ELSEVIER

Cyclooxygenase-2 expression and oxidative DNA adducts in murine intestinal adenomas: Modification by dietary curcumin and implications for clinical trials

R.G. Tunstall, R.A. Sharma*, S. Perkins, S. Sale, R. Singh, P.B. Farmer, W.P. Steward, A.J. Gescher

Cancer Biomarkers and Prevention Group, Departments of Cancer Studies and Biochemistry, University of Leicester, Leicester LE2 7LX, UK

ARTICLE INFO

ABSTRACT

The natural polphenol, curcumin, retards the growth of intestinal adenomas in the Apc^{Min+} mouse model of human familial adenomatous polyposis. In other preclinical models, curcumin downregulates the transcription of the enzyme cyclooxygenase-2 (COX-2) and decreases levels of two oxidative DNA adducts, the pyrimidopurinone adduct of deoxyguanosine (M_1dG) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG). We have studied COX-2 protein expression and oxidative DNA adduct levels in intestinal adenoma tissue from Apc^{Min+} mice to try and differentiate between curcumin's direct pharmacodynamic effects and indirect effects via its inhibition of adenoma growth. Mice received dietary curcumin (0.2%) for 4 or 14 weeks. COX-2 protein, M_1dG and 8-oxo-dG levels were measured by Western blot, immunochemical assay and liquid chromatography–mass spectrometry, respectively. In control Apc^{Min+} mice, the levels of all three indices measured in adenoma tissue were significantly higher than levels in normal mucosa. Lifetime administration of curcumin reduced COX-2 expression by 66% ($P = 0.01$), 8-oxo-dG levels by 24% ($P < 0.05$) and M_1dG levels by 39% ($P < 0.005$). Short-term feeding did not affect total adenoma number or COX-2 expression, but decreased M_1dG levels by 43% ($P < 0.01$). COX-2 protein levels related to adenoma size. These results demonstrate the utility of measuring these oxidative DNA adduct levels to show direct antioxidant effects of dietary curcumin. The effects of long-term dietary curcumin on COX-2 protein levels appear to reflect retardation of adenoma development.

© 2005 Elsevier Ltd. All rights reserved.

1. Introduction

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; diferuloylmethane], the major constituent of the yellow spice turmeric derived from the rhizomes of *Curcuma spp.*, has been shown to prevent cancer in the colon, skin, stomach, duodenum, soft palate and breast tissues of rodents following oral administration [1–3]. In the Apc^{Min+} mouse, a model of human familial adenomatous polyposis (FAP) characterised by an *adenomatous polyposis coli* (*APC*) gene defect, 0.1% and 0.2% dietary curcumin have been shown to inhibit adenoma growth, reflected by total number of adenomas and mean adenoma size [4,5]. Adenomas are generally regarded as precursor lesions of colorectal cancer; their histological development has been linked to carcinogenesis [6]. Dietary consumption of turmeric up to 1.5 g per person per

day, equating to approximately 150 mg/day of curcumin, is not associated with adverse effects in humans in certain South East Asian communities [7]. Similarly, pharmaceutical preparations of curcumin appear to be well tolerated by patients with advanced cancer at doses up to 3.6 g in the UK [8,9] and by patients with pre-invasive malignant or high risk pre-malignant conditions up to 8 g daily in Taiwan [10]. The systemic availability of curcumin is poor because of its propensity to undergo avid metabolism [11]. However, daily ingestion of 3.6 g curcumin resulted in levels in the human gastro-intestinal mucosa in the 10^{-5} M range [10], concentrations sufficient to exert biochemical changes in cells in vitro relevant to cancer chemoprevention [12].

Curcumin acts as an extraordinarily potent antioxidant at neutral and acidic pH [13], as reflected by its ability to decrease levels of oxidative DNA adducts, e.g., the pyrimido-purinone adduct of deoxyguanosine (M_1dG) in the colonic mucosa of rats after 14-day dietary administration [14]. Varying levels of DNA damage arising from the products of endogenous and exogenous oxidative stress have been measured in human tissues [15]. Oxidative DNA adducts have been implicated in early stages of carcinogenesis in a number of tissues [reviewed in 15], but their detection in pre-malignant adenoma tissue has not been reported. Two examples of such adducts are M_1dG and 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxo-dG). M_1dG can be generated by oxidative DNA damage or by malondialdehyde (MDA), an intracellular product of lipid peroxidation [16]. M_1dG adducts have been detected in human blood leukocytes, liver, pancreas and breast tissues, and they are associated with mutagenesis in mammalian cells [15]. In one clinical study, median M_1G levels in normal breast tissue from patients with breast cancer were almost threefold higher than those from women without cancer [17]. The formation of M_1dG adducts in human cells has been demonstrated in the absence of MDA, via base propenals resulting from oxidative DNA damage [18]. 8-Oxo-dG, the most extensively studied adduct arising from oxidative DNA damage, is generated by reactive oxygen species, particularly the hydroxyl radical, which can bind to or abstract hydrogen atoms from DNA bases [15]. 8-Oxo-dG is associated with mutagenesis in bacterial and mammalian cells [19] and with the induction of G to T transversions in human DNA, lesions frequently seen in tumour suppressor genes such as the APC gene [20,21]. Defects in the APC gene are found in 80% of sporadic colorectal adenomas and carcinomas [21].

