RESEARCH ARTICLE

Chemopreventive effect of dietary curcumin on inflammation-induced colorectal carcinogenesis in mice

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Scope: Curcumin is a polyphenol with a variety of pharmacologic effects. We evaluate the effect of dietary curcumin on the severity of repeated colitis-associated colorectal cancer.

Methods and results: Six-week-old C57BL/6 mice were randomized into two dietary groups: standard diet and curcumin at 0.6% diet. The mice were exposed to 15 cycles of 0.7% dextran sodium sulphate for 1 week followed by distilled water for 10 days. After curcumin diet, the disease activity index presented a statistical reduction in the last cycles, macroscopic tumors were not seen and the microscopic study showed minor neoplasic lesions with respect to standard diet-group. β-Catenin translocation to the cytoplasm and/or nucleus was observed in the tumor tissue, but this translocation and its intensity were significantly minor in the curcumin diet-DSS animals. Cytokines as tumor necrosis factor-α and IFN-γ were significantly diminished in DSS-animals fed with curcumin. Conversely, non-modification of p53 expression was observed and cyclo-oxygenase-2 and inducible nitric oxide synthase were significantly reduced in the curcumin diet-DSS group.

Conclusion: We demonstrate the protective/preventive effect of curcumin in the progression of colorectal cancer associated to colitis, which was correlated with a lowered immunoreactivity of ß-catenin, a non-modification of p53 expression, a reduction of proinflammatory cytokine levels and a decrease of inflammatory protein overexpression.

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1 Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal tract malignancies worldwide, principally in the western and westernized countries. Most common risk factors for CRC include genetic predispositions (adenomatous polyposis coli (APC) and hereditary nonpolyposis colon cancer) and exposure to radiation. But intestinal inflammation (ulcerative colitis and Crohn's disease) also drastically increases the risk of developing colon cancer and the degree of inflammation correlates with cancer risk [1].

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Abbreviations: AOM, azoxymethane; **APC,** adenomatous polyposis coli; **COX-2,** cyclooxygenase-2; **CRC,** colorectal cancer; **DAI,** disease activity index; **DSS,** dextran sulphate sodium; **IBD,** inflammatory bowel disease; **iNOS,** inducible nitric oxide synthase; **PGES,** prostaglandin E synthase; **TNF,** tumor necrosis factor

Numerous mechanisms have been described to explain the progress and expansion of colitis-associated CRC. Nevertheless, the main genomic instability that contributes to colon carcinogenesis is the chromosomal instability, which results in damage of genetic material and, consequently, the loss of p53 tumor suppressor and *APC* gene function. The APC protein transferred from the *APC* gene binds and degrades the β-catenin protein, an important cancer target that plays a key role in both cell adhesion and intracellular signalling. However, mutated APC protein, by mutation in the *APC* gene, cannot bind and degrade β-catenin. As a result, β-catenin translocates to the nucleus and, finally, promotes cell proliferation [2]. p53 protein regulates growth and apoptosis; loss of p53 gene function occurs late and is believed to be the defining event that drives the adenoma to carcinoma [3].

Overall, chronic inflammation is the most predisposing key factor to CRC from inflammatory bowel disease (IBD). Proinflammatory cytokines and growth factors released during the inflammation by immune and non-immune cells may influence the carcinogenesis process [4]. It has been proposed that these cytokines contribute to carcinogenesis by influencing the

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survival, growth, mutation, proliferation, differentiation and movement of tumor and stromal cells and by regulating angiogenesis [5]. Moreover, the inducible isoforms of cyclo-oxygenase (COX-2) and nitric oxide synthase (iNOS) are the main enzymes involved in the inflammatory process [6]. In several types of cancer, particularly, gastric carcinoma and colon adenoma, COX-2 is upregulated generating protumorigenic eicosanoids, in particular, prostaglandins that can promote cell growth, angiogenesis and suppression of immunity. iNOS produces large amounts of nitric oxide involved in the initiation, promotion and progression of tumors [7].

