Chemoprevention of azoxymethane-initiated colon cancer in rat by using a novel polymeric nanocarrier–curcumin

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Abstract

Curcumin is a potential natural anticancer drug with limited bioavailability due to the lack of solubility in aqueous solvents. The present study is designed to investigate the preventive effects of polymeric nanocarrier–curcumin (PNCC) on colon carcinogenesis in an azoxymethane-induced rat tumor. Forty rats were divided into control, curcumin- and PNCC-treated groups. Animals received azoxymethane (AOM) as a carcinogenic agent (15 mg/kg, s.c.) weekly for two consecutive weeks. They were given curcumin 0.2% and PNCC two weeks before till 14 weeks after the last injection of AOM. In the end, post euthanasia, the entire gastrointestinal tract was scrutinized for tumors, and the rest of the body for metastatic deposits. Tumor number, size and location were characterized. The histopathological and immunohistochemistry examinations were also performed on colon tissue. In vivo, curcumin nanoparticles inhibited colon cancer growth in animal model. The tumors incidence and number decreased by nanocurcumin comparison with control. Furthermore, the nuclear/cytoplasmic ratio, epithelial stratification, nuclear dispolarity, goblet depletion, structural abnormality, and the expression of Beta-catenin and Bcl-2 proteins were reduced in PNCC compared to others groups (P < 0.05). In addition, Bax protein expression was significantly increased in PNCC in comparison with control and curcumin-treated groups (P < 0.001). The present study demonstrated the potential anticancer effects of PNCC in a typical animal model. The results provide evidence that nanopolymeric curcumin exerts a significant chemopreventive effect on AOM-initiated colon cancer through cell proliferation inhibition and apoptosis induction. More investigations are needed to confirm its safety for human use.

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1. Introduction

Colorectal cancer is a malignant tumor recognized as the third most common cancer worldwide with high morbidity and mortality (Haggar and Boushey, 2009). Hence, new chemo-preventive and chemo-therapeutic approaches are required to reduce its mortality. Some natural compounds such as curcumin can potentially prevent colon cancer development with low side effects (Kelloff et al., 2000). Curcumin (1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione; diferuloylmethane) is a yellow, natural, lipid-soluble compound extracted from plant Curcuma Longa with no discernible toxicity (Aggarwal et al., 2007). Recent studies have shown that curcumin, either alone or in combination with other anticancer agents, has potent anticancer effects. It is also a potent tumor-inhibitory agent with chemopreventive properties against intestinal and colon cancers (Sa and Das, 2008; Kwon and Magnuson, 2009). Curcumin feeding (0.2% of diet) can inhibit azoxymethane (AOM)-induced tumor development and pre-neoplastic lesions of colon cancer (Kawamori et al., 1999; Rao et al., 1999). Administration of curcumin (40 mg/kg, i.v.) into rats resulted in complete plasma clearance after 1 h (Ireson et al., 2001). Rapidly metabolized and conjugated in the liver, curcumin has limited systemic bioavailability (Anand et al., 2007). Thus, more appropriate methods with higher efficacy and lower side effects are needed. Many methods were tested to overcome this defect such as using a nanopolymeric carrier (OA-400) (Sarbolouki et al., 2000).

A unique feature of curcumin nanoparticles (polymeric nanocarrier–curcumin; PNCC) is the ease with which the structure

