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Preliminary study on the recombinant endostatin engineering *Lactococcus lactis*

Research Article

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Abbreviations: 1, 2-dimethylhydrazine, (DMH); *Lactococcus lactis*, (*L lactis*), Luria-Bertani, (LB); tumor volume, (TV)

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Summary

Endostatin is a specific inhibitor of endothelial proliferation and agiogenesis from the COOH-terminal portion of human collagen XVIII. In order to examine the effect on *Lactococcus lactis* (*L. lactis*) and endostatin for curing cancer, rat endostatin gene was isolated by RT-PCR from rat kidney and cloned into the plasmid of *L lactis* and expressed in *Lactococcus lactis* NZ9000. And the effects were observed by orally *L .lactis* and recombinant *L lactis* expressing endostatin for colorectal cancer-induced rats with 1, 2-dimethylhydrazine (DMH) through both the survival and histopathological examination of the rats. The results showed that recombinant endostatin *L lactis* had a significant effect on the Duke's stage of the experimental rats (P<0.05). Furthermore, the mean survival of the rats taken orally with recombinant *L lactis* was longer than that of the rats treated with DMH alone. The study would lay a theoretical foundation for an application of *L lactis* and endostatin to the anti-tumor.

I. Introduction

Numerous studies have shown that both primary tumor and metastatic growth are angiogenesis dependant (Folkman et al, 1990,1992; Kim et al, 1993; Millauer et al, 1994). Therefore, the tumor vascular system has become an important target for cancer therapy. An increasing number of antiangiogenic factors have been discovered. One of the factors responsible for this inhibition was named endostatin, and it was proved that NH_2–terminal sequence of endostatin corresponds to the COOH-terminal portion of collagen XVIII (O'Reilly, 1997). It is reported that a recombinant form of this protein expressed in baculovirus-infected insect cells could inhibit the growth of metastases in the Lewis lung tumor model and an insoluble E. coli derived form of this protein was also shown to be efficacious in preventing primary tumor growth in several tumor models (Boehm et al, 1997; O'Reilly et al, 1997). Additionally, it is known that In curing cancer, the application on endostain was very expensive and needed injecting into blood. These barriers have hampered the widespread translation of endostatin research to clinical practice. There is therefore a great need to increase the yield and to reduce the cost of the production of recombinant endostatin that is suitable for clinical use. Our strategy centered on the optimization of a probiotic strain, *lactococcus lactis* expression system because of its probiotic efficiency in expressing foreign proteins as compared with the other systems. At present, few reports have been seen by oral giving medicine using *L lactis* as a vector. Additionally, lactic acid bacteria (LAB) expression system selected was because of its ability to express heterologous protein in *vivo* and unnecessary to isolate the protein.

II. Materials and methods

A. Bacterial strains and growth conditions

E. coli strains Top10 or TG1 were incubated at 37℃under aeration, and rendered competent to take up DNA using a $CaCl_2$ method. *Lactococcus lactis* strains NZ9000 as host bacteria were grown at 30°C in M17 broth (Merck, Darmstadt, Germany) supplemented with 0.5% glucose (GM17). Antibiotics were used at the following concentrations for *E. coli*: Ampicillin (Am), 100 ug/ml. For *L. lactis*, the concentrations were as follows:

erythromycin (Em), 5 μg/ml; chloramphenicol (Cm), 10 μg/ml. Growth kinetics were determined in GM17(M17 medium in which 1%(wt/vol) glucose) broth as follows. Culture tubes containing 5 ml of prewarmed medium were inoculated with 2% of an overnight culture and incubated at 30°C without shaking in a water bath. Bacterial growth was monitored by spectrophotometric measurements of the optical density at 600 nm (model UV-1205; Japan) every 30 min until the culture reached the stationary phase. The recombinant *L lactis* production was achieved in either 10% (wt/wt) culture media. All media were heat pasteurized at 90°C for 45 min. Two liters of media was inoculated at 1% (vol/vol) from a fresh GM17 culture. Then the ferments were enlarged for incubation at 30°C in 30-liter BIOTECH-30JS(Shanghai). After finishing fermentation, the strains fermented were deposited through centrifuge and freeze-dried for the experiment.

