Antitumoral Activity of Slime-forming, Encapsulated *Lactococcus lactis* subsp. *cremoris* isolated from Scandinavian Ropy Sour Milk, "viili"

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Abstract This study was conducted to investigate the antitumor activity of slime-forming encapsulated *Lactococcus lactis* subsp. *cremoris* KVS 20 (*L. cremoris* KVS 20) isolated from Scandinavian ropy sour milk, "viili". Intraperitoneal (i.p.) injection of lyophilized *L. cremoris* KVS 20 cells resulted in the growth inhibition of ascitic and solid tumors of Sarcoma-180 (S-180) in ICR mice. Tumor growth was significantly retarded, and the weight mean survival time significantly increased when the preparation was injected at a dose of 50 mg/kg for 9 successive days from the day after tumor inoculation. On the other hand, *L. cremoris* KVS 20 did not exhibit direct cytotoxicity against S-180 tumor cells. These findings suggest that the effectiveness of *L. cremoris* KVS 20 in preventing tumor growth is induced through enhanced host-mediated immunity.

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Key words: ropy sour milk, *Lactococcus lactis* subsp. *cremoris*, antitumor activity, host-mediated immunity

Recently, it has been reported that non slime forming dairy lactic lactococci have exhibited antitumor activity against transplantable tumors in mice⁸⁾. Previous work in our laboratory demonstrated that the lyophilized preparation of Scandinavian ropy sour milk, produced using a starter culture containing slime forming encapsulated lactococci, reduced the number of Lewis lung carcinoma in C57 BL/6 mice⁹⁾ and inhibited the growth of the Sarcoma-180 (S-180) solid tumor in ICR

mice¹⁰⁾. The maximum inhibition of tumor growth was obtained via Finnish ropy sour milk, "viili". In addition, the preparation restored the delayed type hypersensitivity response in mice which was lowered by bearing tumor⁹⁾. On the other hand, milk components did not show any activities. These findings strongly suggest that some kinds of lactic lactococci in ropy sour milk, "viili" may have preventive potential against tumor growth.

The present study was designed to find out if

microorganisms present in "viili" would prevent the growth of the S-180 tumor in ICR mice. The major microorganism in "viili" which has a strong slime-forming nature was isolated and the antitumor properties were evaluated.

Materials and Methods

Mice

Pathogen free male ICR mice aged 5 weeks (24 ± 1 g) were procured from Charles River Japan, Inc, Atsugi, Kanagawa, Japan.

Tumor

Sarcoma-180 (S-180) adapted to ICR mice was obtained from Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan. The tumor was maintained in ascitic form by serial intraperitoneal transfer.

In these experiments, the mice received 1×10^6 tumor cells in 0.1 ml of saline. The tumor cells were injected intraperitoneally (i.p.) in ascitic form and intradermally (i.d.) in solid form for the ascitic and solid experimental systems, respectively.

Isolation and identification of slime-forming encapsulated *Lactococcus lactis* subsp. *cremoris* KVS 20

The agar medium (pH 6.8) for isolation of slime-forming lactococcus was prepared by mixing with 5 g of trypticasepeptone (BBL^R), 5 g of agar (Oxoid, Agar No. 1) and 490 ml of permeate obtained by dialysis of 20% (w/v) reconstituted skim milk against the same volume of water (abbreviated as DWA agar medium). Different dilution of "viili" (Valio, Finland) was spread on the DWA agar medium and incubated at 20°C for 3 to 6 days under anaerobic conditions. A strong slime-forming colony was taken and purified by transferring on another DWA plate containing kabicidin (Wako Pure Chem., Co., Ltd., Tokyo, Japan).

Taxonomic properties of the slime-forming *Lactococcus* subsp. were examined according to the methods of HARRIGAN *et al.*⁶⁾. The test strain was classified to be a ropy variant of *L. cremoris* and numbered KVS 20 according to the results summarized in Table 1. This strain seems to be the same strain isolated from "viili" by FORSÉN *et al.*³⁾, because of analogy in the taxonomic properties. *L. cremoris* KVS 20 was cultured in the DWA medium without agar at 18–20°C for 24 h, harvested by centrifugation ($15,428 \times g$, 30 min), washed with sterile distilled water and lyophilized. The lyophilized cell yield was about 550 mg per (4.36×10^7 cells/mg). The cells were killed by heating in them a saline suspension at 100°C for 15 min prior to use.