of their monomers can be varied in order to provide suitable inert drug porters for target cells (Sadeghizadeh et al., 2008). The polymeric nanocarrier (OA-400), a neutral, amphiphatic and biodegradable nanomaterial, previously introduced by our group, is capable of safely delivering genes into different cell lines (Sarboliuki et al., 2000; Sadeghizadeh et al., 2008). Biodegradable drug nanocarriers have promising applications in solving the issue of poor water solubility of hydrophobic drugs. The invention involves the synthesis of non-ionic biocompatible polymeric carriers to enhance the solubility of curcumin as an anticancer drug. It is a new type of compatible polymeric carriers taken from plant fatty acids, and can sustain curcumin size to 80 nm (Sarboliuki et al., 2000). This increases curcumin solubility and can enhance its effectiveness as an anticancer drug. Experimental production and use of OA-400 in cancer treatment was originally introduced by Sarboliuki et al. (2000). The biocompatibility and efficacy of OA-400 has further been proven by researchers at Tarbiat Modarres University and Biophysics and Biochemistry Research Center of Tehran University (Sadeghizadeh et al., 2008; Babaei et al., 2012). The feasibility of preparation and use, size fitness, environmental sustainability, non-toxicity and shell viability are all important factors to be considered for a suitable carrier (Ledley, 1995). Our previous experimental data shows that the polymeric carrier (OA-400) has the required characteristics of a suitable carrier (Sarboliuki et al., 2000). Microscopy results show easily placed polymeric nanocarriers (OA-400) within the host bacteria (100–4000 nm) because of their small size (10–100 nm) (Pourasgari et al., 2009). Thus, the present study is designed to investigate the preventive effects of polymeric nanocarrier–curcumin on colon carcinogenesis in an AOM-induced rat tumor.

2. Materials and methods

2.1. Materials

Curcumin (> 95%) and azoxymethane were purchased from Sigma Aldrich Co. (St Louis, MO). Monoclonal mouse antiRat/Rabbit Beta-catenin, and polyclonal mouse antiRat/Rabbit Bax and polyclonal mouse antiRat/Rabbit Bcl-2 antibodies were purchased from Dako Co. (DAKO Corporation, USA). The polymeric nanocarrier (OA-400) was locally produced in our lab (Patent Number: 71753).

2.2. Animals

Male Wistar rats (100–120 g) were kept in a temperature-controlled environment on a 12:12 h light/dark cycle with free access to food and water. The procedures were in accordance with the guidelines for the care and use of laboratory animals of Tehran University of Medical Sciences.

2.3. Preparation of polymeric nanocarrier–curcumin

Different w/w ratios of polymeric nanocarrier/curcumin in PNCC ranging from 50:1 to 10:1 were tested before settling an appropriate proportion of 25:1 (Babaei et al., 2012). Briefly, curcumin was dissolved in various amounts of polymeric nanocarrier (OA-400) and checked for absorbance spectra by UV spectrophotometry (TECAN, Switzerland). The appropriate mixture of polymeric nanocarrier (OA-400) and curcumin was then evaluated for excitation/emission value in comparison with curcumin dissolved in PBS and 1% methanol as control samples. Subsequently, PNCC was sterilized by 0.22 µm filter. Aliquot solution was stored in a dark place at 4 °C (Babaei et al., 2012).

2.4. Study design

Forty rats were used to study the preventive effects of PNCC on AOM-induced tumor in colon cancer model. According to the study protocol, animals were divided into three groups: (i) control (n=10), (ii) curcumin treated (0.2%) (n=15) and (iii) PNCC treated (n=15). In treated groups, curcumin and PNCC were given for 18 weeks. Here, we did not include the nanocarrier group because its toxic effects were reported in our previous study (Babaei et al., 2012). AOM-induced (15 mg/kg, s.c.) colon cancer was performed in two consecutive weeks. Nutrition with PNCC and curcumin were started two weeks before AOM administration.

2.5. Characterization of colon tumors

Rats were weighed weekly and observed for evidence of rectal bleeding. They were killed at the moribund or at the end of 18th weeks. A thorough necropsy was then made. The entire gastrointestinal tract was scrutinized for lesions and the rest of the body for metastatic deposits. Tumor number, size and location were characterized.

2.6. Histological assay

The colorectal tumoral and adjacent non-tumoral mucosal tissues from the specimens were fixed in 10% formaldehyde, passaged and embedded in paraffin. The paraffin blocks were then sectioned by 3–5 µm thickness for H & E staining. For each case, nine serial sections were used for hematoxylin and eosin (H & E) and immunohistochemical stains.