B. Cloning and expression of rat endostatin in *L.lactis*

E.coli Top10 and TG1 cloning efficiency cells were all prepared by ourselves as competent cells and were ready for transformation using the standard protocol.

The total RNA was isolated from a rat kidney tissue using the RNA extracting kit (Promeg). And the sequence encoding the carboxy terminal portion of rat collagen XVIII was got by the method of RT-PCR. AMV reverse transcriptase, Tag DNA polymerase and other reagents were purchased from Boehringer Mannheim or Sigma. The primers used were: TTT GAA TTC GCC CAC ACC CAC CGC GAC TTC CAG CCG and AAA AGC GCG CGC CTA CTT GGA GGC GGC AGT CAT GAA GCT bases. RT-PCR was carried out using standard conditions. The amplified fragment was purified using the QIAquick PCR purification kit, and digested with *EcoRI* and *NotI*. The plasmids pLa165 and 148 were gifted by Dr Gruss in France. At first, the resulting fragment was ligated into a pre-digested *Lactoccus lactis* expression and secretion plasmid (pLA165). This plasmid carried a signal peptide based on secretion of the staphyiocuccal nuclease and also contained a nisin-residue promotor from lactic acid bacteria. Additionally, the fragment was also ligated into a pre-digested *L.lactis* expression plasmid (pla148) without secreting signal peptide of nuclease-residue. Plasmid DNA was purified from *E.coli* using the alkaline lysis method and was isolated from *L.lactis* as described previously (Ruyter et al, 1996). Restriction endonucleases, T4 DNA ligase, Tag polymerase and other chemicals used in the test were purchased from Boehringer Mannheim or Sigma and they were operated according to the recommendations of the manufacturer. Transformation of *L.lactis* NZ9000 was performed by electroporation and selection for recombinants were plated on GM17 agar plates containing the adequate antibiotic (van de Guchte et al, **1989**; Wells et al, 1993).

Plasmid DNA can be isolated by a number of different methods and using commercially available kits (Promega Wizard Miniprep kit). The kits follow the manufacturer's suggested protocol for plasmid DNA isolation. DNA samples were sequenced by Shanghai Shenggong Company. The purified plasmid DNA was used for further restriction enzyme digestion with *EcoR*I and *Not*I and ligated, additional subcloning.

The endostatin expressed in these cells was monitored by SDS-PAGE. Expressions of endostatin were as follows: Small-scale expression of endostatin in *E. coli* strain TOP10 harboring the plasmid of interest is grown at 37°C in LB medium with shaking in an air incubator. When growth is monitored at OD600 until it reaches a value of 1.0 it was induced by a concentration of 0.2 mmol/L Isopropyl-beta-D-thiogalactopyranoside (IPTG) for additional three or four hours. Then 1 mL aliquots of the culture were removed for analysis of protein content by boiling the pelleted cells, treating them with reducing buffer and electrophoresis via SDS-PAGE. The culture was centrifuged (5000 x g) to pellet the cells. Otherwise, The strains carrying endostatin were grown overnight at 30°C in GM17-Cm, diluted 50-fold in the same medium added with 1 ng/ml of nisin (Sigma), and allowed to grow at 30°C to an optical density at 600 nm of 1.0, about 3-4 h of incubation (Steidler et al, 1995, 2000; Sambrook et al, 1989) and then the recombinant *L lactis* strains were harvested by centrifugation (3000g, 10 min, 4ºC, washed with PBS, resuspended in 1 ml of 10 mM Tris-HCl (pH 7.5), and disrupted with a French press (Bioritech). The cell suspension was centrifuged (10000g, 10 min, 4ºC) to remove cell debris. The samples were mixed in Laemmli buffer and subjected to12% SDS-polyacrylamide gel electrophoresis.