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a polyphenol found in this dietary spice, derived from dried rhizomes of the perennial herb *Curcuma longa* Linn., a member of the ginger family. Interest in this dietary polyphenol has grown in recent years because of its vast array of beneficial pharmacological effects including chemosensitizing, radiosensitizing, wound healing activities, antimicrobial, antiviral, antifungical, cholekinetic, antioxidant [8], anti-inflammatory and immunomodulatory [9, 10] benefits. Several studies, including ours, have identified curcumin as a protective agent in the control of inflammatory disorders such as arthritis and IBD [11]. Because of its ability to interact with multiple molecular targets affecting the multifaceted process of carcinogenesis, curcumin is being investigated as a new promising anticancer agent. In fact, the preclinical studies of curcumin have shown beneficial effects in various types of cancer, including breast, cervical, gastric, hepatic, leukemia, oral epithelial, ovarian, pancreatic, prostate and CRCs [12]. Indeed, curcumin is able to block the carcinogenesis at various stages: tumor initiation, promotion, proliferation, angiogenesis and development of metastasis. Also, curcumin is able to stimulate apoptosis in cancer cells, by interfering with multiple signaling pathways [13]. Therefore, curcumin is a suitable candidate to be incorporated in our study in order to address the inhibition of the progression of colitis-associated neoplasia.

In rodents, cyclic administration of dextran sulphate sodium (DSS) in drinking water results in the establishment of chronic colitis and the development of colorectal dysplasias and cancers with pathological features that resemble those of human colitis-associated neoplasia. The DSS colitis model shows the ''inflammation-dysplasia carcinoma-sequence'' of CRC development, as well as the interplay between the causative factors and background genetics [14]. The aim of this study was to first study the chemopreventive effect of dietary curcumin administered during the promotion/progression stage of colon carcinogenesis in female C57BL/6 mice by means of macro and microscopic techniques. Then, since the translocation of β-catenin from the cell membrane to the cytoplasm or nucleus is an important early event in colorectal carcinogenesis, we also evaluated β-catenin involvement in our animal model. Third, as mentioned above, cytokines released during the inflammation by immune and non-immune cells may influence the development and growth of colitis-associated CRC, thus changes in the colonic tumor necrosis factor (TNF)-α, IFN-γ and IL-6 levels were also investigated. Finally, studies of expression from COX-2, prostaglandin E synthase (PGES)-1, iNOS and p53 will also be analyzed in the colonic mucosa.

2 Materials and methods

2.1 Experimental animals

A total of 84 female C57BL/6 mice (6 weeks of age) were obtained from Charles River (Barcelona, Spain). After weaning and during the experiments, the mice were kept in cages in groups of five or six and maintained in an air-conditioned quarters with a room temperature of 24–25°C, constant humidity and an alternating 12-h light/dark cycle. The mice were randomized into two dietary groups: one was fed with a standard diet and the other one received a diet enriched with curcumin at 0.6% (Supporting Information Table). The dose of curcumin was chosen based on the analyses described in the literature [15]. Curcumin group consumed an average of 3 g of diet at day resulting in a dose of 18 mg of curcumin ingested at day by mouse. The components of the diet (an AIN76A diet) were supplied by Harlan Iberica SA (Barcelona, Spain). The standard AIN76A diet contains 490 mg iron/kg diet, but since previous studies have confirmed that iron may increase disease activity in colitis and this is associated with oxidative stress and neutrophilic infiltration [16], both diets were supplemented with two times the amount of iron in the modified control AIN76A diet (900 mg iron/kg diet). The diets were prepared every week by mixing the respective compounds. The animals were fed with the corresponding diet during two weeks previous to the colitis induction and during the experiment. Body weights, food and drink consumptions were monitored once per week throughout the experiment (data not shown).

Experiments followed a protocol approved by the Animal Ethics Committee of the University of Seville and all experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC).

2.2 Induction of colitis

Chronic ulcerative colitis was induced by the repeated administration of DSS (0.7% w/v; MW: ≈40 000; catalogue number DB001, obtained from TdB Consultancy AB, Uppsala, Sweden), according to the method described by Yeo *et al.* [14]. Each dietary group was divided into two other groups: one group of 30 mice was exposed to 15 cycles of DSS (DSS group) and a second group of 12 mice was administered ordinary tap water throughout the experiment (sham group). Each cycle consisted of 7 days of 0.7% DSS w/v in the drinking fluid, followed by 10 days of ordinary tap water. Animals were sacrificed at the end of treatment (37 weeks) by an overdose of i.p. chloral hydrate.

