SHORT COMMUNICATION

Curcumin modifies *Apc^min* apoptosis resistance and inhibits 2-amino 1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) induced tumour formation in *Apc^min* mice

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Curcumin, the active ingredient of the rhizome of *Curcuma longa*, promotes apoptosis and may have chemopreventive properties. This study investigates the effects of curcumin on apoptosis and tumorigenesis in male *Apc^min* mice treated with the human dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Intestinal epithelial apoptotic index in response to PhIP treatment was approximately twice as great in the wild-type C57BL/6 APC$^{+/+}$ strain than in *Apc^min* mice (3.7% *Apc*$^{+/+}$ versus 1.9% *Apc^min*; P < 0.001). PhIP promoted tumour formation in *Apc^min* proximal small intestine (4.6 tumours per mouse, PhIP treated versus 2.1 tumours per mouse, control untreated; P < 0.05). Curcumin enhanced PhIP-induced apoptosis (4.0% curcumin + PhIP versus 2.1% PhIP alone; P < 0.01) and inhibited PhIP-induced tumorigenesis in the proximal small intestine of *Apc^min* mice (2.2 tumours per mouse, curcumin + PhIP versus 4.6 tumours per mouse PhIP alone; P < 0.05). This study shows that the *Apc^min* genotype is associated with resistance to PhIP-induced apoptosis in intestinal epithelium. Curcumin attenuates *Apc^min* resistance to PhIP-induced apoptosis and inhibits PhIP-induced tumorigenesis in proximal *Apc^min* mouse small intestine.

Introduction

Heterocyclic amines (HCA) are formed by the cooking of meat and comprise an important class of human dietary carcinogens. PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) is the most abundant HCA in cooked meat (1) and is bioactivated to its genotoxic N-hydroxy derivative (2) by cytochrome P4501A2 (3). The intestinal or colonic epithelia form a primary interface with dietary PhIP, are metabolically competent for PhIP bioactivation (4) and may be particularly susceptible to genotoxic injury (2).

DNA damage resulting from dietary PhIP exposure may be converted to characteristic mutations in the adenomatous polyposis coli (*APC*) tumour suppressor gene (5). *APC* mutation is an early event in the evolution of familial adenomatous polyposis (FAP) (6) and sporadic human colorectal cancer (7),

Abbreviations: APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; HCA, heterocyclic amines; ISEL, *in situ* end-labelling; NF κB, nuclear factor kappa B; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; TNFα, tumour necrosis factor alpha.

and promotes intracellular accumulation of β-catenin, increased expression of the AP-1 transcription complex (8) and a phenotype with altered apoptosis (9). The *Apc^min* heterozygous mouse carries a germline nonsense mutation in codon 850 of *Apc* (10) and mutational inactivation of the second *Apc* allele promotes adenoma formation (11). These mice develop, on average, >50 adenomas over the entire length of the intestinal tract. Like FAP, *Apc^min* is an autosomal dominant trait. Intraperitoneal injection of PhIP has been shown to significantly increase the numbers of small tumours and cystic crypts in the proximal section of the small intestine of male *Apc^min* mice, and the number of aberrant crypt foci in the colons of both males and females (12). In addition, neonatal exposure to PhIP directly or via breast milk resulted in an increase in tumour number in both small intestine and colon of *Apc^min* mice (13).

Regulatory pathways of apoptosis may provide novel molecular targets for chemoprevention (9,14). Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl) 1,6-heptadiene-3,5-dione], the major pigment derived from turmeric, inhibits the growth of colon adenocarcinoma cell lines HT29 and HCT15 (15), suppresses activation of the transcription factor NFκB (16,17) and promotes apoptosis in neoplastic cells *in vitro* (18,19) and in rat colonic epithelium *in vivo* (20). Administration of curcumin in the diet suppresses azoxymethane-induced tumour incidence and multiplicity in male F344 rats (21) and inhibits benzo[*a*]pyrene-induced forestomach tumours and 12-*O*-tetradecanoylphorbol-13-acetate-induced skin tumours in mice (22). In addition, dietary curcumin has been shown to promote apoptosis and inhibit spontaneous tumour formation in *Apc^min* mice (23). Development of a role for curcumin in clinical chemoprevention may be enhanced by studies in carcinogenesis models which mimic human disease. In spite of differences in the distribution of tumours between FAP patients and *Apc^min* mice, the *Apc^min* model is considered a useful one for the study of both FAP and sporadic intestinal neoplasms and has previously been used to assess the chemopreventive potential of agents such as aspirin (24) and sulindac (25).