C. Purification and Western-blotting of Endostatin from *L. lactis*

A purification procedure for recombinant endostatin from *L. lactis* has been described previously (O'Reilly et al, 1997). Briefly, bacteria pellet was collected with low-speed centrifugation, followed by lysis with 8 M urea. The lysate was then applied to a heparin column (Qiagen). After washing with 8 M urea containing 10 mM imidazole, endostatin was eluted with 8 M urea containing 250 mM imidazole. Quantification of the endostatin protein before dialysis was performed using the Bio-Rad protein dye method as described by the manufacturer. Finally, the endostatin product was dialyzed against 1xPBS at 4°C. The polyclonal antiserum was prepared according to the routine protocol from the immunized rabbits with the simply purified endostatin expressed in *E.coli*. The gel after running SDS-PAGE was transferred onto a nitrocellulose membrane with a Bio-Rad electro-blotter. The blots were developed with BCIP/NBT (Sigma) developing buffer (Sambrook et al, 1989).

D. Fermentation for the Genetic Engineering of *Lactococcus lactis*

Stock cultures of *L. lactis* strains NZ9000 and the recombinant *L. lactis* strains were prepared by mixing 10 ml of a fresh culture with 25 ml of 20% skim milk and 25 ml of a 20% glycerol solution and then freezing the mixture at -70°C in 1-ml sterile cryovials. Working cultures were prepared by inoculating 100 ml of LM17 broth with 1 ml of a thawed stock culture, and incubating this mixture at 30°C for 24 h. The growth of *L. lactis* strains in LM17 broth was evaluated by automated spectrophotometry with a Powerwave unit (Bio-Tek Instrument, Winooski, Vt.) as described previously (Champagne et al, 1999). Starter production was achieved in either 6% (wt/wt) nonfat dry milk. All media were heat pasteurized at 90°C for 45 min. Two liters of media was inoculated at 1% (vol/vol) from a fresh LM17 culture. The ferments were enlarged for incubation at 30°C in 30-liter BIOTECH-30JS (Shanghai). Agitation was kept at 60 rpm, and fermentations were stopped when the pH of the medium

reached 4.7. The time required to complete the various fermentations was registered and will be referred to hereafter as the "fermentation time." Expression and induction of the fermentations were done as above mentioned. The fermentations were precipitated by centrifugation and freeze-dried for tests.

E. Experimental animals and Experimental protocol

40 male Wistar rats at 5 weeks of age were purchased from the Institute of Animal, Chinese Academy of Medical Sciences, Beijing in China and housed in plastic cages with wood chips in an animal room with a 12 h light/dark cycle at 22±2ºC and 44±5% relative humidity. Rats were fed the basal diet, and water was available. Body weight and food consumption of the rats were measured once a week. DMH was purchased from Tokyo Kasei Co. (Tokyo, Japan). The experimental design is shown in **Figure 1**. Colorectal cancer inducing was performed as follows. After the first week acclimatization, forty rats at 6-week-old were randomly divided into 4 groups, 10 rats each group. The rats of 4 groups were given subcutaneous injections of DMH dissolved in normal saline solution, and the dosage is 40mg/kg body weight (wt) once a week for 10 weeks. The fourth group rats were only injected with 0.9% normal saline (vehicle) at the same time. After the last DMH attacking, the animals in group 1 were fed with $1x10^8$ recombinant *L.lactis* secreting endostatin protein, the animals in group 2 with the same amount of *L.lactis* no endostatin gene but containing the plasmids once a day for 22 weeks, and the rats in group 4 were fed with the same amount of solvent without *L.lactis* (the vehicle control).

Group 3 was taken as a carcinogen control. The length of treatments differed slightly with each other group. The rats were sacrificed under ether anesthesia and checked at week 22^{nd}.

F. Experimental observation

The rats treated with DMH-induced colorectal cancer could characteristically develop multiple tumors, and each tumor would be at a different histological stage (Pozharisski, 1975). Therefore, the animals in this experiment were staged (Duke's stage) with reference to a single index tumor, defined as the largest macroscopically and histologically identifiable colorectal tumor (Dukes et al, 1958).