2.3 Evaluation of the severity of clinical colitis

The clinical activity of colitis was evaluated during the experimentation phase in order to determine the disease activity index (DAI) as described by Gommeaux *et al.* [17]. The presence of diarrhea, rectal bleeding and weight loss were registered at the beginning, in the middle and at the end of the DSS treatment as well as during the phase when the animals were on a pure-water diet. The latter was done for each different cycle, separately graded on a scale (Table 1). The average of the three values constituted the DAI.

2.4 Macroscopic and histopathological evaluation

After killing the animals, colons were removed, slightly cleaned in physiological saline to remove fecal residues, weighed and measured. Macroscopic damage was evaluated by an independent observer who was unaware of the treatment. Weight/length ratio of the animal's colon was also determined as an indirect marker of inflammation.

Photographs taken from the colon samples were digitized using Kodak D290 Zoom camera (Eastman Kodak, Rochester, NY, USA). Pieces of the colon were collected and frozen in liquid nitrogen to measure biochemical parameters.

The colon was divided into three segments: proximal, middle and distal. Tissue samples of the region with polypoid or the flat-elevated lesion were excised out of every segment, fixed in 4% buffered formaldehyde, dehydrated by increasing concentrations of ethanol and embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin. Mucosal integrity was observed histologically in a blinded experiment and dysplasia was analyzed and diagnosed as low-grade or high-grade of dysplasia and adenocarcinoma, according to the established criteria [18]. Tumor incidence was calculated as the number of macroscopic tumor-bearing mice divided by the total number of mice and tumor multiplicity as the number of tumors divided by the number of tumor-bearing mice [14]. Histopathological evaluation was determined by a pathologist who was unaware of the experimental protocol.

Table 1. Scoring of DAI in DSS-induced colitis

Score	Bleeding	Weight loss (% of initial wt.)	Stool consistency
0	None	<1	Normal stools
1	Small spots of blood in stool; dry anal region	1–4.99	Soft pellets not adhering to the anus
2	Large spots of blood in stool; blood appears through anal orifice	5–10	Very soft pellets adhering to the anus
3	Deep red stool; blood spreads largely around the anus	>10	Liquid stool on long streams; wet anus

Criteria from the work of Gommeaux *et al.* [17].

2.5 Immunohistochemical evaluation of β-catenin

β-Catenin staining was carried out using a streptavidin–biotin-peroxidase method as described by Sánchez-Fidalgo *et al.* [19]. For immunohistochemical analysis, the pathologist quantified β-catenin by means of the observation of three arbitrary high power fields from distal colon from eight animals that had neoplasic lesions. The number of positive cells in 100 cells from each field was counted and the average of these values was calculated. Therefore, β-catenin staining was expressed as the percentage of cells in each group showing cell membrane or cytoplasmic and/or nucleus expression [20].

2.6 Colonic cytokine levels

TNF-α, IL-6 and IFN-γ concentrations in the colonic tissue were measured by the quantitative enzyme immunoassay kits according to the manufacturer's protocol (Diaclone, Besançon, France). Briefly, the colon samples were weighed and homogenized, after thawing, in 0.3 mL PBS solution (pH 7.2) 1% BSA at 4°C, and were centrifuged at $12\,000 \times g$ for 10 min. The concentrations of cytokines were determined by duplication. TNF-α, IL-6 and IFN-γ values were expressed as pg/mg tissue.

2.7 Isolation of cytoplasmic proteins and Western blot analysis

The frozen colonic tissues were weighed and homogenized in an ice-cold buffer (50 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM EGTA, 0.5 mM EDTA, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, 1 mM PMSF and 250 mM NaCl. Homogenates were centrifuged ($12\,000 \times g$, 15 min, 4°C) and the supernatants were collected and stored at −80°C. The protein concentration of the homogenate was determined following the Bradford colorimetric method [21]. Aliquots of supernatants containing equal amounts of protein (50 μg) were evaluated to determine p53, COX-2, PGES-1 and iNOS proteins by Western blot as described by Sánchez-Fidalgo *et al.* [19].