Biological mechanisms by which curcumin may prevent cancer are also thought to involve inhibition of activation of the transcription factor NF-κB [22], which may lead to suppression of expression of the enzyme cyclooxygenase-2 (COX-2) [12,23]. In human colon cancer, as well as adenomas of FAP and Apc^{Min+} adenomas, COX-2 is often induced [24–26]. Dietary administration of celecoxib, an inhibitor of COX-2 enzymatic activity, has been shown to dramatically decrease adenoma number in Apc^{Min+} mice [27].

In a preclinical platform of development of biomarkers of chemopreventive efficacy for intervention trials with curcumin, we investigated COX-2 expression and levels of oxidative DNA adducts in intestinal adenomas of Apc^{Min+} mice. The

results advocate the measurement of M_1dG as a biomarker of curcumin's pharmacodynamic effects in adenoma tissue. In contrast, effects on COX-2 expression in this tissue appear to have a complex relationship with adenoma size.

2. Materials and methods

2.1. Apc^{Min+} mouse model

Animal experiments were carried out under project licence PPL 40/2496, granted to University of Leicester by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards outlined in the UKCCCR guidelines [28]. A breeding colony was established from C57BL/6J Min/+ mice (Jackson Laboratory, Bar Harbor, ME), and the Apc^{Min+} genotype was confirmed by polymerase chain reaction [29]. All animals were randomly allocated into treatment or control groups and had access to food and water ad libitum.

The curcumin used in this study (purchased from Apin Chemicals, Abingdon, UK) was extracted from turmeric. It contained 85% curcumin and 15% desmethoxycurcumin and bisdesmethoxycurcumin, confirmed by HPLC analysis. Two experimental protocols were investigated. In the first, mice received either standard diet (AIN93G high protein, Dyets Inc., Bethlehem, PA, USA) or standard diet containing 0.1% or 0.2% w:w curcumin ($n = 4$ in each group) from weaning (at approximately 4 weeks of age) to week 18. In the second, mice received standard diet from weaning to week 12, after which they either remained on standard diet (control) or were switched to diet containing curcumin 0.2% (w:w, $n = 4$ in each group) for 4 weeks until the end of the experimental period (week 16). The age of the mice at the end of the experimental period was not identical for the two experiments since direct comparison of the parameters measured in the two independent experiments was not intended. Mice were killed by terminal anaesthesia using halothane. The gastrointestinal tract was flushed with phosphate-buffered saline (pH 7.4) and adenomas were micro-dissected under visual magnification. Normal mucosa was scraped using a blunt spatula. Histological examination ensured accurate scraping of intestinal mucosa only. All tissue samples were frozen in liquid nitrogen and stored at −80 °C.

2.2. Biomarker measurements

COX-2 levels were analysed by Western blot as previously described using a polyclonal antibody against COX-2 (Santa Cruz Biotechnology Inc., CA). Blots were stripped and re-analysed for α-tubulin using a polyconal anti-α-tubulin antibody (Oncogene, San Diego, CA) to control for protein loading and transfer. Semi-quantitation of Western blots was performed using a GeneGenius gel documentation system. Band density was quantitated with GeneTools software (Syngene, Cambridge, UK). Extraction of genomic DNA and analysis of M_1dG adduct levels by immunoslot blot were performed as previously described [8,14,30]. The detection limit for M_1dG adducts was 2.5 adducts per 10^8 nucleotides. To avoid artifactual formation of 8-oxo-dG during sample collection and DNA extraction, desferoxamine mesylate (5 mM, Sigma–Aldrich,

Poole, UK) was added to the tissue homogenisation and DNA extraction buffers prior to DNA extraction. 8-Oxo-dG was determined by an immunoaffinity column purification LC-MS/MS method [31]. Prior to enzymatic digestion, a stable isotope [^{15}N$_5$]8-oxo-dG internal standard was added to each DNA sample. Following enzymatic digestion, an aliquot (1 μg) of the digested DNA was analysed by HPLC to determine the level of deoxyguanosine. Digested DNA was subjected to immunoaffinity column purification prior to positive ion LC-MS/MS multiple reaction monitoring analysis using a Micromass Quattro-BioQ instrument (Manchester, UK) with an electrospray interface [31]. The limit of detection of the LC-MS/MS multiple reaction monitoring for 8-oxodG was 25 fmol on column.

