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World J Gastroenterol 2005;11(46):7242-7247
World Journal of Gastroenterology ISSN 1007-9327

Effect of oral *Lactococcus lactis* containing endostatin on 1, 2-dimethylhydrazine-induced colon tumor in rats

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Received: 2005-02-17 Accepted: 2005-07-04

Abstract

AIM: To investigate the effects of oral *Lactococcus lactis* (*L lactis*) containing endostatin on 1, 2-dimethylhydrazine (DMH)-induced rat colorectal cancer.

METHODS: Recombinant endostatin was produced by the expression of *L lactis* NZ9000. Sixty male Wistar rats were injected with DMH (40 mg/kg body weight) subcutaneously once a week for 10 wk to induce colorectal cancer. The rats were gavaged with 1 mL of endostatin at a dose of 1×10^8/d and fed with the basal diet. The animals were killed after 22 wk for histopathological examination. The total time of experimental observation was 58 wk.

RESULTS: Rat endostatin protein was expressed in *L lactis*. Recombinant endostatin exhibited a significant effect on colorectal cancer ($P<0.05$). Furthermore, the mean survival time of the rats treated with endostatin was longer than that of the animals treated with DMH. There was no statistically significant difference between the rats treated with endostatin and those treated with DMH. The results showed that endostatin could not result in complete cure.

CONCLUSION: Oral endostatin exerts an influence on the progression of chemically induced colon tumors.

Key words: Endostatin; DMH; Tumors

Li W, Li CB. Effect of oral *Lactococcus lactis* containing endostatin on 1, 2-dimethylhydrazine-induced colon tumor in rats. *World J Gastroenterol* 2005; 11(46):7242-7247
http://www.wjgnet.com/1007-9327/11/7242.asp

INTRODUCTION

There are lines of evidence that angiogenesis is essential for the growth and persistence of solid tumors and their metastases[1,2]. Tumor angiogenesis is regulated by the balance between proangiogenesis and antiangiogenesis factors, and this balance varies in different organ environments[3]. Systemic administration of recombinant endostatin potently inhibits angiogenesis and maintains metastases at a microscopic size, resulting in a strong anti-tumor activity[4-6]. Endostatin has been shown in some studies to inhibit the formation or growth of lung and liver metastases[7,8]. Endostatin, an angiogenesis inhibitor produced by hemangioendothelioma, is a 20 kDa carboxy-terminal fragment of collagen XVIII[5]. The efficacy of endostatin in colon environment is not well established. To our knowledge, there are no published reports on the efficacy of endostatin against chemically induced colon tumor progression.

An autochthonous colon cancer model is useful to evaluate the clinical therapeutic efficacy of drugs for colorectal cancer[9,10]. As DMH model is known to closely parallel the human disease in terms of disease presentation, gross and microscopic pathology[11], it is anticipated that DMH-induced colon tumors respond to chemotherapeutic drugs. Drugs such as 5-fluorouracil (5-FU) can inhibit the growth of DMH-induced colon tumors and prolong the survival of their rodent hosts[12]. Therefore, DMH-induced colon tumors at present are the most popular models to study the morphology, pathogenesis, prevention, and treatment of colorectal cancer[11,13]. Though 5-FU derivatives have been tested in the DMH model, whether this colon cancer model of rats can be applied to the evaluation of the effect of endostatin on colon tumors remains unknown. The aim of the present study was to investigate the effects of recombinant endostatin on the progression of DMH-induced colon tumors in rats.

MATERIALS AND METHODS

Animals and chemicals

Five-week-old male Wistar rats were provided by the Laboratory Animal Center, Chinese Academy of Medical Sciences, Beijing, China, and housed in plastic cages in a 12-h light/dark cycle at 22±2 ℃ and 44±5% relative humidity. Rats were fed with the basal diet with free access to water. Body weight and food consumption were measured weekly during the experiments. DMH was purchased from Tokyo Kasei Co. (Tokyo, Japan).

Preparation of recombinant endostatin

Endostatin expression experiments were performed with *L lactis* NZ9000 (donated by Institute National de la Research Agromique, France). All cloning steps were done with *E coli* Top 10. *E coli* (stored in our laboratory, China) was grown on Luria-Bertani (LB) medium and incubated at 37 ℃. *L lactis* was grown on M17 medium containing 0.5% (wt/vol) glucose and incubated at 30 ℃. When appropriate, chloramphenicol was added at a final concentration of 10 μg/mL and ampicillin was supplied at a concentration of 100 μg/mL. Expression of the endostatin gene was induced by nisin promotor. *L lactis* was cultured overnight and then transferred into a fresh medium at a dilution of 1:50. After 3-4 h of incubation, 1 μg/mL of nisin (Sigma) was added to the culture and incubated for 3-4 h.