When the experimental rats were fed till the termination all rats were autopsied. The colons were cut out, flushed with saline and opened along the longitudinal median axis. And then the tumor width (*W*) and length (*L*) were measured with calipers. The tumor volume (*TV*) was determined by the following formula: $TV = (L * W^2)/2$. After the gross pathologic changes (number, dimensions and distribution of the tumors) were recorded, the colons were fixed flat between pieces of filter paper soaked in 10% phosphate-buffered formalin. And the liver and kidneys were excised and weighed. Other major organs (stomach, small intestine, spleen, lungs and lymph nodes) were also excised and then fixed in 10% phosphate-buffered formalin solution. Afterward, all tissues were embedded in paraffin and stained with routine hematoxylin and eosin. And then the histopathological analysis was carried out for the correlative colonic tissues.

G. Statistical analysis

Statistical analysis was carried out using SPSS 9.0 (Statistical Package for the Social Science) software in a computer. The difference between the average values of the groups was analyzed using Cochran's two-tailed Student's *t*-test. And the difference of lesion incidences between the groups was assessed by chi-square test, and the rat mortality was also counted by the Log Rank method (Peto et al, 1977).

III. Results

A. Construction of the expression plasmid

The gene *endostatin* was isolated as about a 0.8kb *EcoRI* and *NotI* fragment from rat kidney through RT-PCR. And the fragment was introduced into plasmid T-easy vector, and the resulting plasmid, pT-endo, was transferred to *E. coli* TOP10. The sequence analysis revealed that endostatin was a complete open reading frame. To clone *endostatin* in *L. lactis* NZ9000, the fragment cut with *EcoR*I and *Not*I was introduced into the vector pLa165. The resulting plasmid, pLa165-endo, was transformed to *L. lactis* NZ9000 by electroporation. Transformants were screened by PCR and restriction enzyme analysis. The resulting recombinant strain, containing plasmids pLa165-endo and pLa148-endo, were designated. The plasmid DNA was reisolated and subjected to a restriction analysis. The resulting restriction pattern was identical to the pattern obtained with the plasmid of *L. lactis*. The result showed that correct coding endostatin sequence has been constructed. Other constructive procedures were done as above mentioned. As a result, a recombinant *L. lactis* clone containing endostatin from rat was obtained (**Figures 2, 3**).

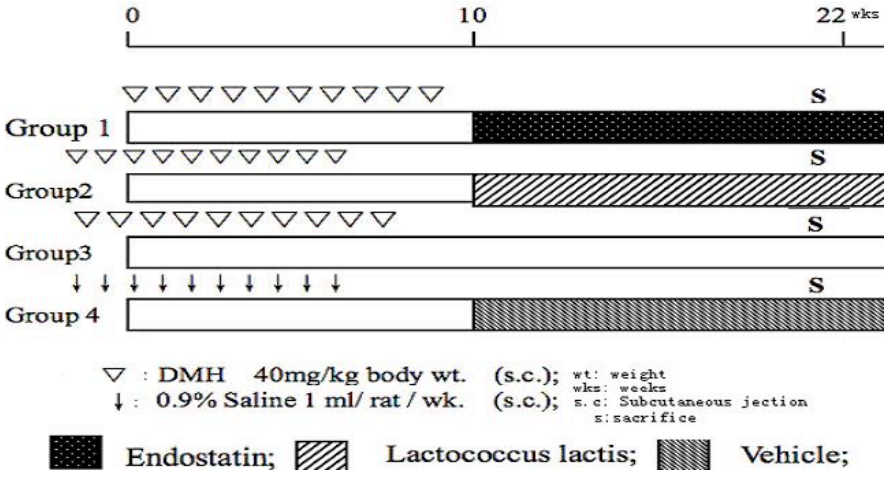

Figure 1. Experimental design.

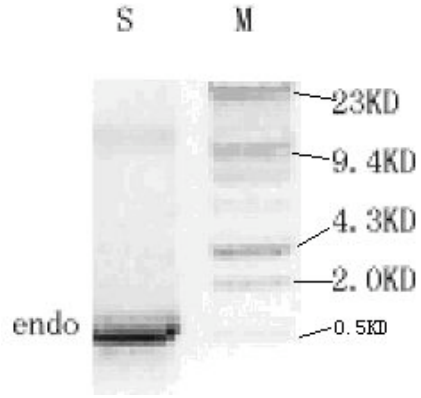

Figure 2. Rat endostatin gene by PCR. S: endostatin gene by PCR, Endo:endostatin gene, M: marker.