2.3. Measurement of COX-2 mRNA by RT-PCR

Apc^{Min+} mice received AIN93G diet from weaning to 18 weeks of age, after which time intestinal adenoma and mucosa tissue were collected on ice. Adenomas were sorted by size, and all tissues stored in RNALater (Qiagen, Sussex, UK) at −80 °C. Total RNA was extracted from tissues using the RNeasy mini extraction kit (Qiagen) following the manufacturer's instructions. RNA purity was assessed by absorbance spectroscopy. Reverse transcription of RNA (2 μg) was performed using the Omniscript RT kit (Qiagen), Oligo (dT) 12–18 Primers and RNaseOUT ribonuclease inhibitor (Invitrogen, Paisley, UK), as detailed in the manufacturer's instructions. COX-2 cDNA transcript was amplified by polymerase chain reaction (PCR) using a COX-2 mouse primer set (Maxim Biotech Inc., CA, US). To allow normalisation of COX-2 mRNA levels between samples, levels of GAPDH mRNA were determined using a commercially available primer set (Maxim Biotech). An aliquot (1 μl) of the cDNA template was added to each PCR reaction, and reactions were carried out as described by the manufacturer. The optimum number of amplification cycles was determined for both COX-2 and GAPDH. As a control for contamination and primer degradation, all samples were run with a paired control in which water was substituted for cDNA template. Amplified PCR products were resolved through a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and viewed (UV light) using a GeneGenius gel documentation system. Band density was quantified using GeneTools software (Syngene).

2.4. Statistical evaluation

Biomarker analysis results were subjected to analysis of variance (ANOVA) and Tukey's post hoc testing for statistical significance using SPSS software Version 11 for Windows 2000. Statistical significance was indicated by $P < 0.05$.

3. Results

3.1. Effect of curcumin on levels of COX-2 in Apc^{Min+} mouse adenomas

Mice received curcumin at 0.2% in the diet from weaning (∼300 mg/kg body weight per day), an intervention which has been previously reported to decrease adenoma number by 39% [5]. At week 18, adenoma tissue and normal mucosal samples were resected, pooled and investigated for COX-2 protein content by Western blotting. Fig. 1 shows that whereas COX-2 expression in normal mucosa was very low, intestinal adenomas contained high levels of this protein, consistent with previous reports [24–26]. In mice that had consumed curcumin throughout their lifetime, COX-2 expression in adenoma tissue was decreased compared to mice on the control diet: Semi-quantitation by densitometry of the blots suggested that COX-2 levels were 44 ± 19% of those in adenomas of control mice ($n = 4$, $P = 0.01$). An effect of curcumin could not be discerned on normal mucosa because of the undetectable COX-2 expression (Fig. 1).

In a separate experiment, the hypothesis was tested that COX-2 levels in Min adenomas were decreased after short-term administration of curcumin. Apc^{Min+} mice received curcumin (0.2%) for 4 weeks from week 12. This treatment did not influence the total number of adenomas counted at 16 weeks of age: 35.9 ± 4.6 total adenomas in curcumin-fed mice compared to 34.5 ± 6.8 in control animals ($n = 8$). COX-2 protein levels in intestinal adenomas from mice exposed to curcumin were not lower than those in mice on control diet (result not shown).

3.2. Dependency of COX-2 expression on stage of adenoma development

COX-2 expression in murine adenomas has previously been shown to depend on adenoma size [32,33]. We therefore
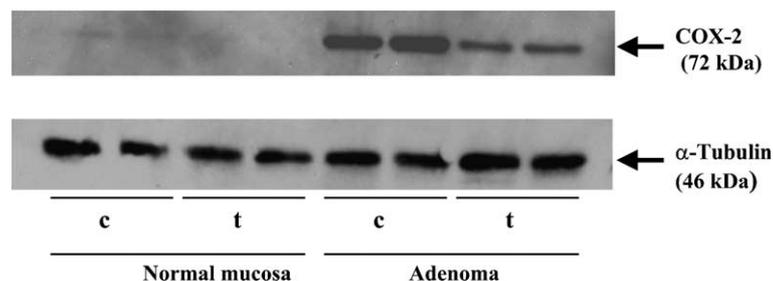