2.7. Hematoxylin and eosin examinations

Histological evaluations were performed at first by routine H & E staining. The grade of histological abnormality was semi-quantitatively scored using the following five parameters: (a) nuclear/cytoplasmic ratio (< 25%: 0, 25–50%: 1, > 50%: 2); (b) epithelial stratification (none: 0; mild: 1, severe: 2); (c) nuclear dispolarity (none: 0; mild: 1, severe: 2); (d) goblet cell depleteness (null to mild: 0, moderate: 1, severe: 2); (e) structural abnormality (none: 0, mild: 1, severe: 2). At least five sections were examined for grading. Two evaluators independently read the slides without having access to the cases. The agreement between their results was greater than 90%, and the discordant cases were jointly reevaluated by the pathologists, reaching a decision through consensus. The total score of each parameter was regarded as the score of histological abnormality. Almost all colon cancers were scored as 8–10 points while normal samples earned 0.

2.8. Immunohistochemistry examinations

Immunohistochemistry was carried out on 5 µm tissue sections from formalin-fixed paraffin blocks using the avidin–biotin immunoperoxidase method. Sections were stained by monoclonal mouse antiRat/Rabbit Beta-catenin, polyclonal mouse antiRat/Rabbit Bax and polyclonal mouse antiRat/Rabbit Bcl-2 antibodies (DAKO Corporation, USA) according to the manufacturer’s instructions. Briefly, the paraffin sections were deparaffinized with xylene and rehydrated through a series of descending graded ethanol solutions. Endogenous peroxidase activity was blocked by incubation for 15 min in a 0.3% H2O2 buffer. Biotinylated secondary antibody and an avidin–biotin complex with horseradish peroxidase were applied, followed by addition of the chromogen 3,3’-diaminobenzidine (DAB) (Sigma Chemical). Finally, slides were counterstained with hematoxylin and observed with a light
microscope. Slides of histological sections, previously confirmed to be positive for these markers, were used as positive controls. The same slide was used as negative control by subtracting the primary antibody from the reaction. The two evaluators read the same slides as previously described in H & E study.

The criteria used to evaluate the Beta-catenin, Bax and Bcl-2 markers were based on the estimated proportion of positive cells and estimated average staining intensity of positive cells in cytoplasm (for Bax) and membrane, cytoplasm or nucleus (for Beta-catenin and Bcl-2). The semi-quantitative score was adopted as follows (no staining: 0, faint/barely staining in at least 1/3 of cells: 1, moderate staining in at least 1/2 of cells: 2 and strong staining in almost all cells: 3).

2.9. Data analysis

Analysis of variance (ANOVA) and tukey test were used for comparison among groups. Differences in tumor incidence (% animals with colon adenocarcinomas) were analyzed by Fisher’s exact probability test. Ratio comparison was determined by Chi-square test. Values represented mean ± S.E.M. P < 0.05 was considered to be statistically significant.

3. Results

3.1. General observations

Four of 44 rats died due to azoxymethane’s toxicity two to three weeks after AOM injection in control (n=2), curcumin (n=1) and PNCC (n=1) groups. Animals’ weight in azoxymethane, curcumin and nanocarrier–curcumin treated cases increased with no significant changes compared to each other. Body weight rose steadily to reach a plateau after about 15 weeks in all rats, but showed a slight fall in AOM group in the last few weeks as tumors developed. There were no significantly differences in food intake (g/day) in control and treatment groups. Curcumin and PNCC fed animals demonstrated no evidence of weight loss, and no gross organ changes were seen at necropsy. No behavioral changes were observed in the rats during the course of administration, or in the ensuing follow-up period.

3.2. Tumor type

The lesions varied from normal colonic mucosa, mild to severe dysplasia and colonic adenocarcinoma.

3.3. Metastases

Of 40 rats in the 18th week, only 11 had metastases in control (n=6), curcumin (n=4) and PNCC (n=1) groups. Most frequent metastases were seen in epicolic and para-aortic intra-abdominal lymph nodes.