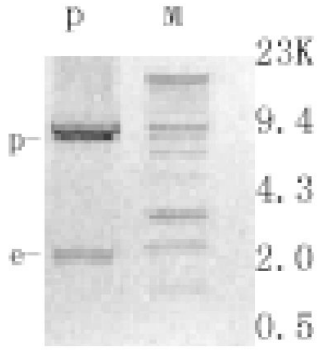

Figure 3. Expressive plasmid by restriction enzyme. p: expressive plasmid cut by restriction enzyme, e: endostatin gene, M: marker.

B. Expression of rat endostatin gene in *L.lactis*

The recombinant lactic acid bacteria were incubated and induced by *nisin* in M17-Glu for 6 hours. The endostatin protein was examined by SDS-PAGE and was identified by Western-blot with the polyclonal endostatin antibody from rabbits. The results showed that rat endostatin protein were expressed obviously in *L. lactis* as an included body form, and expression of that in superment was much few through examination (**Figures 4, 5**). The quantity of expression was estimated about 0.1mg/ml .

C. Experiment on animals

All rats of groups lived out the termination and maintained a relatively healthy appearance throughout the experiment. No signs of severe toxicity were observed for all the rats after given endostatin, and no tumors were found on the rats treated with normal saline (vehicle). By the end of 22nd week, final average body weights of the rats treated with DMH alone as well as the animals additionally received either endostatin or *L. lactis* were decreased significantly ($p<0.05$) comparing with the vehicle control. Relative liver and kidney weights as well food consumption had no significant differences among the groups (**Table 1**).

Histo-pathological examinations were summarized in **Table 2**. Adenomas and carcinomas of the rats would be analyzed according to the colonic epithelial lesions. By the end of 22nd week, the examinations indicated that endostatin did not affect the incidence of colon tumors of the rats. However, owing to receiving endostatin, tumor volume of the rats decreased apparently but no significant differences statistically comparing with DMH-treated alone group ($p>0.05$). But it was found that there was a significant difference in Duke's stage between the rats treated with DMH alone and with endostatin ($p<0.05$). Additionally, liver lesions and lymph nodes metastases of about 30% rats were observed in third group (group 3).

At the termination, the observation and statistics indicated that all the rats treated with endostatin had about 30% survival rates (**Figure 6**). The macroscopically visible metastases changes were found in their lungs and livers of the rats through the investigation. And all of the rats injected with saline were alive well by the end of the experiment, but none of the rats with DMH-treated alone could survive cancer-induced.

Table 1. Final average body weight, relative liver and kidney weights determination (22wk)[a]

Group- dividing No.	number n	Final Body Wt, g	Relative Liver Wt, g	Relative Kidney Wt, [b]g
1 (DMH+ Endostatin)	10	379.0±24.9*	2.94±0.26	0.56±0.12
2 (DMH+ *L.Lactis*)	10	395.0±36.5 *	3.05±0.25	0.56±0.08
3 (DMH)	10	383.5±19.2 *	3.10±0.40	0.55±0.07
4 (Saline+ vehicle)	10	439.5±39.3	3.09±0.35	0.56±0.12

a: Values are means±SD;
b: Kidney weight values are totals for both kidneys.
*: $P < 0.05$ (t-test) compared with Group 4.
Wt: weight; g: gram; wk: week.

Table 2. Incidence of colon tumor, classification, multiplicity, tumor volume and stage in rats treated

Disposal		Incidence	Adenoma	Carcinoma	Diversity[a]	Tumor volume	Duke's stage [b]		
	n	n (%)	n (%)	n (%)	No.	mm^3	A	B	C
DMH+ Endostatin	10	5 (50)	5 (50)	2.51±1.80	2.37±1.84	1.0	4	-*	
DMH+ L.lactis	10	9 (90)	2 (22)	7(78)	2.66±1.47	2.53±2.00	4	3	-*
DMH	10	10 (100)	5 (50)	5 (50)	4.00±2.96	4.30±4.56	2	-	3

a: Number of tumors/tumor-bearing rat.
b: Number of rats with carcinoma for any of three tumor stages.
*: P < 0.05 (Chi-Square) compared with DMH–treated group alone.