2.8 Statistical methods

All values in the figures and text are expressed as arithmetic mean ± standard error (SEM). The data were evaluated with the Graph Pad Prism® version 2.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way ANOVA, using Tukey–Kramer multiple comparisons test as post hoc test. The descriptive values have been evaluated by chi-square

test. p values of <0.05 were considered statistically significant. In the experiment involving histology, immunohistochemistry or Western blot, the figures shown are representative of at least six experiments performed on different days.

3 Results

3.1 General observations after long-term DSS experiment

The clinical findings in the two dietary groups during 15 cycles of DSS administration in the drinking water were evaluated in the disease course. Both dietary groups, the standard and the curcumin diets, showed evident clinical signs of diarrhea and rectal hemorrhage. The evolution of the body weight and consumption of food and drink during this period did not show significant differences (data not shown).

Stool consistency was gradually increased from the 4th-DSS cycle and the bleeding score also increased from the 12th cycle in the standard diet-DSS group. The highest levels of diarrhea and visible fecal blood were found on cycles 12 and 13 respectively and this group always showed significant higher values than the curcumin diet-DSS group in the last cycles (data not shown).

In this line, the DAI analysis in the course of 15 cycles of DSS treatment showed a statistical increase ($p<0.05$) from cycles 12 to 15 in the standard diet-DSS group *versus* the curcumin diet-DSS group (Fig. 1).

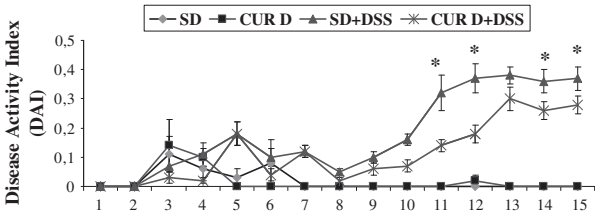

Figure 1. Effect of curcumin diet at 0.6% on disease activity index (DAI) combining the scores of bleeding, weight loss and stool consistency divided by 3. Data are expressed as the means ± S.E.M. (*) $p<0.05$ *vs.* curcumin diet-DSS animals.

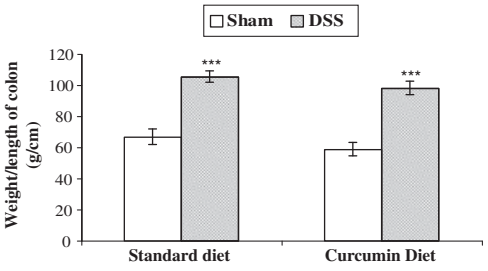

Figure 2. Effect of curcumin diet at 0.6% on weight/length of the colon in the experimental animal model of dextran sodium sulphate (DSS)-induced colon cancer. Data are expressed as the means ±S.E.M. (***) $p<0.001$ *vs.* Sham group.

At the end of the treatment, the weight/length of the mice colon in both diets augmented significantly after DSS administration in contrast with their sham groups ($p<0.001$), without differences between both dietary-DSS groups (Fig. 2).

3.2 Development of cancer in an experimental model of repeated DSS-induced colitis

The number, size and location of detectable tumors were examined macroscopically. None of the animal controls, in the standard or curcumin diets, which were not subjected to the cycles of DSS, showed inflammation and/or injury in the colon. Among the mice that received 15 cycles of DSS and were fed with the standard diet was observed a tumor incidence of 6/20 and a value of tumor multiplicity of 1 (Table 2). Macroscopic tumor areas showed nodular and polypoid tumors that appeared mainly in the middle/distal portion of the large intestine. On the contrary, none of the animals fed with the curcumin diet had any macroscopic lesions, since a tumor incidence of 0/20 was observed ($p<0.01$ *versus* standard diet-DSS group).

3.3 Histopathological analysis of UC-associated colorectal tumors

Histopathological study of the inflammation in DSS-treated animals from both the experimental diets showed mild-to-moderately severe inflammation mainly in the middle and distal sections without differences between them. It was characterized by crypt abscess and inflammatory lymphocytic infiltration with the glandular destruction and regenerative atypia, and ulcerations were not observed. All the slides showed zones with inflammation-limited surface epithelium, zones with focal inflammation limited to mucosa and other zones with focal transmural inflammation.