Cloning and expression of rat endostatin in L lactis

Total RNA was extracted from rat kidney tissue using the SV total RNA isolation systems kit (Promega) and reverse-transcribed by reverse transcription system kit (Promega). The mixture was incubated at 25 ℃ for 10 min, at 42 ℃ for 60 min, at 95 ℃ for 5 min, and at 4 ℃ for 5 min. The two gene-specific primers were designed by the sequence encoding the carboxy terminal portion of rat collagen XVIII. The primer sequences were 5'-TTT GAA TTC GCC CAC ACC CAC CGC GAC TTC CAG CCG-3' and 5'-AAA AGC GGC CGC CTA CTT GGA GGC GGC AGT CAT GAA GCT-3'. PCR was performed in a total volume of 50 μL of reaction solution and 2 μL of RT template. The PCR conditions were as follows: denaturation at 94 ℃ for 5 min, then 25-35 cycles at 94 ℃ for 0.5 min, at 56 ℃ for 0.5 min, at 72 ℃ for 1 min, a final extension at 72 ℃ for 5 min and a DNA fragment was obtained. The amplified fragment was purified using the QIAquick PCR purification kit (QIAGEN Inc.) and digested with *EcoRI* and *Not*I. The resulting fragment was respectively ligated to nisin promotor plasmids pLA141 and pLA151 digested with *EcoRI* and *Not*I. The pLA141 plasmid carried a signal peptide Usp45. The recombinant plasmid DNA was transferred into *L lactis* NZ9000 by electroporation and transformants were plated on GM17 agar plates containing chloramphenicol according to the method of Wells *et al*.[15]. The recombinant plasmid DNA was isolated from *L lactis* as described previously[15,16] and the nucleotide sequence was further determined.

Western blotting

Total protein was prepared from exponentially growing cultures. The bacteria were harvested by centrifugation at 3 000 r/min for 10 min at 4 ℃, washed with PBS, resuspended in 1 mL of 10 mmol/L Tris-HCl (pH 7.5) and disrupted with a French press (Bioritech). The cell suspension was centrifuged at 10 000 g for 10 min at 4 ℃ to remove cell debris. The samples were mixed in *Laemmli* buffer and subjected to SDS-12% polyacrylamide gel electrophoresis. The protein was transferred onto nitrocellulose membranes with a Bio-Rad electroblotter. The blots were developed with BCIP/NBT developing buffer (Sigma).

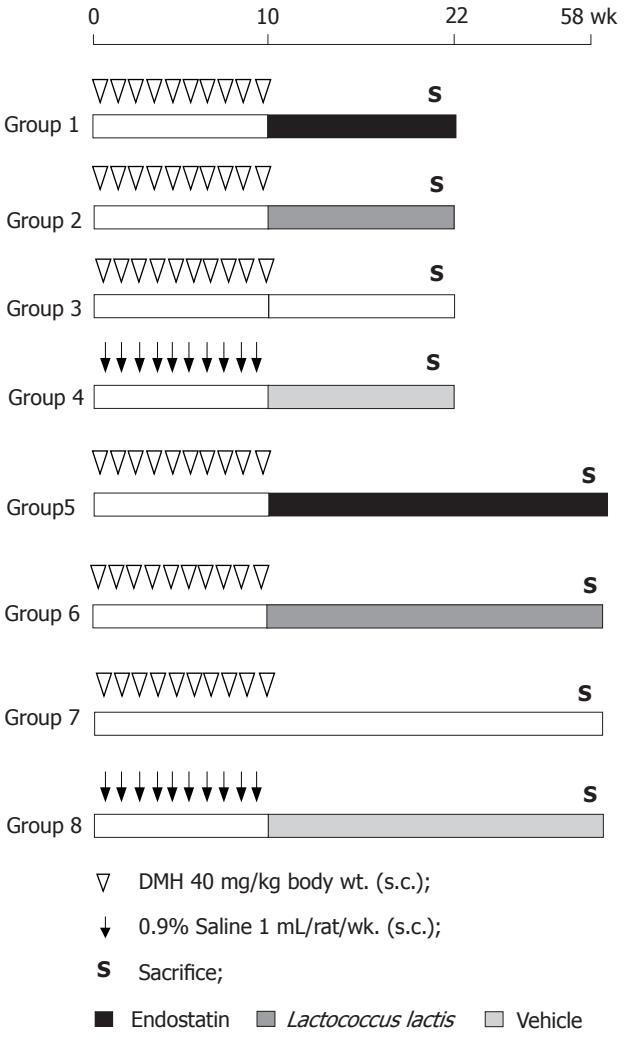

Figure 1 Experimental design.