In this study we assessed the effects of PhIP and curcumin on tumorigenesis and regulation of apoptosis in the *Apc^min* mouse intestine. Our findings further support a role for curcumin in chemopreventive strategies against colorectal cancer.

Materials and methods

Chemicals and reagents

PhIP was obtained from NARD Pharmaceuticals (Amagasaki, Japan) and curcumin was obtained from Sigma-Aldrich (Dorset, UK). All other reagents were readily available commercially.

Animals

Four-week-old *Apc^min* heterozygous C57BL/6J male mice and their male wild-type *Apc*$^{+/+}$ littermates were used in the study. Calculations of animal numbers were based upon previous studies of curcumin effects on azoxymethane colonic tumorigenesis where curcumin treatment reduced tumour number by 40% (20). On the assumption of comparable chemoprevention by curcumin in a PhIP tumorigenesis model, 10 animals in each group were calculated to provide 80% power for detection of a 40% difference of polyp number, with

95% confidence. Animal numbers were kept to this minimum in accordance with the United Kingdom Coordinating Committee on Cancer Research guidelines (26).

Genotyping

Tail DNA genotyping was carried out by allele specific PCR (27). Tail tips were finely minced and suspended in 1 ml PCR digestion buffer (50 mM KCl, 10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.45% Nonidet P40, 0.45% Tween 20, 0.1 mg/ml gelatin). Aliquots of 10 µl of 20 mg/ml proteinase K were then added before digestion at 55°C for 2 h. Proteinase K was inactivated by incubation at 95°C for 10 min, then samples were centrifuged at 12 000 *g* for 1 min and the supernatants removed and stored at –20°C until PCR amplification. Genomic DNA (2 µl of the proteinase K digest) was amplified in a 25 µl reaction volume containing final concentrations of 12.5 µM APC-specific primers (fwd, 5′-TGATACTTCTTCCAAAGCTT-TGGCTAT; rev, 5′-TCTCGTTCTGAGAAAGACAGAAGC), 50 mM Tris–HCl, 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.01 U/µl *Taq* polymerase. Amplification was carried out for 4 min at 94°C followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min before a final extension at 72°C for 10 min. An aliquot (10 µl) of the PCR reaction was then digested with *Hin*dIII (1 U) in a final volume of 20 µl at 37°C for 1 h. Wild-type (123 bp) and mutated (144 bp) bands were separated on an 8% acrylamide gel and visualized after staining with ethidium bromide.

Treatment regimes

The pelleted AIN-76 diet which lacks carcinogens was used as control and vehicle diet for delivery of curcumin (2000 p.p.m.) and PhIP (300 p.p.m.). Four groups of 10 Apc^{min} and wild-type $Apc^{+/+}$ C57BL/6J mice received (i) control, (ii) curcumin only, (iii) PhIP only and (iv) curcumin + PhIP diets, respectively for 10 weeks. Animals were weighed weekly and checked daily for signs of ill health. Each animal was given 6 g diet daily. At 70 days, animals were killed by CO_2 inhalation and their intestinal tracts were removed and flushed with phosphate-buffered saline (PBS). The small intestine was divided into three equal sections, by length, opened longitudinally and polyps were counted immediately using an illuminated magnifier and their diameters were measured. Sections of intestine bearing polyps were then fixed overnight in 10% neutral buffered formalin and embedded in paraffin blocks for histochemical analysis or assessment of apoptosis.

Assay of apoptosis in intestinal tissue by in situ end-labelling (ISEL)

Tissue sections were deparaffinized, rehydrated and incubated with 40 µg/ml proteinase K for 30 min at 37°C. After washing in ddH_2O, 70 and 100% ethanol for 2 min each, the sections were incubated with 10 µM each dCTP, dGTP, dTTP and biotinylated dATP and 5 U/ml Klenow fragment in 50 mM Tris–HCl (pH 8.0) containing 10 mM $MgCl_2$ and 50 mM NaCl at 37°C for 30 min. The sections were rinsed in ddH_2O and blocked in 0.8% H_2O_2 in PBS for 15 min at room temperature. After washing in PBS the slides were maintained at room temperature for 30 min, followed by a further wash in PBS and colour development using DAB. The sections were then counterstained in haematoxylin, dehydrated and mounted in DPX mounting medium. Apoptotic cells were identified under the light microscope by their brown colouration and morphological criteria, as previously defined. The apoptotic index for tissue assays was defined as the percentage of ISEL positive cells exhibiting morphological apoptotic features. A total of 300 cells were counted per section in adjacent crypts in randomly chosen fields, as described by Samaha *et al.* (20). The observer was blinded to the animal treatment groups. Approximately 84% of intestinal sections upon which assays for apoptosis were carried out were longitudinal crypt sections, enabling assessment of apoptosis along the entire length of the crypt. Other sections contained a mixture of longitudinally and transversely sectioned crypts.