Incidence of UC-associated neoplasic lesions were analyzed and diagnosed as it is shown in Table 2. A total of 11 out of 20 of the mice that received 15 cycles of DSS and were fed with the standard diet developed adenocarcinomas (Supporting Information Fig. 1A) and 17 from 20 exhibited high-grade of dysplasia (Supporting Information Fig. 1B). Nevertheless, the number of adenocarcinomas and high-grade dysplasia were reduced, although not significantly, to 12 and 7 out of 20 respectively in curcumin-fed animals. About 100% of the animals treated with 15 cycles of DSS independently of the diet used experimented low-grade dysplasia (Supporting Information Fig. 1C).

3.4 Translocation of β-catenin into the nucleus and overexpression in colitis-induced colon cancer

Other studies have demonstrated that β-catenin is a useful biomarker for colitic cancer in mice with DSS-induced colitis.

Table 2. Incidence of ulcerative colitis-associated neoplasic lesions in animals treated with 15 cycles of DSS (0.7% v/v) and fed with different diets: standard diet and curcumin-enriched diet

Diet	Low-grade dysplasia	High-grade dysplasia	Adenocarcinoma	Tumor incidence	Tumor multiplicity
Standard	20/20	17/20	11/20	6/20	1
Curcumin	20/20	12/20	7/20	0/20**	0

Neoplasic lesion incidence: number of malignant lesions-bearing mice/total number of mice per group [14]. **$p<0.01$ *versus* standard group.

Table 3. Immunohistochemical evaluation of β-catenin in sham animal and treated with 15 cycles of DSS (0.7% v/v) and fed with different diets: standard diet and curcumin-enriched diet

Group		β-Catenin staining pattern (%)	
		Cell membrane	Cytoplasmic and/or nucleus
Standard	Sham	79.3±4.3	0
	DSS	90.2±5.5	58.3±3.5***
Curcumin	Sham	81.0±5.0	0
	DSS	85.0±5.6	22.8±2.8** +++

Data are expressed as the mean±SEM. **$p<0.01$ and ***$p<0.001$ *versus* respective sham group; +++$p<0.001$ *versus* standard diet-DSS animals.

Translocation of this protein from the cell membrane to the cytoplasm or nucleus is an important early event in human colorectal carcinogenesis. In our study, positivity of β-catenin was mainly localized at the membranes of cell–cell borders in normal colon epithelial cells from both diets (Table 3) (Supporting Information Fig. 2A). However, its translocation from the membrane to the cytoplasm and into the nucleus was observed in the tumor tissue of DSS-treated mice. Moreover, positive reaction against β-catenin was significantly higher in samples from animals fed with standard diet ($p<0.001$) than in those from DSS-curcumin animals, where also the intensity of β-catenin expression was weaker (Supporting Information Fig. 2B and C).

3.5 Proinflammatory cytokine levels in UC-associated colorectal carcinogenesis

To investigate the role of TNF-α, IFN-γ and IL-6 in CRC associated with chronic colitis, we analyzed the production of selected cytokines in the colonic tissues homogenates after 15 cycles of DSS treatment (Fig. 3). Production of these three cytokines was significantly enhanced to the end of DSS treatment in the animals fed with the standard diet ($p<0.05$) *versus* sham group. However, TNF-α and IFN-γ levels were significantly diminished ($p<0.05$) in DSS-animals fed with the curcumin diet with respect to standard diet. IL-6 levels augmented after 15 cycles of DSS although no differences between the diet groups were observed.

3.6 Identification of differentially expressed proteins in UC-associated colorectal carcinogenesis

p53, COX-2, PGES-1 and iNOS protein expressions were determined by Western blotting analysis. p53 is a potent inhibitor of cell growth and tumor growth, and its inactivation and/or mutation is considered a prerequisite for tumor formation. As shown in Fig. 4A, in this experimental model, p53 protein diminished after the DSS treatment, although this downregulation was significant only in the standard diet-fed animals ($p<0.05$ *versus* sham), whereas the same levels remained for its sham group in curcumin-fed mice.