Fig. 1 – COX-2 protein levels in intestinal mucosa and adenoma tissue of Apc^{Min+}mice that received control diet (c) or diet with 0.2% curcumin (t). Adenomas from each animal were pooled (number and size distribution as in Ref. [5]). Analysis was performed by Western blotting using COX-2 and α-tubulin (46 kDa) antibodies; the latter was included to ensure equal loading and protein transfer. Semi-quantitation of densitometry of blots of adenoma tissue from four mice suggested that COX-2 protein levels were reduced by 66 ± 19%, compared to controls ($P = 0.01$).

explored the relationship between COX-2 protein levels and adenoma size in the mice used in the study described here. To that end, small intestinal adenomas were excised from control Apc^{Min+} mice at 12 or 16 weeks of age and separated into small (<1 mm), medium (1–3 mm) and large tumours (>3 mm) prior to analysis for COX-2 protein expression. Consistent with previous results [32,33], COX-2 protein levels varied with tumour size, with the highest levels observed in the subset of the largest polyps (Fig. 2), although the differences did not reach significance. Furthermore, the question was addressed whether the developmental regulation of COX-2 expression is the corollary of altered COX-2 gene transcription. Messenger RNA extracted from intestinal adenomas of differential size was subjected to RT-PCR and transcript levels were corrected by comparison to GAPDH transcription. Fig. 3 shows that COX-2 transcript levels were low in mucosa and

high in adenomas. There was no difference in transcript levels between small, medium-sized and large adenomas.

Taken together with the results from the curcumin feeding experiments, these observations suggest that: (1) COX-2 protein expression in Apc^{Min+} mice is upregulated at a transcriptional level from early stages of adenoma development; (2) intervention with dietary curcumin from week 12 onwards does not affect COX-2 levels, nor total adenoma number; and (3) lifetime intervention with curcumin at a dose which decreases total adenoma number attenuates COX-2 levels measured at the end of the experiment. It is therefore likely that the observed decrease in adenomatous COX-2 levels in mice fed with curcumin represents an indirect effect, i.e., the consequence of curcumin-induced retardation of adenoma growth.

3.3. Effect of curcumin on levels of oxidative DNA adducts in Apc^{Min+} mouse adenomas

Levels of M_1dG adduct were determined by immunoslot blot analysis in murine intestinal adenomas and normal mucosa. M_1dG levels in adenomas from mice on control diet were threefold higher than those in mucosa (12.8 ± 2.9 vs. 4.0 ± 1.5 adducts per 10^7 nucleotides, respectively, $P < 0.01$, $n = 4$–6). In mice that received curcumin (0.2%) for their lifetime, M_1dG adduct levels in adenomas were 39% below
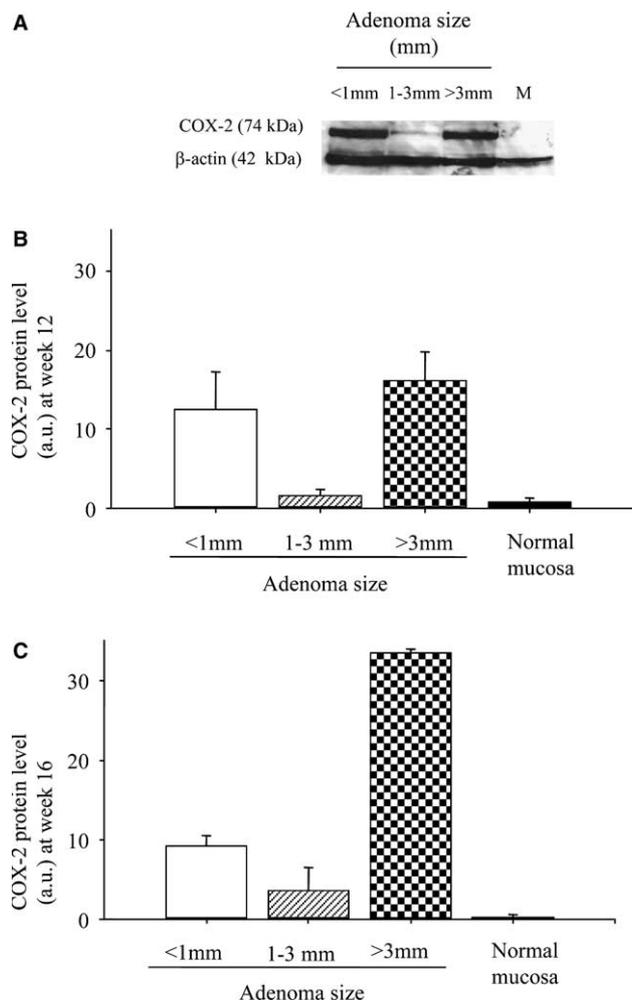