3.4. Tumor incidence

The percentage lesions incidence in rats was higher in the distal colon compared to the transverse and proximal colons. Rats in control and curcumin groups had higher lesions induction compared to the PNCC groups. Among the treatment groups, reductions in tumor incidence (%) in curcumin fed rats ranged from 70 to 85 compared to control. However, PNCC fed rats had the lowest lesions incidence (33%).

3.5. Tumor number

In vivo application of a polymeric nanocarrier–curcumin inhibited colon cancer growth in animal model. Control diets fed rats had highest tumor numbers in both proximal (9) and distal colon (29). No proximal tumors were seen in PNCC fed rats. Results showed that the incidence of colon adenocarcinoma was 80% in control group. Administration of PNCC alone significantly inhibited incidence of colonic adenocarcinomas by about 33% (P < 0.05) compared with control group. The percentage inhibition of colon adenocarcinomas by curcumin and PNCC was 78% and 33% (P < 0.05), respectively, compared with control.

3.6. Tumor size

Compared to control fed rats, rats treated with PNCC had smaller lesions (mm) in the distal colon. Rats in control group had larger lesions (mm) in distal than proximal colon. However, curcumin fed rats and curcumin+nano particle combination had smaller lesions (mm) in distal colon in comparison with control. Reductions (%) in lesions size (mm) in rats fed with curcumin and PNCC was 20 and 76.7 (P < 0.05), respectively, compared with control group.

3.7. Tumor histology

In hematoxylin and eosin examinations, the mean number of lesions, nuclear/cytoplasmic ratio, epithelial stratification, nuclear dispolarity, goblet depletion and structural abnormality were declined in PNCC group compared to the others groups (P < 0.05) (Table 1, Fig. 1).

In immunohistochemistry examinations, AOM reduced the expression of Bax protein whereas increased the expression of Beta-catenin in the colon tissue. Proapoptotic Bax protein expression was increased by PNCC in comparison with control and curcumin groups (Table 2, Fig. 2). Antiapoptotic Bcl-2 protein expression in colonic mucosa was expectedly found to be over-expressed after AOM therapy. Furthermore, Beta-catenin and

<table>
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<th>Groups</th>
<th>Parameters</th>
<th>Number of lesions</th>
<th>Nuclear/cytoplasmic ratio</th>
<th>Epithelial stratification</th>
<th>Nuclear dispolarity</th>
<th>Goblet depletion</th>
<th>Structural abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2.5 ± 1.27</td>
<td>1.5 ± 0.53</td>
<td>1.4 ± 0.52</td>
<td>1.3 ± 0.48</td>
<td>1.5 ± 0.53</td>
<td>1.4 ± 0.52</td>
</tr>
<tr>
<td>Curcumin</td>
<td></td>
<td>1.53 ± 0.91</td>
<td>1.27 ± 0.46</td>
<td>0.93 ± 0.6*</td>
<td>1.07 ± 0.59</td>
<td>1.27 ± 0.7</td>
<td>1.07 ± 0.7</td>
</tr>
<tr>
<td>Nanocarrier/c</td>
<td></td>
<td>0.57 ± 0.5*</td>
<td>0.76 ± 0.36*</td>
<td>0.4 ± 0.5*</td>
<td>0.57 ± 0.5*</td>
<td>0.93 ± 0.6*</td>
<td>0.6 ± 0.55*</td>
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The grade of histological abnormality was semi-quantitatively scored using the following five parameters: (a) nuclear/cytoplasmic ratio (< 25%: 0, 25–50%: 1, > 50%: 2); (b) epithelial stratification (none: 0, mild: 1, severe: 2); (c) nuclear dispolarity (none: 0, mild: 1, severe: 2); (d) goblet depletion (null to mild: 0, moderate: 1, severe: 2); (e) structural abnormality (none: 0, mild: 1, severe: 2). Values represented mean ± S.E.M.

* P < 0.5 compared to control group.

** P < 0.5 compared to curcumin group.
Bcl-2 proteins expression and Bcl-2/Bax ratio were dramatically decreased in PNCC in comparison with other groups \( (P < 0.001) \) (Table 2, Figs. 2 and 3).