The expression of inflammatory proteins, COX-2 and iNOS, in the colon were also examined (Fig. 4B and D respectively). COX-2 and iNOS expressions significantly augmented in the standard diet-fed group treated with DSS ($p<0.05$ *versus* sham). On the contrary, these proteins did not show significant changes in the animals fed with the curcumin diet and after 15 cycles of DSS treatment *versus* its respective sham group. Moreover, this protein reduction was significant when compared with the standard diet-DSS group ($p<0.05$). The study of PGES-1 expression did not show statistical differences between the control groups and the mice treated with 15 cycles of DSS solution (Fig. 4C).

4 Discussion

Induction and maintenance of remission and mucosal healing might be one of the primary therapeutic goals in ulcerative colitis in order to decrease the likelihood of cancer developing, since IBD patients are among the highest risk groups for developing CRC. Therefore, the focus of this study has been to deepen in the potential protective role of curcumin administration by diet in the development of dysplasia and/or cancer from a chronic colitis in a DSS mouse model.

Earlier studies have demonstrated that 0.2% curcumin in the diet during the initiation period can inhibit azoxymethane (AOM)-induction of aberrant crypt foci and colon tumors in F344 rats [22–24]. In similar studies, feeding of 2% curcumin resulted in decreased numbers of chemically induced colon tumors in CF-1 mice [25]. Pereira *et al.* [26] reported that administration of 0.8 and 1.6% curcumin continuously during the initiation and post-initiation phases significantly inhibited development of AOM-induced colonic adenomas in rats. Curcumin by diet (0.2–0.5%) has also demonstrated a chemopreventive activity in a model germane to human colorectal carcinogenesis involving *Apc* mutations [27].

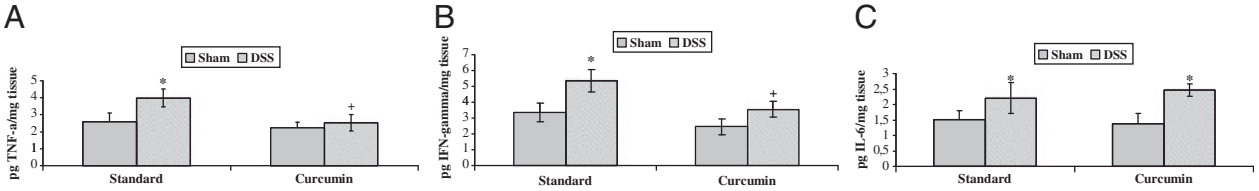

Figure 3. Effect of curcumin diet at 0.6% on tumor necrosis factor alpha (TNF-α) (A), interferon gamma (IFN-γ) (B) and interleukine-6 (IL-6) (C) in the colon tissue after 15 cycles with DSS 0,7% (v/v). Data are expressed as the means plusmn; S.E.M. (*) $p<0.05$ *vs.* Sham group and (+) $p<0.05$ *vs.* standard diet-DSS animals.

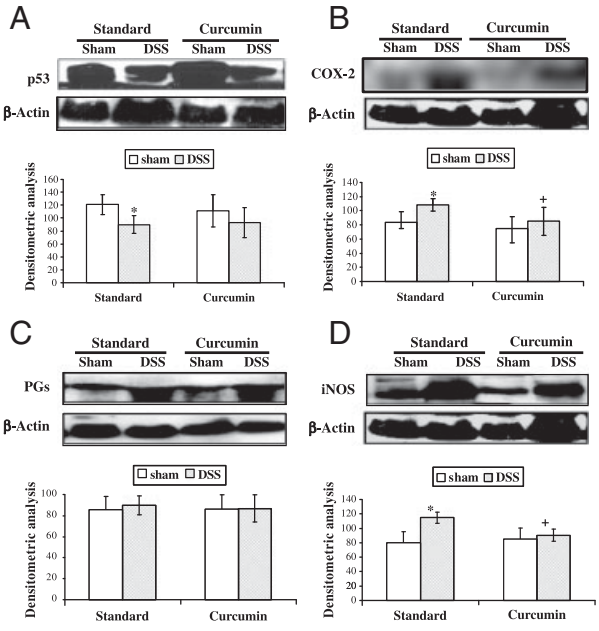

Figure 4. Effects of curcumin diet at 0.6% on expression of different proteins in the colon tissue after 15 cycles with DSS 0.7% (v/v). Western blotting using antibodies against p53 (A), cyclooxygenase (COX)-2 (B), prostaglandin E synthase (PGES)-1 (C) and inducible nitric oxide synthase (iNOS) as described in section Materials and methods. Data are expressed as the means ±S.E.M. (*) $p<0.05$ *vs.* Sham group; (+) $p<0.05$ *vs.* standard diet-DSS animals.