Antitumor activity of slime-forming encapsulated *L. cremoris* KVS 20

The lyophilized preparations of "viili" and *L. cremoris* KVS 20 were injected i.p. daily at a concentration of 10 and 50 mg/kg for 9 successive days starting on the day after tumor inoculation. The controls were injected with sterile saline and reconstituted skim milk. For tumor growth in ascitic form, a weight mean survival time (WMST)¹⁾ was determined through the experimental period of 45 days. In the solid tumor experiment, the tumors were excised and weighed on the 21st day of the tumor inoculation. The percent inhibition of tumor growth (TGI) was calculated by the formula,

$$\text{TGI (\%)} = (1 - \text{mean tumor weight of experiments} / \text{mean tumor weight of controls}) \times 100$$

Direct cytotoxicity to tumor cells by *L. cremoris* KVS 20

Approximately 1×10^5 tumor cells were suspended in 1 ml of MEM medium supplemented with 2% fetal calf serum and were co-cultured with various concentrations of the lyophilized *L. cremoris* KVS 20 cells in 24 well

Antitumoral Activity of *L. cremoris*

Table 1. Properties of ropy strain KVS 20 isolated from ropy sour milk, "viili"

Biochemical tests	Ropy strain	Reference strains		
	KVS 20	GIFU 8591 ^{T1}	NCDO 176 ²	GIFU 8590 ^{T1}
Growth at				
10.0°C	+ ³	+	+	+
39.5	— ⁴	+	+	—
45.0	—	—	—	—
Growth in				
2% NaCl	+ ³	+	+	+
4	— ⁴	+	+	—
6.5	—	—	—	—
Hydrolysis of arginine	— ⁵	+ ⁶	+	—
Growth at pH 9.2	— ⁴	+ ³	+	—
CO ₂ from citrate	— ⁷	—	+	—
CO ₂ from glucose fermentation	— ⁷	—	—	—
Type of lactic acid formed	L(+)	L(+)	L(+)	L(+)
Identification	<i>L. cremoris</i>	<i>L. lactis</i>	<i>L. diacetylactis</i>	<i>L. cremoris</i>

¹GIFU 8591^T(=NCDO 604, *L. lactis*) and GIFU 8590^T(=NCDO 607, *L. cremoris*) were obtained from Laboratory of Microbiology, Faculty of Medicine, Gifu University.

²NCDO 176 (*L. diacetylactis*) was obtained from AFRC Institute of Food Research, Reading Laboratory, Shinfield, Reading, England.

³Growth, as recorded by absorbance ≥ 0.27 at 520 nm.

⁴No growth, as recorded by absorbance at 520 nm.

⁵No ammonia production from arginine.

⁶Ammonia production from arginine.

⁷No gas production.

plate (MS-80240, SUMILON, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After 24, 48 and 72 h, the growth was evaluated by the number of viable cells which were measured by the dye exclusion method of trypan blue¹²⁾.

Statistics

Mean tumor weight and survival time were determined by the Student's *t* test. The difference in the number of survivors was calculated by χ^2 test.

Results

Preliminary studies had shown that S-180 have a doubling time of 24 h as an ascites in ICR mice. All mice died progressively with growing of the tumor after an injection of 10^6

cells. The WMST was 9.7 ± 2.9 days after an injection of 10^6 cells.

In the ascitic tumor experiments, different doses of *L. cremoris* KVS 20 in relation with "viili" were used to inhibit the tumor growth. Fig. 1 shows the representative time course of survivors, indicating that the mice treated with *L. cremoris* KVS 20 and viili survived longer than those treated with skim milk and untreated controls. However, no differences were found between *L. cremoris* KVS 20 and viili treatment. The data in Table 2 revealed that the WMST of mice given *L. cremoris* KVS 20 had significantly increased as compared with that of the controls. The degree of increased WMST was not significantly different with the doses administered and was almost the same as

those in the case of treatment with viili. Thus, it appears that *L. cremoris* KVS 20 has the ability to inhibit ascitic S-180 tumor *in vivo*.

Further experiments were performed to confirm the antitumor effects of *L. cremoris*

Fig. 1. Antitumor activity of *L. cremoris* KVS 20 cells on ascitic S-180 tumor in mice.

■—■; Control (saline), □—□; Skim milk powder (50 mg/kg), ●—●; Lyophilized viili (10 mg/kg), ▲—▲; Lyophilized viili (50 mg/kg), ○—○; *L. cremoris* KVS 20 cells (10 mg/kg), △—△; *L. cremoris* KVS 20 cells (50 mg/kg).

KVS 20 in the solid tumor system since significant effect had been observed in the ascitic tumor system. The solid tumors were palpable after 5 days of tumor inoculation. Initial growth of the tumors in the mice administered with *L. cremoris* KVS 20 and the control was almost identical. On the 21st day of tumor inoculation, all animals bearing palpable tumors were sacrificed and their tumors were removed and weighed. Mice given *L. cremoris* KVS 20 demonstrated inhibition of tumor growth as compared to untreated mice (Table 3). In particular in those mice administered with a dose of 50 mg/kg of *L. cremoris* KVS 20, the mean tumor weight was 0.78 g which is significantly lower than the untreated control mice which bore tumors with a mean weight of 1.51 g. The results indicate that *L. cremoris* KVS 20 has the ability to inhibit the growth of both ascitic and solid S-180 tumors.