Treatment protocol

The experimental design is shown in Figure 1. After 1 wk of acclimatization, 80 six-week-old rats were randomly divided into 8 groups (10 rats/group). Animals in groups 1-3 and 5-7 received subcutaneous injections of DMH dissolved in normal saline solution (40 mg/kg body weight) once a week for 10 wk. Rats in groups 4 and 8 were injected with 0.9% normal saline (vehicle) at the same time. After the last DMH treatment, the animals were additionally gavaged with 1 mL of *L lactis*-secreted endostatin protein (groups 1 and 5) and *L lactis* without endostatin gene but containing plasmids (groups 2 and 6) once a day for 12 wk or until they were killed after 58 wk. The dose of endostatin and *L lactis* was 1×10^8 daily. Groups 4 and 8 were gavaged with 1 mL of the solutions not containing *L lactis* (the vehicle control). Groups 3 and 7 served as a carcinogen control. The time of treatment differed slightly in each experiment. All animals that survived were killed under either anesthesia at wk 22 (groups 1-4) or to end of the experiment. The total time of experimental observation was 58 wk.

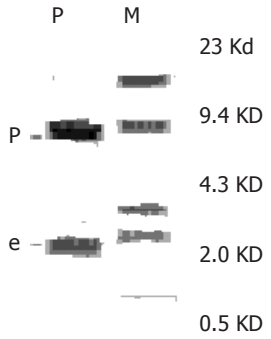

Figure 2 Expression plasmid obtained by restriction enzyme analysis. P: expression plasmid cut by *Eco*RI and *Not* I; e: endostatin gene; M: molecular weight marker.

Autopsy

The colons were removed, flushed with saline and opened along the longitudinal median axis. Tumor width (*W*) and length (*L*) were measured with calipers. The tumor volume (TV) was determined by the following formula: $TV = (L \times W^2)/2$[8]. After the gross pathologic changes (number, dimensions, and distribution of the tumors) were recorded, the colons were fixed flat in 10% phosphate-buffered formalin. The liver and kidneys were removed and weighed. Other major organs (stomach, small intestine, spleen, lungs, and lymph nodes) were also excised and fixed in 10% phosphate-buffered formalin. All tissues were embedded in paraffin, cut into sections and stained with hematoxylin and eosin. The proximal, intermediate and distal segments of the colon were examined for histopathological analysis.

Tumor staging

Animals with DMH-induced colon cancer developed multiple tumors and each tumor had a different histological stage[14]. Consequently, the animals were staged (Duke's stage) with reference to a single index tumor, defined as the largest macroscopically and histologically identifiable colon tumor.

Statistical analysis

Statistical analyses were completed with SPSS 9.0 software. The significance of differences between the average values of the groups was analyzed using Cochran's two-tailed Student's *t*-test. The significance of differences in lesion incidence between the groups was assessed by χ^2 test. Rat mortality was analyzed by the log-rank method of Peto *et al*.[17].

RESULTS

Construction of expression plasmid

The plasmid containing the endostatin gene was pla148, identified as the expression plasmid I. Recombinant *L lactis* was obtained by PCR and restriction enzyme analysis when the recombinant plasmid was transformed into *L lactis* by electroporation (Figure 2).

Expression of rat endostatin gene in L lactis

Recombinant lactic acid bacteria were incubated and induced by *nisin* in M17-Glu for 6 h. Endostatin protein was identified by SDS-PAGE and Western blot with the antibody prepared from rabbits immuned with human endostatin protein. Rat endostatin protein was expressed in *L lactis* (Figures 3A and 3B).