Statistical analysis

The Kruskal–Wallis H test was used for analysis of variance of non-parametric data among all test groups. The Mann–Whitney *U* test was applied to assess differences of non-parametric data between paired independent groups. Values of $P < 0.05$ were considered significant.

Results

Effects of dietary PhIP on intestinal epithelial apoptosis in wild-type $Apc^{+/+}$ and Apc^{min} mice

Rates of ISEL detected apoptosis were compared in proximal, middle and distal thirds of $Apc^{+/+}$ and Apc^{min} mouse small intestine. No differences of apoptosis were observed between untreated $APC^{+/+}$ and Apc^{min} mucosa (Table I). Kruskall–Wallis analysis of variance demonstrated significant differences of apoptosis between treatment groups ($P < 0.001$). PhIP induced epithelial apoptosis in all three anatomical levels of the small intestine in both mouse strains (Figure 1), although higher levels were observed in $APC^{+/+}$ than in Apc^{min} mice ($P < 0.001$). Lower levels of PhIP-induced apoptosis were observed in Apc^{min} adenomas than in Apc^{min} unaffected mucosa (Table I) ($P < 0.001$).

Effects of dietary PhIP on intestinal tumour formation in wild-type $Apc^{+/+}$ and Apc^{min} mice

Dietary PhIP increased tumour formation in the proximal third of Apc^{min} mouse small intestine ($P < 0.05$; Table II). Tumour size was greater in PhIP-treated than control Apc^{min} mice ($P < 0.001$; Table III). No tumours were seen in the intestines of any wild-type $Apc^{+/+}$ mice.

Effects of curcumin on PhIP-induced apoptosis and tumour formation

Curcumin treatment enhanced PhIP-induced apoptosis ($P < 0.01$; Table I) and inhibited PhIP-induced tumorigenesis in the proximal small intestine of Apc^{min} mice. Tumour number ($P < 0.05$) was lower in curcumin + PhIP versus PhIP-treated Apc^{min} mice (Table II). No effects of curcumin treatment were observed on PhIP-induced apoptosis or tumour formation in Apc^{min} mid or distal small intestine. No effects of curcumin treatment were observed on PhIP-induced apoptosis in $Apc^{+/+}$ mouse small intestine.

Discussion

Much of the variation of human cancer incidence may be associated with dietary differences (28). Meat intake has been implicated in the aetiology of colorectal cancer and heterocyclic amines, including PhIP, have come under close scrutiny (3). Cancer risk may also be ameliorated by dietary agents. Improved understanding of interactions between pro and anticarcinogens may enhance chemoprevention strategies.

In this study we show that dietary administration of curcumin inhibits PhIP-induced tumorigenesis and promotes apoptosis in the proximal section of the small intestine. In addition, induction of apoptosis following administration of PhIP is shown to be more pronounced in the normal intestinal mucosa of wild-type mice than in that of Apc^{min} mice, suggesting that the *Apc* status of a cell may have an effect on its ability to respond to PhIP-induced DNA damage by inducing apoptosis. Furthermore, we show that in proximal small intestine this resistance to PhIP-induced apoptosis in the Apc^{min} mouse can be reversed by administration of curcumin in the diet.