As shown above, the ability of curcumin to interfere with colon carcinogenesis in chemical and genetic rodent models has already been studied. However, it is interesting to see how our results show a preventive/protective role for dietary curcumin in our experimental model where an ''inflammation-dysplasia-carcinoma sequence'' typical of ulcerative colitis-associated CRC is shown. This model, where animals were subjected to repeated administration of DSS with an iron supplemented diet, has demonstrated its feasibility to induce dysplasia and carcinoma in the colon, albeit slowly and with a low incidence since it is caused by the chronic inflammation, without additional activation of signalling pathways by a chemical carcinogen [28].

Our results demonstrate that curcumin, a naturally occurring anti-inflammatory and antioxidant agent, given as a dietary supplement, shows antitumor activity. The group that received the curcumin diet presented a minor incidence of ulcerative colitis-associated colonic neoplasms. This positive effect is well related with better DAI index values during the last cycles, aberrant accumulation of β-catenin, characteristic of deficient APC function and a lack of modification of p53 expression in the colonic tissue at the end of the experimental model. Moreover, a reduction in proinflammatory cytokine levels and COX-2 and iNOS protein expressions were also observed. These data suggest the potential usefulness of this agent as a chemopreventive agent for individuals at high risk for colon cancer development, such as patients with ulcerative colitis.

Additionally, curcumin appears not to affect the number of low-grade dysplasia suggesting that most of chemopreventive efficacy of this agent is achieved during the promotion/progression phase in this model. These results are in accordance with other authors who showed chemopreventive activity of this compound during the promotion/progression phase in AOM-induced CRC [22].

In normal conditions, the binding of β-catenin by APC protein targets β-catenin for degradation, thereby generating and maintaining low levels of β-catenin in cells [29]. However, the loss of wild-type APC abrogates this pathway, resulting in increase in this protein. Translocation of β–catenin from the cell membrane to the cytoplasm or nucleus is an early event in colorectal carcinogenesis in human [2] and in our experimental model, it is also involved in the intestinal carcinogenesis process. Previous investigations found that a decrease in tissue β-catenin expression by immunohistochemistry was correlated with effective tumor inhibition. Therefore, β-catenin has been suggested to be an important target for drug design for preventing the growth and metastasis of cancer cells through multiple pathways [30]. Our results have shown that one of the beneficial mechanisms of curcumin-induced tumor suppression in this model involves diminution of β-catenin. These results are consistent with the previous studies where curcumin decreased β-catenin in the enterocytes of the Min/+ mouse, which develops multiple intestinal adenocarcinomas as a result of a germline mutation in one APC allele [31], in the HCT-116 cell line that carries a wild-type *APC* gene [32], and in HT-29 [33], observations previously associated with an antitumor effect.

CRC arises as a result of sequential episodes of activating mutations in oncogenes, such as ras, and inactivating mutations, truncations or deletions in the coding sequence

of several tumor suppressor genes, including p53 [34] among others. In normal cells, p53 is inactive but several stimuli can activate it, resulting in anticancer responses [15] and loss of p53 gene function occurs late and is believed to be the defining event that drives the adenoma to carcinoma [35]. Our study shows that p53 expression was diminished in response to 15 cycles of DSS treatment, which may deactivate apoptotic pathway driving to the propagation of DNA damage. On the contrary, the curcumin diet did not modify its expression significantly, suggesting a p53-dependent mechanism for colon cancer regulation in this model by curcumin. Revising the literature, several published studies suggest that curcumin may beneficially induce apoptosis in part through its induction of p53 expression [36], although other studies suggest that curcumin may instead have a deleterious, antiapoptotic effect by downregulating p53 [37, 38]. Therefore, the role of p53 in cell cycle arrest induced by curcumin has not yet been completely elucidated and further studies should be required in order for a better understanding of this concept.