Fig. 2 – COX-2 protein levels in Apc^{Min+} mouse intestinal mucosa and adenomas of size <1 mm, 1–3 mm and >3 mm in week 12 (A, B) or week 16 (C), (A) shows a Western blot, representative of week 12, (B) and (C) show values obtained from semi-quantitation of densitometry of blots from adenomas from three separate mice on week 12 (B) or week 16 (C) corrected for protein loading using β-actin expression. Values show mean ± SD (n = 3). M, mucosa, a.u., arbitrary units.
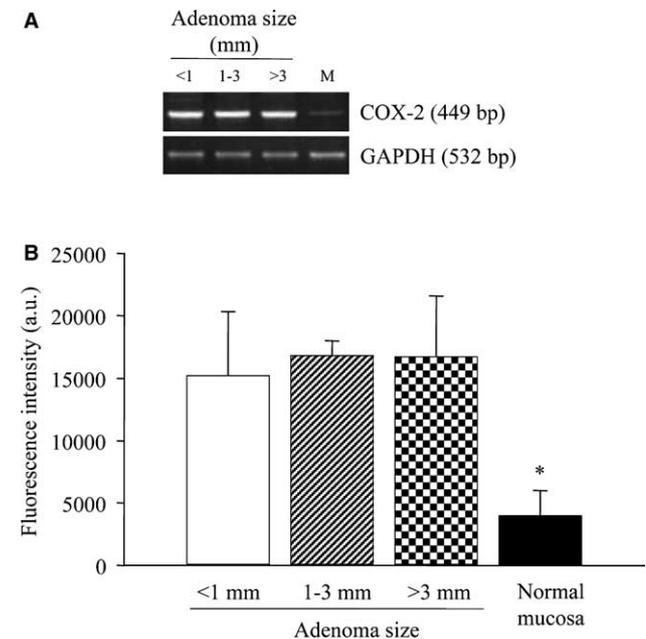

Fig. 3 – COX-2 mRNA levels in Apc^{Min+} mouse intestinal mucosa and adenomas of size <1 mm, 1–3 mm and >3 mm from 16-week old mice. COX-2 mRNA levels were determined semi-quantitatively by reverse transcription-PCR, (A) shows a representative fluorograph of tissue from one mouse, (B) shows semi-quantitation by densitometry of fluorographs from samples of four separate mice, normalised to mRNA levels of the housekeeping gene GAPDH. Values show mean ± SD. Star indicates mucosal COX-2 mRNA levels are significantly lower than those in adenomas of any size group ($P < 0.01$). M, normal mucosa, a.u., arbitrary units.

Table 1 – M_1dG adduct levels in Apc^{Min+} mouse intestinal adenomas following (A) lifetime dietary intervention with 0.1% or 0.2% curcumin from 4 weeks of age and (B) 28 days dietary intervention, started in week 12 with 0.2% curcumin

	Intervention group[a]	M_1dG levels (adducts per 10^7 nucleotides)[b]
A	Control	12.8 ± 2.9
	Curcumin 0.1%	$7.1 \pm 2.1^{**}$
	Curcumin 0.2%	$7.6 \pm 2.9^{**}$
B	Control	13.4 ± 4.9
	Curcumin 0.2%	$5.1 \pm 2.2^{*}$

a Control mice received diet alone. Adenoma tissue from each animal was pooled, DNA extracted and M_1dG adducts assessed by immunoslotblot.

b Values represent mean ± SD ($n = 4$–6). Statistically significant differences compared to control mice are indicated by $^{*}P < 0.01$ or $^{**}P < 0.005$ (by ANOVA).

those in animals on control diet (Table 1). The theory that the decrease in M_1dG adduct levels represented an antioxidant pharmacodynamic effect was substantiated by measurement of a separate oxidative DNA adduct, 8-oxo-dG. Curcumin (0.2%) abolished the excess of 8-oxo-dG adduct seen in adenomas of untreated mice over those in mucosa, in that adduct number per 10^6 nucleotides in adenomas of control mice was 50 ± 38 and in curcumin-treated mice 38 ± 9 ($P < 0.05$, $n = 4$), whilst the respective mucosal levels were 38 ± 19 and 35 ± 16.