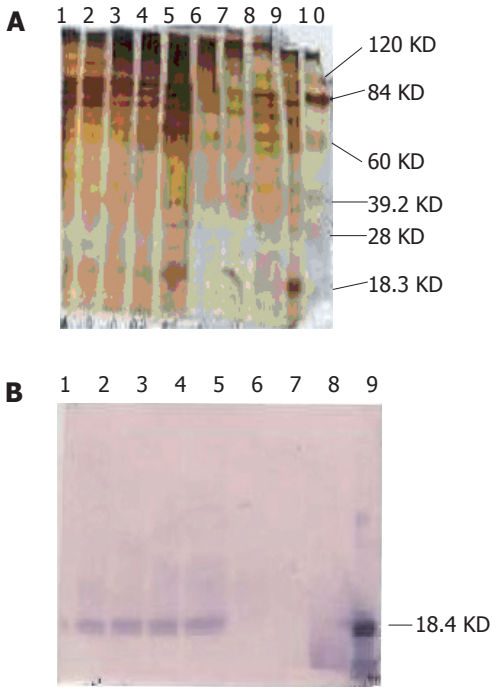

Figure 3 Expression of rat endostatin gene in *L lactis*. **A:** Silver-strained SDS-PAGE of expressed endostatin in L lactis; **B:** Western blot. Lanes 1-6: engineered *L lactis* 1-6 h after induction; lane 7: *L lactis* without endostatin gene; lane 8: rat sera from groups after oral recombinant *L lactis*; lane 9: endostatin protein expression in *E coli* cells.

Animal experiment

All rats in groups 1-4 and 8 survived until the final termination and were relatively healthy throughout the experiment. No signs of severe toxicity were observed in all the animals that were given endostatin. No tumor was found in vehicle-treated animals. By the end of wk 22, the average body weights of the rats treated with DMH or endostatin or *L lactis* were significantly decreased compared to the vehicle control ($P<0.05$). Relative liver and kidney weights and food consumption did not significantly differ among the groups (Table 1). Macroscopically, the distribution of colon tumors in the proximal and middle colon at the end of wk 22 had no significant difference among the groups (data not shown).

Histopathological findings are summarized in Table 2. Colon epithelial lesions were divided into adenomas and carcinomas. At the end of wk 22, the incidence of colon tumors was not significantly affected by endostatin. The mean tumor incidence in a single tumor-bearing rat was 2.50 in group 1 and 4.00 in group 3. Tumor volume was decreased in rats receiving endostatin. However, it did not differ from that in DMH-treated group. In addition, there was a significant difference in Duke's stage between the animals treated with DMH and those treated with

Table 1 Average final body weight, relative liver and kidney weights, and food consumption data (mean±SD)

Group	Treatment	*n*	Final body Weight, g	Relative liver Weight, g	Relative kidney Weight, g	Food consumption (g/rat/d)
1	DMH+endostatin	10	379.0±24.9[a]	2.94±0.26	0.56±0.12	18.02
2	DMH+*L lactis*	10	395.0±36.5[a]	3.05±0.25	0.56±0.08	18.00
3	DMH	10	383.5±19.2[a]	3.10±0.40	0.55±0.07	18.48
4	Saline+vehicle	10	439.5±39.3	3.09±0.35	0.56±0.12	20.07

[a]$P<0.05$ *vs* group 4.

Table 2 Colon tumor incidence, classification, multiplicity, tumor volume, and stage in rats treated with DMH with or without endostatin (mean±SD)

Treatment		Incidence	Adenoma	Carcinoma	Multiplicity	Tumor volume	Duke's stage		
	n	*n* (%)	*n* (%)	*n* (%)	number	mm^3	A	B	C
DMH+endostatin	10	10(100)	5 (50)	5 (50)	2.50±1.80	2.35±1.84	1	4	-a
DMH+*L lactis*	10	9(90)	2 (22)	7 (78)	2.67±1.47	2.54±2.00	4	6	-a
DMH	10	10 (100)	5 (50)	5 (50)	4.00±2.96	4.31±4.56	2	-	3

[a]$P<0.05$ *vs* DMH-treated rats.

Table 3 Tumor classification, distribution, and differentiation in rats treated with DMH with or without endostatin

Treatment	Tumor	Classification (%)		Distribution in colon (%)			Differentiated carcinoma (%)		
	number	Adenoma	Carcinoma	Proximal	Middle	Distal	Well	Moderately	Poorly
DMH+endostatin	25	16 (64)	9 (36)	12 (48)	9 (36)	4 (16)	6 (66.7)	3 (33.3)[a]	0
DMH+ *L lactis*	24	15 (62.5)	9 (37.5)	10 (41.7)	12 (50)	2 (8.3)	6 (66.7)	3 (33.3)[a]	0
DMH	40	27 (67.5)	13 (32.5)	15 (37.5)	19 (47.5)	6 (15)	3 (23.1)	10 (76.9)	0

[a]$P<0.05$ *vs* DMH-treated rats.