Our group has also shown a strong correlation between colitis-associated cancer and TNF-α, IFN-γ and IL-6 production in repeated colitis with 15 cycles of DSS (0.7% w/v) administration [28, 38], suggesting that inflammation plays a causative role. Cytokines are recognized to play a major role in the immunopathogenesis of IBD and a crucial role in promoting neoplastic transformation [4, 5, 39]. Pharmacologic blockade of TNF-α with monoclonal antibodies has demonstrated great efficacy in the treatment of colorectal carcinogenesis associated with chronic colitis [39]. In our study, mice fed with 0.6% of curcumin presented a significantly lesser induction of TNF-α and IFN-γ cytokines respect to standard diet-DSS animals, without any change in IL-6 levels. These results indicate that curcumin appears to be able to exert anti-inflammatory and growth inhibitory effects on CRC development by reducing this TNF-α and IFN-γ increase. Accumulating evidence suggests that curcumin has a diverse range of molecular targets, among them are transcription factors, growth factors and their receptors, cytokines, enzymes and genes regulating cell proliferation and apoptosis [40]. Curcumin inhibited the expression of TNF-α-induced IL-1β, IL-6, and TNF-α, but not IL-8, in TNF-α-treated HaCaT cells [41].

Earlier evidence indicates the important role of inflammatory proteins, as COX-2 and iNOS, and how the inhibition of both reduces colon carcinogenesis [42]. Their over expression is associated with colon tumor formation and/or promotion by a number of potential mechanisms [43, 44]. COX-2 is up-regulated generating prostaglandins that can promote cell growth, angiogenesis and suppression of immunity, and iNOS is responsible for the production of large amounts of NO, which is able to cause DNA-damage and at the same time inhibits DNA repair mechanisms [15, 45]. Indeed, in chronic inflammation NO stimulates COX-2 activity and increases p53 mutations contributing to clone cellular expansion and genomic instability.

The analysis of these protein expressions in our model of colitis-associated tumorigenesis demonstrated that all of them significantly increased during the course of this disease [19, 28]. However, the curcumin diet reduced significantly both COX-2 and iNOS expression *versus* standard diet. Several studies have shown that curcumin downregulates the expression of COX-2 protein in different tumor cell lines, most likely through the downregulation of NF-kB activation [13, 40]. Curcumin was also shown to inhibit colon carcinogenesis during the post-initiation stage through the modulation of COX activity [24]. More recently, long-term dietary curcumin administration demonstrated a reduction of COX-2 expression by 66% in intestinal adenoma tissue from ApcMin+mice [46] and an increase to compensate AOM-induced reduction of COX-1 expression [47].

iNOS downregulation by curcumin in our model is also consistent with other authors who have demonstrated that curcumin possesses anti-inflammatory activity by being a potent inhibitor of iNOS [48] and inhibited AOM-induced colonic mucosal iNOS by ~40% [49].

The efficacy of curcumin in the experimental model used in this study that mimics the progression of the colonic inflammation to cancer may be more a consequence of its anti-inflammatory effect rather than an anticarcinogenic effect. In fact, some studies have also shown its potent intestinal anti-inflammatory effect. For instance, recently Nones *et al.* [50] have showed that addition of the curcumin to the diet (0.2%), monitored from the age of 7–24 weeks, alleviated the colonic inflammation in mdr1a$^{-/-}$ mice. They concluded that the observed beneficial effects could be explained *via* an up-regulation of xenobiotic metabolism and a down-regulation of proinflammatory pathways. Besides, Arafa *et al.* [51] have demonstrated as prior administration of 100 mg/kg (i.p.) of curcumin during 7 days mitigated the injurious effects of DSS and ameliorated all the altered biochemical parameters. Additionally, a recent report also showed that treatment with 0.5, 2.0 or 5.0% curcumin in the diet suppressed proinflammatory cytokine messenger RNA expression as well as CD4 (+) T-cell infiltration and NF-kappaB activation in the colonic mucosa [52].

In summary, the ability of curcumin to prevent the development of the colonic carcinogenesis was demonstrated by a better DAI index, a lower immunoreactivity of β-catenin and a non-modification of p53 expression. We suggest that these beneficial effects could be mediated mainly through the anti-inflammatory mechanisms, such as reduction of the proinflammatory cytokine levels and a decrease of COX-2 and iNOS protein overexpression in the colonic tissue.

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