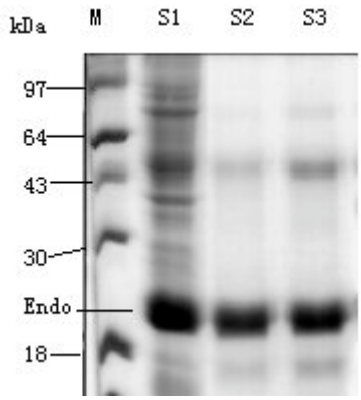

Figure 4. Rat endostatin protein by SDS-PAGE. S1: from E.coli s2 and s3: purified recombinant. endostatin from *L.Lactis*; M: marker. Endo: position of endostatin

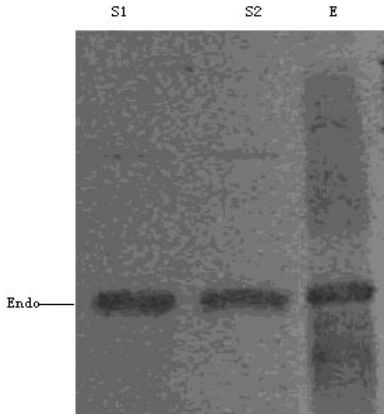

Figure 5. Rat endostatin protein by western-blot. E: from E.coli S1 and s2 from *L lactis*. Endo: position of endostatin

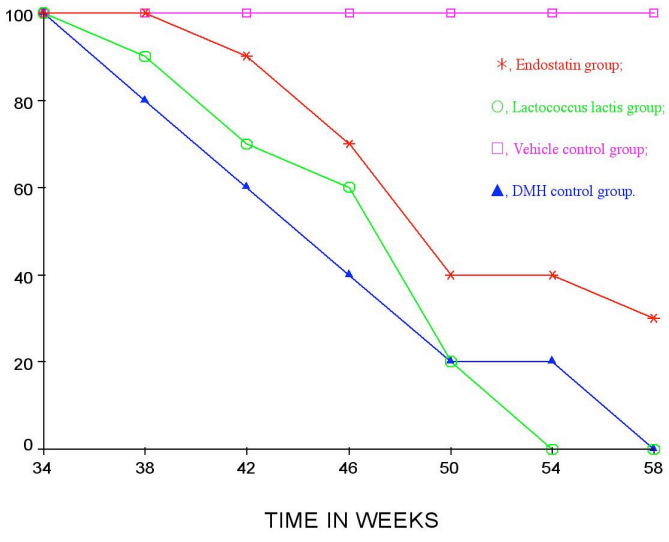

Figure 6. Survival rates of the experimental rats in each groups.

Though the survival period of rats administered with DMH could be more long than 22 weeks, the mean survival rates of the group treated with endostatin were higher than one with DMH alone (**Figure 6**), however, the survival rates had no significant differences in statistics (p>0.05).

IV. Discussion

With the aim of studying the functions of the *L. lactis* and endostatin, well as whether the presence or absence of the recombinant endostatin genes in *L lactis* would influence the survival or nutrition characteristics of *L lactis*, we proposed to experimentally construct a probiotic

recombinant strain either exerting *L lactis* or endostatin effect. The design of rational approaches to metabolic engineering and/or natural selection with such an aim requires an in-depth understanding of the pathway, the genes involved, and their regulation. As a result, the industrially recombinant endostatin *L. lactis* had been obtained and the effects on the experimental animals had been observed.