The increase in tumour number in only the proximal part of the small intestine of Apc^{min} mice after exposure to PhIP is in accordance with a previous study which showed that administration of PhIP by intraperitoneal injection resulted in a similar region-specific effect, although only an increase in the number of small tumours was observed (12). We also show that PhIP is associated with increased tumour size, an effect again only seen in proximal small intestine. Mechanisms responsible for this regional specificity of PhIP are unclear. Our results suggest that it is not due to regional differences in the mutagenic effect of PhIP since the rate of PhIP-induced apoptosis is not significantly altered along the length of the small intestine of both Apc^{min} and $Apc^{+/+}$ mice. Indeed, it has previously been shown that there is no difference in mutability of PhIP between the proximal and distal section of the small intestine of mice exposed to the carcinogen (29). PhIP-induced
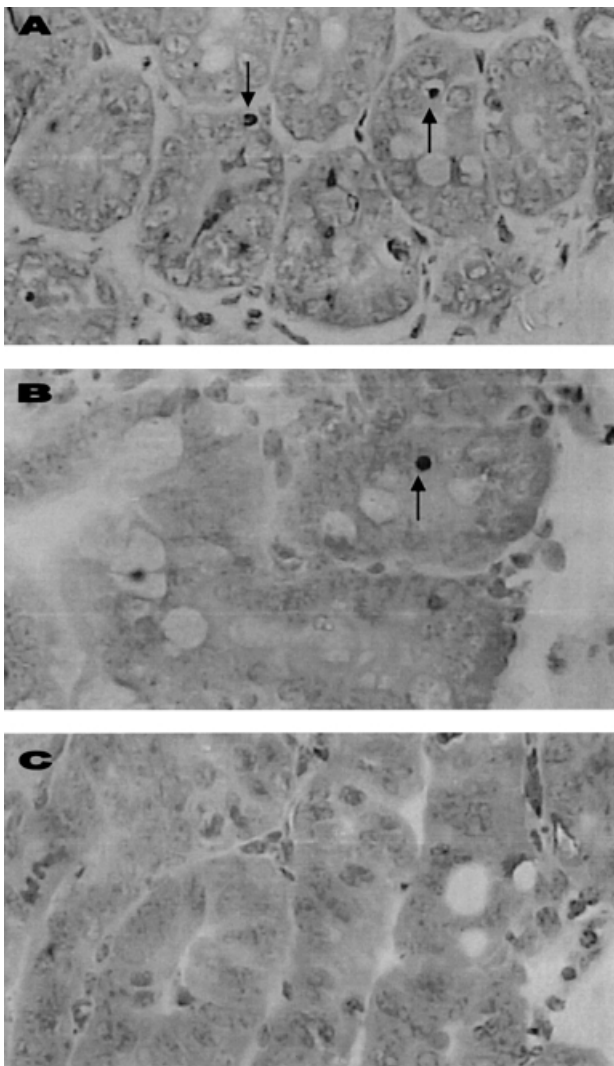
Table I. Apoptotic indices of normal mucosa and adenomas of $Apc^{+/+}$ and Apc^{min} mice

Genotype	Diet	Proximal SI	Middle SI	Distal SI	Total SI	Adenomas
$Apc^{+/+}$	Control	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.2	–
$Apc^{+/+}$	Curcumin	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	–
$Apc^{+/+}$	PhIP	3.7 ± 2.0[a]	3.9 ± 1.4[a]	3.4 ± 2.0[a]	3.7 ± 1.7[a]	–
$Apc^{+/+}$	Curcumin + PhIP	3.7 ± 1.8	2.7 ± 1.0	2.9 ± 1.4	3.1 ± 1.4	–
Apc^{min}	Control	0.2 ± 0.3	0.2 ± 0.2	0.4 ± 0.4	0.2 ± 0.2	0.3 ± 0.3
Apc^{min}	Curcumin	0.4 ± 0.6	0.3 ± 0.1	0.1 ± 0.2	0.2 ± 0.3	0.1 ± 0.1
Apc^{min}	PhIP	2.1 ± 1.7[a]	1.6 ± 0.6[a]	2.2 ± 1.0[a]	2.0 ± 0.9[a]	0.4 ± 0.3[b]
Apc^{min}	Curcumin + PhIP	4.0 ± 1.3[c]	2.3 ± 1.4	2.0 ± 1.4	2.9 ± 1.6	0.6 ± 0.4

[a]PhIP treatment induced higher levels of apoptosis throughout the small intestine in $Apc^{+/+}$ versus Apc^{min} intestinal mucosa ($P < 0.001$ by Mann–Whitney test).
[b]PhIP treatment induced higher levels of apoptosis in uninvolved Apc^{min} intestinal mucosa than in Apc^{min} adenomas ($P < 0.001$ by Mann–Whitney test).
[c]Curcumin treatment enhanced PhIP apoptosis in Apc^{min} proximal small intestinal mucosa ($P < 0.01$ by Mann–Whitney test). Apoptosis values expressed as percentages.

Fig. 1. Apoptosis in $Apc^{+/+}$ and Apc^{min} mucosa. Apoptosis in (**A**) unaffected APC$^{+/+}$ mucosa, (**B**) Apc^{min} mucosa, (**C**) Apc^{min} adenoma after PhIP treatment. Apoptosis was detected by *in situ* end labelling. Arrows show apoptotic cells.

