence (Fig. 4B) analysis. However, as altered protein expression is difficult to meaningfully quantify using these techniques we additionally tested *XRCC3* expression by immunohistochemical analysis using ADP (Fig. 5). Here a significant increase in XRCC3 protein expression was noted upon 1 mM aspirin treatment for 48 h compared to untreated samples. In contrast, the reduction in *BRCA1* and *ATR* and the increase in *GADD45α* mRNA signal were not accompanied by a relative decrease or increase in protein levels as analysed by immunoblotting (Fig. 3) and immunofluorescence (Fig. 4A). A subtle redistribution of ATR was apparent in aspirin treated cells, with fluorescence becoming more diffuse in treated cells, possibly reflecting aspirin treatment altering protein translocation/localization.

Discussion

The molecular basis for the specific cytotoxic activity of aspirin (and indeed other NSAIDs) to colorectal cancer cells, despite vigorous and long-standing investigation has resulted in a plethora of competing hypotheses. Whilst expression profile analysis of colorectal cancer cells in response to aspirin or sulindac has been previously carried out (24,29,31), to our knowledge this is the first study that has used PCR array methodology to analyse gene expression upon aspirin treatment. We report that when SW480 cells are exposed to 1 mM aspirin for 48 h, a significant decrease in the transcription of *ATR*, *BRCA1* and *MAPK12* and a significant increase in the transcription of *XRCC3* occurs. Most trans-

criptional changes were not significant but the overall trend as shown in Fig. 2 was down-regulation, possibly reflecting the toxic effect of aspirin on the transcriptional machinery.

The down-regulation of transcription of the tumour suppressor gene *BRCA1* upon aspirin treatment (Table I) is of interest as recently published data indicate that regular NSAID and aspirin intake is associated with an increase in breast cancer (37). However, immunoblotting and immunofluorescence analysis of BRCA1 and ATR proteins (Figs. 3 and 4) did not show dramatic decreases in protein expression in SW480 cells treated with 1 mM aspirin for 48 h and so did not correlate with changes in mRNA expression as seen by PCR array analysis. It is possible that the cells are able to compensate at the protein level for the possible transcriptional effects that are mediated by aspirin treatment, especially if the transcriptional changes are rather subtle. This may reflect alterations in mRNA stability or translation in addition to the stability of the protein product or altered subcellular locations, for example as shown by NF-κB (9,14).

The up-regulation of *GADD45α* upon aspirin treatment seen in the present study has been previously reported (29,31) and thus, can be interpreted to act as a positive control validating our RT-PCR array data. However, we did not find concordance between *GADD45α* gene transcription and protein expression (Figs. 3 and 4). Conclusions drawn from studies where only *GADD45α* expression at the transcript level has been measured but not demonstrated explicitly at the level of protein expression have to be viewed cautiously. Notwithstanding such concerns, there is substantial evidence of an involvement of GADD45α in the action of non-steroidal anti-inflammatory drugs (NSAIDs): treatment with NSAIDs resulted in the up-regulation of the cytokine melanoma differentiation associated gene-7/interleukin-24 (IL-24) in a number of different cancer cell types which led to an overexpression of GADD45α resulting in cell cycle arrest (32). In addition, experiments utilising RNAi to knock down *GADD45α* expression showed apoptosis to be prevented in cells when treated with NSAIDs (32). Using microarray analysis in prostate cancer cell lines Zerbini *et al* identified a role for IL-24 as a mediator for NSAIDS in inducing growth arrest and apoptosis (32). Intriguingly, IL-24 was previously reported to exercise cytotoxicity through activation of the p38 MAPK pathway and concomitant coordinated overexpression of the *GADD* family of genes in human melanoma cells (33). Thoms *et al* have also reported the activation of p38 MAPK pathway by aspirin in SW480 cells (38). However, we have shown in this study that the transcript level of an isomer of p38, MAPK12, is significantly down-regulated upon aspirin treatment.

XRCC3 is a Rad51 paralog and is involved in homologous recombination and the repair of DNA double strand breaks. We have shown that XRCC3 protein expression is increased in SW480 cells upon 1 mM aspirin treatment after 48 h compared to untreated cells by immunoblotting, immunofluorescence and also immunohistochemical analysis. It is important to note that whilst the overexpression of XRCC3 shown here is not necessarily indicative of a direct role of XRCC3 in aspirin-mediated cell death, this may have implications for the sensitivity of cells to chemotherapeutic agents, particularly in light of the ubiquitous use of aspirin in healthcare. Indeed, overexpression of XRCC3 in the breast cancer cell line MCF7 induced cisplatin resistance (39). In contrast, depletion of *XRCC3* transcription by siRNA in MCF7 cells inhibited cell proliferation, led to accumulation of DNA breaks and triggered activation of the p53-dependant cell death (40). XRCC3 deficient HCT116 cells have shown increased sensitivity to cisplatin and mitomycin C (42). Although some studies have shown no association between polymorphisms in *XRCC3* and colorectal cancer risk (43-46) other studies have (47,48), and in addition *XRCC3* polymorphisms have also been associated with breast and lung cancer susceptibility (41,49).

The present study highlights the importance of testing changes in protein expression to confirm gene expression analysis. We have shown changes in mRNA levels in DNA repair genes and signaling pathways in response to aspirin exposure (*ATR*, *BRCA1*, *MAPK12*, *XRCC3* and *GADD45α*) but expression of *ATR*, *BRCA1* and *GADD45α* did not correlate at a protein level. It may be that previous microarray research has highlighted changes in mRNA but these changes are not relevant post-transcriptionally and therefore make very little difference in actual disease pathogenesis and/or in cytotoxicity. Similarly Goel *et al* (27) reported an up-regulation in the expression of the DNA mismatch repair proteins hMLH1, hMSH2, hMSH6 and hPMS2 in a colorectal cancer cell line exposed to aspirin whilst showing no significant alteration in transcription as assessed by quantitative multiplex RT-PCR.

In conclusion, our results demonstrate via RT^2 Profiler Array analysis that aspirin causes a decrease in *ATR*, *BRCA1* and *MAPK12* and increases in *XRCC3* and *GADD45α* gene transcription. Protein expression analysis suggests that changes in transcript levels of these genes do not necessarily correlate with changes in protein expression. This finding has relevance to microarray studies that solely rely on analysis of message and thus raises the concern of the validity of such studies that do not provide evidence of changes at a protein level. However, XRCC3 expression is significantly up-regulated as both transcript and protein in the SW480 cancer cell line as a result of exposure to aspirin. Aspirin may have the capacity to dysregulate XRCC3 expression and thus function, with important implications for the response of host cells to chemotherapeutic agents. The significance and utility of this study is further underlined by the recent finding that a metabolite of aspirin (2,3-dihydrobenzoic acid) was capable of inducing oxidative damage and DNA strand breaks in the PANC-1 human pancreatic cancer cell line (50).

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