Endostatin, a 20kDa protein factor responsible for this inhibition has been expressed correctly in *E. coli* and *L lactis*. The result was consistent with previous studies in its molecular weight (O'Reilly et al, 1997). **Figures 4** and **5** illustrate the presence in which the endostatin gene could be expressed by using a nisin-inducible controlled expression system. In *L. lactis*, expression of endostatin could contribute to anti-tumor as for previously report (O'Reilly et al, 1997). Furthermore, this expression could not affect the survival of *L lactis*. Previous work had shown that *L. lactis* was a probiotic bacterium and could be successfully used in a milk-based medium (O'Reilly et al, 1997), indicating the potential usefulness in fermented dairy foods. It had also been suggested that yogurt bacteria might apply to cure some diseases (Kelkar et al, 1988). The present study indicated that recombinant engineering strain from *L. lactis* NZ9000 as a model strain could have either adminstrative heteroprotein into the body or simple application by oral way in spite of its relatively small quatity of expression for endostatin in *L lactis* comparing with *E. coli*. It is important to note that the genetic modifications of the endostatin-producing strains (being either chemically induced or genetically engineered) did not appear to affect their acid production during growing period as an important attribute in fermentation of foods. Furthermore, they have a considerable advantage over the latter since such chemically induced strains are much easier to proliferate from existing industrial strains and are much more likely to be accepted by the public. The present results are thus an important step in the development of recombinant endostatin engineering *L lactis* application. Though the quantity of endostatin produced from *L. lactis* entering the stomach and guts have not been known, the preliminary effect of anti-tumor on endostatin in vivo has been identified by the experiment which the rats treated with endostatin sequentially after DMH-treated could prolong the survival effectively. Additionally, animals with less advanced disease (stage A) survived significantly longer than those with more advanced (stage B and C), irrespective of treatment. The Duke's staging system for human colorectal cancer could provide accurate prognostic information, moreover, in our study, there was not only a significant difference in the levels of differentiation and metastases (Duke's stage) between the groups treated with DMH alone and with endostatin added but also the rats treated with endostatin showed an elongated survival comparing with that of untreated rats. Additionally, influences of survival for the rats could be also proved by the results of decreasing invasion degree and maintaining highly differentiated malignant tumors.

Therefore, the experiments would, at least in part, explain that the endostatin had a potential antitumor effect. Furthermore, it is likely to be directly to attribute to induce tumor stabilization and its ability to inhibit specifically endothelial proliferation in endostatin-treated animals through observing the improved survival of the rats. However, the paper only introduced that oral recombinant *L. lactis* carrying endostatin could only prolong the survival of tumor-bearing rats but did not introduce whether endogenous endostatin could be produced by nisin induction and no complete cure result. It could be deduced that lack of the significant difference in our study may be small numbers of animals. Instead, the finding more demonstrated that achieving regression of established tumors would be more difficult to be taken on than tumor formation to be inhibited.

One of the most important issues in endostatin therapy was the treatment period. At a typical dose level (20 mg/kg/12 h) previously showed to be active in a large number of studies (Dhanabal et al, 1999). The ultimate goal of antiangiogenic therapy would be to make long-term tumor stabilization (Fortier et al, 1999). The data from the non-primates study indicated that endostatin may be administered for long periods without producing toxicity (Dhanabal et al, 1999), and our experimental result is consistent with the report. Although endostatin prepared from a yeast system is being used in ongoing Phase I clinical trials, the low yield and high cost of the system have made it difficult to produce in quantities that are realistic for comprehensive clinical evaluation and application. Our results presented in this report offer an alternative method that will prove valuable in helping to determine the clinical activity of endostatin. Obviously, it will be of great interest and importance to adopt an effective method given drug in curing the patients with tumors.

Therefore, we propose that this agent might be evaluated in clinical trials as a consolidation drug for the patients who have achieved remission. But it should be not neglected that the production of recombinant endostatin and metabolic activities of intestinal flora of experimental animals were significantly different from those of humans. The exact change in survival of the animals may be crucial for a sensitive determination of anticancer drugs. And we believe that the effect on endostatin in combination with *L. lactis* on DMH-induced colon tumor progression was strongly correlated with endostatin other than *L. lactis*. The study introduced only the effect of oral *L lactis* containing endostatin on the experimental rats and not referred to the increase in colonic expression of endostatin. Therefore, more work needs to be done to investigate the effect of endostatin in recombinant *L. lactis* on antitumour as well as the precise mechanisms. The results only indicated that the recombinant *L lactis* would be a potent administrative vehicle because of a probiotics or bacteria that "favor life, and also the way of administration in curing colorectal cancer would have a potential meaning the reason for this is that the survival rates of rats given the probiotics additionally containing endostatin gene rose than the control. And it was also proved that the rats receiving the *L lactis* containing endostatin had a good effect anti-cancer growth through the preliminary study. Taken together, these results demonstrate the potential use