4. Discussion

The major goal of this study is to develop novel strategies for colon cancer prevention by means of combining potential chemopreventive agents with a polymeric nanocarrier to increase their chemoprevention in otherwise healthy individuals. The present study results show that the tumors incidence and number, the mean number of lesions, structural abnormalities and Beta-catenin were significantly declined in curcumin nanoparticle-treated group. The nanopolymeric curcumin also induced the expression of pro-apoptotic protein Bax and reduced anti-apoptotic Bcl-2 expression and Bcl-2/Bax ratio relative to control and curcumin groups. The present study provides evidence that nanopolymeric curcumin exerts a significant chemopreventive effect on AOM-initiated colon cancer through inhibition of cell proliferation and apoptosis induction.

We observed a decrease in tumor incidence as well as tumor size and number in both proximal and distal sections in rats treated with PNCC compared to control group. Tumor number and tumor size are indicators of proliferation and angiogenesis. Changes in tumor growth characteristics observed in rats fed with PNCC suggests anti-proliferative and anti-angiogenic effects of nanopolymeric curcumin. Lower tumor incidence, smaller tumor size and lesion number may have been attributed to the direct effects of nanocurcumin which acts as anti-proliferative and anti-angiogenic factors or its indirect effects via anti-oxidative enzymes.

Previous studies showed Beta-catenin as a key intracellular messenger in gastrointestinal tract malignancies such as stomach and colon cancers (Polakis, 2000; Oyama et al., 2008), and suggested that activation of the Beta-catenin transcription could play a role not only in initiation of colon carcinogenesis but also in its promotion in mice (Oyama et al., 2008). In our study, the
nanoparticle–curcumin was more active than curcumin in suppressing Beta-catenin activation and other abnormalities. The nanoparticle-curcumin also inhibited the growth of a wide variety of tumor cells in rat colon cancer model. These effects were comparable with that of curcumin alone. These results imply that nanoparticle–curcumin may be superior to curcumin as an antitumor agent.

The role of apoptosis in colon carcinogenesis has been extensively studied, suggesting that resistance to apoptosis in premalignant colonic epithelial cells will lead to the development of colon tumors (Hall et al., 1994). In order to gain insight into mechanisms involved in apoptosis induction mediated by nanoparticle curcumin in colon cancer, we studied the effect of nanoparticle curcumin on the expression of Bcl-2 and Bax proteins under in vivo situations. Many genetic alterations observed in colon cancer led to an imbalance in the pro- and anti-apoptotic members of the Bcl-2 family (Jaattela, 2004), which are considered as a target for anticancer therapy (Baell and Huang, 2002). It has generally been established that oncoprotein Bcl-2 duels with its counteracting twin, a protein known as Bax. Overexpression of Bax promotes cell death; conversely, Bcl-2 functions as a suppressor of apoptosis. A decrease in Bcl-2/Bax ratio has been considered as a reliable indicator of the overall propensity of a cell to undergo apoptosis. In the present study, nanoparticle curcumin decreased Bcl-2/Bax ratio by suppressing Bcl-2 expression and Bax stimulation. This may be indicative of the Bcl-2 family role in apoptosis induction mediated by nanoparticle curcumin in rat colon cancer. These results are consistent with our earlier observations in which we demonstrated a significant increase in anti-tumor property of curcumin by using dendosome, a polymeric curcumin-derived nanoparticle and introduced it as a promising anti-cancer therapeutic agent (Babaei et al., 2012).