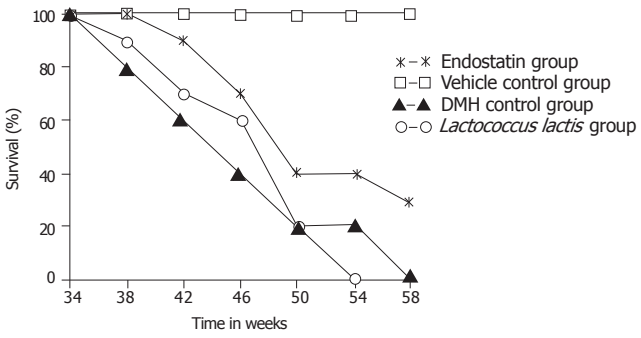

Figure 4 Survival rate of rats injected with DMH with or without endostatin and normal saline.

endostatin ($P<0.05$). Liver lesions and lymph node metastases were observed in about 30% of the animals in group 3.

The survival rates of rats in groups 5-8 are shown in Figure 4. The group that received endostatin had a survival rate of 30%. The survived rats were killed and metastases were found in their lungs and livers. All the saline-injected rats were alive at the end of the experiment. However, none of the DMH-treated rats survived the full duration of the experiment. The mean survival time of endostatin-treated animals was longer than that of DMH-treated rats. The range of ages at death in DMH-treated animals was 38-57 wk (Table 3).

DISCUSSION

Studies using preclinical models of nonhematologic malignancies indicate that antiangiogenic therapies may delay or even abrogate tumor growth[5,18,19]. Endostatin is one of the antiangiogenic drugs and our data indicate that the administration of endostatin after DMH treatment could prolong the survival time of rats. The Duke's staging system for human colorectal cancer provides accurate prognostic information. In other words, animals with less advanced disease (stage A) survive significantly longer than those with advanced disease (stages B and C) irrespective of the treatment. In our study, there was a significant difference in the levels of differentiation and metastases (Duke's stage) between the animals treated with DMH and those treated with endostatin. Endostatin-treated rats had an improved survival compared to untreated rats, indicating that the survival time of rats with colon cancer parallels to that of human beings with this disease. These results can at least in part explain the mechanism of the potent antiangiogenic and antitumor activities of endostatin. Furthermore, the improved survival is directly attributable to the effective induction of tumor stabilization and its ability to inhibit specifically endothelial proliferation in endostatin-treated animals. However, oral administration of endostatin could only prolong the survival time of tumor-bearing rats but not result in complete cure.

There are reports on endostatin against metastases in lung, stomach, and liver[7,8,20]. Recent studies showed that endostatin has an antiangiogenic action[21-24] and can

induce apoptosis in colon cancer cells by inhibiting tumor angiogenesis and inhibit tumor growth and metastases of human colon cancer xenograft in nude mice[24]. Some other mechanisms may be involved in endostatin stabilizing and maturating newly formed blood vessels[21]. Jia *et al*.[22] reported that endostatin could inhibit tumor growth and angiogenesis by blocking Vegf/Flk-1 pathway. In addition to its antiangiogenic activity, endostatin exerts a direct anticancer action that appears to be restricted to some tumor cell lines[25]. At the same time, endostatin has been demonstrated to induce regression of tumors in mice[7,26], but actual regression as opposed to growth inhibition has not been demonstrated in the colon environment. Though our study demonstrated that endostatin could influence rat colon tumor progression, the precise mechanisms by which endostatin exerts effects on colon carcinoma are not well understood.

Long-term administration of endostatin is needed because the inhibition of tumor metastases has not been observed after a shorter endostatin-treated period. The ultimate goal of antiangiogenic therapy is to induce long-term tumor stabilization[27] because data from studies in nonhuman primates indicate that endostatin may be administered for a long time without toxicity[28]. Furthermore, studies *in vitro* and *in vivo* suggest that endostatin gene therapy can effectively suppress angiogenic processes in model systems[29,30]. It was reported that endostatin treatment is not associated with any recognizable vascular changes in tumor samples[31].

Certain strains of lactic acid bacteria have been found to prevent putative preneoplastic lesions induced by carcinogens[32,33]. The antimutagenic activity of lactic acid bacteria is suspected to reside in the cell wall[34] as lactic acid itself has no antimutagenic effect[35]. The findings of the current study do not support the suggestion that the addition of *L lactis* may also prolong the survival time of DMH-treated rats. The reason for this is unclear, but might be explained by the differences in bacterial strains. The antitumor effect of lactic acid bacteria is still controversial[36]. Further study is needed to identify the antitumor effect of endostatin and the precise mechanisms by which these effects are mediated.

In conclusion, long-term administration of endostatin can inhibit the progression of chemically-induced colon tumors and prolong the survival time of rats.

ACKNOWLEDGMENTS

The authors thank Professor Shoji Fukushima, Department of Pathology, Osaka City Medical School (Japan) for valuable discussion and comments, and Professor Yanfeng Zhong, Department of Pathology, Beijing University Medical School for histopathological examination.

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