of recombinant *L lactis* containing antiangiogenic endostatin peptide as a novel therapeutic agent in experimental animals with tumor. The results from this study also opened a new avenue for treatment of cancer and provide a hopeful route for promising to overcome drug resistance.

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References

Boehm T, Folkman J Browder T, O'Reilly MS **(1997)** Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. **Nature** 390, 404-410.

Champagne, C. P., H. Gaudreau, N. Chartier, J. Conway, and E. Fonchy (1999). Evaluation of yeast extracts as growth media supplements for lactococci and lactobacilli using automated spectrophotometry. **J. Gen. Appl. Microbiol**. **45:**17-21.

de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos **(1996)**. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. **Appl. Environ. Microbiol**. 62**,** 3662-3667.

Dhanabal M, Ramchandran R, Volk R, Stillman IE, Lombardo M, Iruela-Arispe ML, Simons M, Sukhatme VP **(1999)** Endostatin: yeast production, mutants, and antitumor effect in renal cell carcinoma. **Cancer Res** 59, 189-197.

Dukes CE, Bussey HJ **(1958)** The spread of rectal cancer and its effect on prognosis. **Br J Cancer** 12, 309-320.

Folkman J (**1990**) What is the evidence that tumors are agiogenesis dependent [J]. **Natl Cancer Inst** 82, 4- 6.

Folkman J.Tumor angiogenesis[A].Holland JF,Frei E,Bast RC,(eds).**Cancer Medicine**(ed 3)[M].Philadelphia:PA,Lea & Febiger,1992.

Fortier AH, Fogler WE, Tomszewski JE, et al **(1999)** Recombinant human endostatin protein in cynomolgus monkey produces no toxicological effects following iv administration for 28 consecutive days. **Clin Cancer Res** 5, 3813s.

Kelkar SM, Shenoy MA, Kaklij GS **(1988)** Antitumor activity of lactic acid bacteria on a solid fibrosarcoma, sarcoma-180 and Ehrlich ascites carcinoma. **Cancer Lett** 42, 73-77.

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N **(1993)** Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. **Nature** 362, 841-844.

Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A **(1994)** Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. **Nature** 367, 576-579.

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J **(1997)** An Endogenous Inhibitor of Angiogenesis and Tumor growth. **Cell** 88, 277-285.

Peto R, Pike MC, Armitage P **(1977)** Design and analysis of randomized clinical trials requiring prolonged observation of each patient. **Br J Cancer** 35, 1-39.

Pgga DR, Kuipers OP, Beerthuyzen MM Boerrigter IA, Devos WM **(1996)** Functional analysis of promoters in the nisin gene cluster of Lactoccus lactis. **J Bacteriol** 178,3434-3439.

Pozharisski KM **(1975)** Morphology and morphogenesis of experimental epithelial tumours of the intestine. **J Natl Cancer Inst** 54, 1115-1135.

Sambrook J, Fritsch EF, Coulson AR **(1989) Molecular Cloning**: A Laboratory Manual, 2nd edu. New York: Cold Spring Harbor Laboratory.

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. **(2000)** Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. **Science** 289, 1352-1325.

Steidler L, Wells JM, Raeymaekers A, Vandekerckhove J, Fiers W, Remaut E **(1995)** Secretion of biologically active murine interleukin-2 by Lactococcus lactis subsp. lactis. **Appl Environ Microbiol** 61, 1627-1629

van de Guchte M, van der Vossen JM, Kok J, Venema G. **(1989)** Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. lactis. **Appl Environ Microbiol** 55, 224-228.

Wells JM, Wilson PW, Lepage RWF (**1993**) Improved cloning vectors and transformation procedure for Lactococcus lactis. **J Appl Bacteral** 154, 1-9.