In previous reports, some polymeric nanovectors were used to deliver curcumin. Bisht et al. (2007) synthesized a polymeric nanoparticle of curcumin (50–100 nm range) in an encapsulated formulation utilizing micellar aggregates of cross-linked and indiscriminate acrylic copolymers of N-isopropylacrylamide (NIPAAm) with N-vinyl-2-pyrrolidone (VP) by radical copolymerization. An ideal drug delivery platform must be biodegradable, biocompatible and not be associated with incidental adverse effects. The nano-carries of Bisht were nondegradable and harmful to health due to NIPAAm, VP and poly(ethylene glycol)-monoacrylate monomers use. We used fatty acids instead to
sensitize our nano-carrier ((Sarbolouki et al., 2000; Sadeghizadeh et al., 2008; Babaei et al., 2012), so, they are biodegradable and more biocompatible than Bish’s nano-carrier and are not harmful to health. On the other hand, the toxicity profiles of Bish’s nanocarriers were studied mostly in vitro cell lines including hepatocytes, mammary epithelial cells, fibroblasts, and athymic mice, but our studies were done both cell lines, and in mice and rat (Sadeghizadeh et al., 2008; Babaei et al., 2012). In some other studies, lipid-based nanoparticles were used to improve intravenous delivery of curcumin into tissue macrophages (Sou et al., 2008). Shaikh et al. (2009) prepared curcumin-loaded poly-lactic-co-glycolic acid (PLGA) nanoparticles and promoted oral bioavailability of curcumin at least 9-fold compared to absorption enhancers like piperine. Anand et al. (2010) used PLGA-polyethylene glycol nanoparticles to deliver encapsulated curcumin with 97.5% efficiency but low drug loading. These nanoparticles enhanced in vitro cellular uptake and bioactivity and improved in vivo bioavailability. Recently, Letchford et al. (2008) found that methoxy-poly (ethylene glycol)-poly-caprolactone (MPEG-PCL) micelles had potential applications in curcumin delivery. Subsequently, Mohanty et al. (2010) prepared encapsulated curcumin within MPEG-PCL micelles and evaluated their in vitro application in cancer therapy.

Although our nanoparticle-curcumin (OA-400) normally carries transport materials into different cells; its efficiency is considerable. Toxicity results in cell culture and animal studies indicate that polymeric nanocarriers (OA-400) are neutral, and in fact, very safe at high concentrations without any signs of destructive effects (Sarbolouki et al., 2000; Sadeghizadeh et al., 2008). The toxicological study results in cell culture and animal models showed that the nanocarrier used in this study is inert, in fact, so much so that even at high concentrations it showed no deleterious effects. In the case of nanocarrier at the very high dose of 5 mg/kg, however, initially some swelling developed under one arm and the animal died after three weeks (Sadeghizadeh et al., 2008). Our data for the first time reveals that nanopolymeric compounds not only boost the solubility of curcumin and its uptake in cell lines but also increase its toxicity on cancer cells rather than healthy ones. Hence, the polymeric nanocarrier (OA-400) can be a useful tool for transferring drug and gene into cells. The results of transfection clearly showed that the polymeric carrier (OA-400) had high-efficiency at very low values (Sarbolouki et al., 2000; Sadeghizadeh et al., 2008; Babaei et al., 2012). This issue with the biodegradable ability shows that the polymeric carriers (OA-400) are unique host cell drug in animal models as per following aspects:

(a) They are inexpensive, neutral, non-toxic, biodegradable and easy to use.
(b) Pharmaceutical agents can create a weak structural combination helping their easy cellular separation.
(c) Compared with some commercial products and bacterial hosts, they are more efficient, and probably the best available pharmaceutical carriers.
(d) Although nanocarriers are biodegradable, five-year experience has proven that the polymeric carriers (OA-400) are stable and resistant not only in dry environments but also in fluids with low temperature.

Taken together, this study exhibits that the main problem with curcumin administration can partly be resolved by using an appropriate carrier such as polymeric carriers (OA-400). This can be a reliable method in taking advantage of natural and inexpensive products like curcumin in prevention and treatment of cancers. Nanocurcumin provides an opportunity to expand the clinical repertoire of this efficacious agent. Future studies utilizing nanocurcumin are warranted in pre-clinical and in vivo models of cancer to show its potential benefit and elucidate the signaling pathways and possible roles of this nanoparticle.

5. Conclusion

The present study demonstrated the potential anticancer effects of PNCC in a typical animal model. The results provides evidence that nanopolymeric curcumin exerts a significant chemopreventive effect on AOM-initiated colon cancer through inhibition of cell proliferation and apoptosis induction.

Acknowledgments

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