Lifetime administration of curcumin at 0.1% dietary concentration ($\sim$150 mg/kg bw per day), which failed to affect Apc^{Min+} adenoma number in previous experiments [5], decreased adenomatous M_1dG adduct levels, by 43% (Table 1). Similarly, reduction in M_1dG levels was observed in adenomas of mice that received curcumin (0.2%) for only 4 weeks from week 12, an intervention which did not affect total adenoma number (vide supra). Adenoma tissue M_1dG adduct levels in these mice were 38% of those measured in mice on control diet ($P < 0.05$, Table 1). M_1dG levels in normal mucosa were not affected by curcumin (result not shown). Comparison of M_1dG adduct levels in different stages of adenoma development was not possible because sufficient DNA could not be obtained from small adenomas to permit reproducible analysis. Similarly, the numbers of adenomas in the large intestine were too low (mean 0.3 per mouse) to permit comparison of effects on adenomas arising in the colon and those arising in the small intestine.

Overall, these results demonstrate the sensitivity of levels of the oxidative DNA adducts, M_1dG and 8-oxo-dG, in adenoma tissue to intervention with dietary curcumin. Significant changes in adduct levels were exerted by dosing regimes which decreased total adenoma number, but also by regimes which failed to affect adenoma multiplicity.

4.   Discussion

Adenoma number is currently used as a primary endpoint to demonstrate the chemopreventive efficacy of an interven-

tional agent in patients with pre-malignant lesions of the lower gastrointestinal tract [34]. Adenoma size is a useful secondary endpoint to indicate retardation of adenoma development. In the study presented here, COX-2 protein and M_1dG were explored as potential markers of the pharmacological efficacy of curcumin. Measurement of both markers by blotting techniques is amenable to incorporation into large-scale clinical trials. The Apc^{Min+} mouse model was selected as an experimental paradigm since oral curcumin has been shown to significantly delay adenoma development, reflected by both total adenoma number and mean adenoma size [4,5]. This model has previously been applied in the prediction of ability of the NSAID sulindac [35] and the COX-2 inhibitor celecoxib [27] to reduce adenoma number in patients suffering from FAP [34,36].

Interesting discrepancies exist when one compares the COX-2 protein expression in Apc^{Min+} mouse adenomas described here with the previously reported size-dependency of COX-2 expression in adenomas of $Apc^{\Delta716}$ mice assessed by immunohistochemistry [32,33]. These authors have suggested that COX-2 expression increases with adenoma size, but that polyps smaller than 1 mm showed "little COX-2 expression". Consistent with these reports, large adenomas in Apc^{Min+} mice in our study expressed the highest COX-2 levels. However, inconsistent with the previous immunohistochemical reports, COX-2 protein levels in our experiments were upregulated over levels in adjacent non-malignant tissue from an early stage of adenoma development (i.e., <1 mm in size). This discrepancy may be explicable by differences in analytical technique. Takeda and Seno and colleagues studied individual adenomas by immunohistochemistry [32,33]. Instead, we pooled tissue from several mice in order to obtain sufficient protein for reproducible analysis by Western blotting. Differences between individual similar-sized adenomas were therefore averaged out in the data presented in Fig. 2. Whilst the methodology described here permits only gross inferences to be drawn regarding COX-2 protein expression changes in relation to adenoma size, it demonstrates that COX-2 protein expression is upregulated from the earliest discernible stages of adenoma development. Intervention after this time with dietary curcumin does not appear to impact significantly on COX-2 expression or total adenoma number. On the basis of these results, we suggest that chemoprevention strategies using curcumin should be implemented before or during the earliest stages of adenoma development to demonstrate effects on adenoma number as a primary endpoint or on COX-2 protein levels as a surrogate endpoint.

According to the data presented above, oxidative DNA adduct levels appear to reflect direct anti-oxidant effects of curcumin, which seem independent of its effects on adenoma number. Particularly noteworthy is the finding that levels of M_1dG and 8-oxo-dG adducts were elevated in Apc^{Min+} adenoma tissue compared to surrounding mucosa. Adduct levels appeared exquisitely sensitive to curcumin exposure, irrespective of whether exposure was for the lifetime of the mice or only short-term. A dose–response relationship was not demonstrated for the two dose levels at the single time-point studied. Further studies will be designed to explore this relationship as part of biomarker validation. The relevance

to later stages of carcinogenesis of diminishing oxidative DNA adduct levels during the initiation/promotion phases can not be studied in the Apc^{Min+} murine model since the animals die during adenoma promotion.