Table II. Small intestinal adenoma formation in Apc^{min} mice

Diet	Proximal SI	Middle SI	Distal SI	Total SI
Control	2.1 ± 2.5	6.0 ± 5.9	6.1 ± 5.2	14.1 ± 11.6
Curcumin	1.5 ± 2.2	6.5 ± 6.2	5.1 ± 6.7	13.2 ± 15.1
PhIP	4.6 ± 2.7[a]	7.8 ± 10.2	4.6 ± 10.3	17.1 ± 22.5
Curcumin + PhIP	2.2 ± 1.5[b]	6.7 ± 6.6	7.4 ± 5.6	16.3 ± 12.3

[a]PhIP promotes adenoma formation in Apc^{min} proximal small intestine ($P < 0.05$ by Mann–Whitney test).
[b]Curcumin inhibits PhIP tumorigenesis in Apc^{min} proximal small intestine ($P < 0.05$ by Mann–Whitney U test).

Table III. Apc^{min} adenoma size

Diet	Proximal SI	Middle SI	Distal SI	Total SI
Control	2.5 ± 0.6	2.0 ± 0.8	1.7 ± 0.6	1.9 ± 0.7
Curcumin	2.9 ± 1.0	2.0 ± 0.7	1.8 ± 0.9	2.1 ± 0.9
PhIP	3.8 ± 1.7[a]	2.1 ± 0.8	2.1 ± 1.0	2.8 ± 1.5
PhIP + curcumin	3.2 ± 1.1	1.9 ± 1.0	1.7 ± 0.6	2.0 ± 1.1

[a]PhIP treatment is associated with increased tumor size in Apc^{min} proximal small intestine ($P < 0.001$ by Mann–Whitney test). Curcumin and PhIP treatment is associated with lower adenoma size than PhIP treatment alone in Apc^{min} small intestine ($P < 0.001$ by Mann–Whitney test).

in the cell. Similarly, expression of APC in human colorectal cancer cells containing endogenous inactive *APC* alleles inhibits cell growth by the induction of apoptosis (9).

Curcumin has previously been shown to inhibit tumorigenesis in animal models (21,22) and to promote apoptosis both *in vivo* and *in vitro* (18–20). Curcumin given to female Apc^{min} mice resulted in a decrease in intestinal tumour number and an increase in apoptosis (23). In the present study we administered a 2-fold higher dose of curcumin to male Apc^{min} mice, but found no effect on spontaneous apoptosis or tumour number. It is possible that the sex of test animals could influence the response to curcumin. However, when given in combination with PhIP, curcumin significantly reduced the number of tumours in the proximal small intestine of Apc^{min} mice compared with PhIP alone, so that tumour numbers were similar to those in mice receiving control diet. This decrease in tumour number was accompanied by a corresponding increase in apoptotic index. In contrast, in the middle and distal sections of the small intestine, apoptotic indices in PhIP/curcumin-treated animals remained unchanged from those

apoptosis was greater in the unaffected intestinal epithelium of wild-type mice than in that of heterozygous mutant Apc^{min} mice which in turn was greater than in homozygous mutant Apc^{min} adenomas, suggesting that resistance to PhIP-induced apoptosis may be related to the mutational status of *Apc* alleles

receiving PhIP alone. This suggests that the protective effect of curcumin against PhIP-induced tumorigenesis is achieved, at least in part, by the reversal of resistance of *Apc^{min}* intestinal epithelium to PhIP-induced apoptosis. Curcumin treatment may inhibit apoptosis resistance associated with *Apc* mutation through alteration of the equilibrium of NFκB and p53. These transcription factors have been shown to regulate each other's ability to stimulate gene expression in a process that is controlled by their relative levels (30). Curcumin inhibits TNF-induced NFκB activation in human intestinal cell lines (31), and has been shown to increase p53 expression in basal cell carcinoma cells (32). Activation of NFκB by DNA damage appears to be mediated through the same signalling intermediates as activation by cytokines such as TNF. Both result in the induction of IκB kinase dependent degradation of IκB in the cytoplasm, resulting in the release and activation of NFκB (33). This pathway may be implicated in curcumin effects on PhIP-induced apoptosis and tumorigenesis in *Apc^{min}* mice.

In conclusion, our findings support a role for curcumin in the inhibition of adenoma formation and progression, from a background of heterozygous APC mutant epithelium. These findings may facilitate chemopreventive strategies, in the human corollary of FAP.

Acknowledgements

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