Although the results from the mouse model used here do not advocate measurement of M_1dG adduct levels in normal colorectal mucosa, it is conceivable that such analysis is useful in humans. M_1dG adduct levels in normal colorectal mucosa have been compared between individuals with and without adenomas [37]: Individuals with macroscopic adenomas showed a trend towards higher M_1dG levels in normal colorectal mucosa, although this difference was not statistically significant. Consumption of legumes, nuts, wholemeal bread, cereals, fruits, vegetables, salads and raw tomatoes decreased M_1dG levels in normal mucosa. The suspicion that M_1dG adduct measurement might be useful in clinical trials has also been fuelled by the results of a recent pilot study of oral curcumin for 7 days prior to surgery in patients with resectable colorectal cancer [9]. In this trial, M_1dG adduct levels were 2.5-fold higher in malignant colorectal tissue than normal colorectal mucosa. Moreover, patients who consumed curcumin capsules (3.6 g daily) displayed significantly lower M_1dG adduct levels in malignant tissue than those observed in biopsy tissue obtained from the same tumour before the 7-day intervention.

What is the potential corollary of the results described here for the clinical development of curcumin in view of the robust ability of curcumin to delay malignancies in murine models of intestinal carcinogenesis [1,4,5]? Since our results suggest that curcumin intervention should occur before or during the earliest stages of adenoma development in order to demonstrate effects on adenoma number or on COX-2 protein levels as a surrogate biomarker, a prudent initial clinical strategy would be the exploration of its potential long-term efficacy in individuals at risk of developing colorectal polyposis, either due to inherited predisposition or due to acquired factors such as history of previous adenoma or previous cancer. In these individuals, M_1dG measurement in normal or adenoma tissue may represent an early biomarker of the antioxidant efficacy of curcumin, permitting the broader relevance of this biomarker to be ascertained. It is emphasised that M_1dG measurement should not be considered a surrogate biomarker for adenoma development. Although COX-2 expression in human intestinal polyps and malignant tissue is known to be highly variable [38,39], measurement of COX-2 protein levels or products of COX catalysis [23] may provide additional information regarding the efficacy of intervention in retarding adenoma development.

Conflict of interest statement

None declared.

Acknowledgements

REFERENCES

1. Rao CV, Rivenson A, Simi B, et al. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res* 1995;**55**:259–66.
2. Kelloff GJ, Crowell JA, Hawk ET, et al. Strategy and planning for chemopreventive drug development: clinical development plans II. *J Cell Biochem* 1996;**63**(26S):54–71.
3. Kawamori T, Lubet R, Steele VE, et al. Chemopreventative effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res* 1999;**59**:597–601.
4. Mahmoud NN, Carothers AM, Grunberger D, et al. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000;**21**:921–7.
5. Perkins S, Verschoyle RD, Hill KA, et al. Chemopreventive efficacy and pharmacokinetics of curcumin in the Min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* 2002;**11**:535–40.
6. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;**319**:525–32.
7. Sharma RA, Gescher AJ, Steward WP. Curcumin: The story so far. *Eur J Cancer* 2005;**41**:1955–68.
8. Sharma RA, Euden SA, Platton SL, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin Cancer Res* 2004;**10**:6847–54.
9. Garcea G, Berry DP, Jones DJL, et al. Consumption of the putative chemopreventive agent curcumin by cancer patients: assessment of curcumin levels in the colorectum and their pharmacodynamic consequences. *Cancer Epidemiol Biomarkers Prev* 2005;**14**:120–5.
10. Cheng AL, Hsu CH, Lin JK, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* 2001;**21**:2895–900.
11. Ireson C, Orr S, Jones DJL, et al. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. *Cancer Res* 2001;**61**:1058–64.
12. Plummer SM, Holloway KA, Manson MM, et al. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. *Oncogene* 1999;**18**:6013–20.
13. Jovanovic SV, Steenken S, Boone CW, et al. H-atom transfer is a preferred antioxidant mechanism of curcumin. *J Am Chem Soc* 1998;**121**:9677–81.
14. Sharma RA, Ireson CR, Verschoyle RD, et al. Effects of dietary curcumin on glutathione S-transferase and malondialdehyde-DNA adducts in rat liver and colon mucosa: relationship with drug levels. *Clin Cancer Res* 2001;**7**:1452–8.
15. Marnett LJ. Oxyradicals and DNA damage. *Carcinogenesis* 2000;**21**:361–70.
16. Sharma RA, Gescher A, Plastaras JP, et al. Cyclooxygenase-2, malondialdehyde and pyrimidopurinone adducts of deoxyguanosine in human colon cells. *Carcinogenesis* 2001;**9**:1557–60.
17. Wang MY, Dhingra K, Hittleman WN, et al. Lipid peroxidation induced putative malondialdehyde-DNA adducts in human

breast tissues. *Cancer Epidemiol Biomarkers Prev* 1996;**5**: 705–10.

18. Dedon PC, Plastaras JP, Rouzer CA, et al. Indirect mutagenesis by oxidative DNA damage: formation of the pyrimidopurinone adduct of deoxyguanosine by base propenal. *Proc Natl Acad Sci USA* 1998;**95**:11113–6.

19. Feig DI, Sowers LC, Loeb LA. Reverse chemical mutagenesis: identification of the mutagenic lesions resulting from reactive oxygen species-mediated damage to DNA. *Proc Natl Acad Sci USA* 1994;**91**:6609–13.

20. Cheng KC, Cahill DS, Kasai H, et al. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes $G \rightarrow T$ and $A \rightarrow C$ substitutions. *J Biol Chem* 1992;**267**:166–72.

21. Nakamura Y, White R, Smits AM, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;**319**:525–32.

22. Singh S, Aggarwal BB. Activation of transcription factor NF-kappaB is suppressed by curcumin (diferulolylmethane). *J Biol Chem* 1995;**270**:24995–5000.

23. Plummer SM, Hill KA, Festing MFW, et al. Clinical development of leukocyte cyclooxygenase 2 activity as a systemic biomarker for cancer chemopreventive agents. *Cancer Epidemiol Biomarkers Prev* 2001;**10**:1295–9.

24. Bamba H, Ota S, Kato A, et al. High expression of cyclooygenase-2 in macrophages of human colonic adenoma. *Int J Cancer* 1999;**83**:470–5.

25. Hull MA, Booth JK, Tisbury A, et al. Cyclooxygenase 2 is up-regulated and localized to macrophages in the intestine of Min mice. *Br J Cancer* 1999;**79**:1399–405.

26. Sinicrope FA, Lemoine M, Xi L, et al. Reduced expression of cyclooxygenase 2 proteins in hereditary nonpolyposis colorectal cancers relative to sporadic cancers. *Gastroenterology* 1999;**117**:350–8.

27. Jacoby RF, Seibert K, Cole CE, et al. The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. *Cancer Res* 2000;**60**:5040–4.

28. Workman P, Twentyman P, Balkwill F, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (2nd ed.). *Br J Cancer* 1998;**77**:1–10.

29. Luongo C, Moser AR, Gledhill S, et al. Loss of Apc (+) in intestinal adenomas from Min mice. *Cancer Res* 1994;**54**:5947–52.

30. Leuratti C, Singh R, Lagneau C, et al. Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay. *Carcinogenesis* 1998;**19**:1919–24.

31. Singh R, McEwan M, Lamb JH, et al. An improved liquid chromatography–mass spectrometry/mass spectrometry method for the determination of 8-hydroxy-2′-deoxyguanosine in DNA samples using immunoaffinity column purification. *Rapid Commun Mass Spec* 2003;**17**:126–34.

32. Takeda H, Sonoshita M, Oshima H, et al. Cooperation of cyclooxygenase 1 and cyclooxygenase 2 in intestinal polyposis. *Cancer Res* 2003;**63**:4872–7.

33. Seno H, Ishima M, Ishikawa T, et al. Cyclooxygenase 2- and prostaglandin E_2 receptor EP_2-dependent angiogenesis in $Apc^{\Delta716}$ mouse intestinal polyps. *Cancer Res* 2002;**62**: 506–11.

34. Steinbach G, Lynch PM, Phillips RKS, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000;**342**:1946–52.

35. Boolbol SK, Dannenberg AJ, Chadburn A, et al. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Res* 1996;**56**:2556–60.

36. Giardiello FM, Hamilton SR, Krush AJ, et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993;**328**: 1313–6.

37. Leuratti C, Watson MA, Deag EJ, et al. Detection of malondialdehyde-DNA adducts in human colorectal mucosa: relationship with diet and the presence of adenomas. *Cancer Epidemiol Biomarkers Prev* 2002;**11**:267–73.

38. Eberhart CE, Coffey RJ, Radhika A, et al. Upregulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994;**107**:1183–8.

39. Uefuji K, Ichikura T, Mochizuki H. Expression of cyclooxygenase-2 in human gastric adenomas and adenocarcinomas. *J Surg Oncol* 2001;**76**:26–30.