The direct cytotoxicity of *L. cremoris* KVS 20 against S-180 tumor cells was evaluated by their co-culture *in vitro* for 72 h. Results are

Table 2. Antitumor activity of *L. cremoris* KVS 20 cells on ascitic S-180 tumor in mice

Group	Dose (mg/kg)	WMST (days, mean $\pm$ S.D.)	No of survivors for 45 days
Control	(saline)	9.7 $\pm$ 2.9	0
Skim milk powder	50	10.6 $\pm$ 2.6	0
Lyophilized viili	10	19.9 $\pm$ 7.8*	0
	50	25.0 $\pm$ 9.1*	1
KVS 20 cells	10	20.5 $\pm$ 9.6*	1
	50	23.6 $\pm$ 14.0*	2

Groups were consisted of 10 mice.

* $p < 0.05$ (against Control).

Table 3. Antitumor activity of *L. cremoris* KVS 20 cells on solid S-180 tumor in mice

Group	Dose (mg/kg)	Tumor incidence	Tumor weight (g, mean $\pm$ S.D.)	TGI (%)
Control	(saline)	10/10	1.51 $\pm$ 0.73	0
KVS 20 cells	10	10/10	1.02 $\pm$ 0.44	32.1
	50	10/10	0.78 $\pm$ 0.58*	48.2

* $p < 0.05$ (against Control).

Fig. 2. Direct cytotoxic activity of *L. cremoris* KVS 20 cells on S-180 tumor.

●—●; Control (saline), ○—○; 100 µg/ml, □—□; 500 µg/ml.

shown in Fig. 2. *L. cremoris* KVS 20 failed to inhibit the growth of the S-180 tumor cells at a concentration of 100 µg/ml. At a high concentration of 500 µg/ml, the number of tumor cells was slightly suppressed, but it was not significantly lower than that of the control. Thus, *L. cremoris* KVS 20 is shown to have no direct killing potential against S-180 tumor cells.

Discussion

The slime-forming lactic acid bacteria isolated from ropy sour milk viili was identified to be *L. cremoris*. This is in conformity with the view that the characteristic ropiness of viili is produced by encapsulated species of *L. cremoris*¹³⁾. The heat killed *L. cremoris* KVS 20 cells showed significant inhibition activity against the growth of the S-180 tumor. This fact suggest that *L. cremoris* KVS 20 cells are the primary component in viili which is potential to prevent the development of S-180 tumor. Since the injection of ropy sour milks in previous studies with the same schedules has shown a reduced number of Lewis lung

carcinoma⁹⁾ and to inhibit the growth of S-180 tumor¹⁰⁾, in this experiments, *L. cremoris* KVS 20 cells were injected i.p. for 9 successive days from the day after tumor inoculation. The highest prevention of tumor growth via *L. cremoris* KVS 20 was achieved when it was injected at a concentration of 50 mg/kg in both forms of ascitic and solid tumors, and the incidence of the effect on both forms of S-180 tumors was almost the same. Thus, *L. cremoris* KVS 20 is effective not only for ascitic but also for solid tumors inoculated in the distant part from the injection site of *L. cremoris* KVS 20, indicating that the antitumor effect of *L. cremoris* KVS 20 may be induced by the indirect host reaction enhanced with *L. cremoris* KVS 20 injection. Several researchers have recognized the antitumor activity of dairy lactic acid bacteria against allogenic and syngenic tumors in mice^{2,4,5,7,8,11,14,16)}. In particular, *Lactobacillus casei* (*L. casei*) has exhibited marked antitumor activity against the ascitic and solid forms of S-180 when it was injected i.p. and intravenously at a concentration of 2 and 10 mg/kg for 5 days after tumor inoculation⁷⁾. It appears that the potential of *L. cremoris* KVS 20 to inhibit the growth of the S-180 tumor is comparable to that of *L. casei* although detailed experiments on the effective doses and administration route are to be done.

L. cremoris KVS 20 had no direct cytotoxicity against S-180 tumor cells in vitro, indicating that the antitumor activity of *L. cremoris* KVS 20 is not dependent on direct growth inhibition. It is known that various microorganisms have an immunostimulating effect which is involved in their antitumor activity. The mechanism by which *L. cremoris* KVS 20 can inhibit tumor growth remains unclear. One of the advanced conceivable mechanism appears to be an enhancement of the immune responses of the host.

It has been demonstrated that a polysaccharide produced by *Lactobacillus helveticus*

var. *jugurti*¹⁵⁾, component (s) synthesized by a yoghurt starter culture⁴⁾ and glycopeptide cell wall fragments of *Lactobacillus delbrueckii* subsp. *bulgaricus*²⁾ induces the growth inhibition of tumors. The slime material produced by *L. cremoris* KVS 20 may also be a principal component in the antitumor factor in "viili".

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スカンジナビアの粘質酸乳“ヴィリー”より分離した莢膜性粘質物産生 *Lactococcus lactis* subsp. *cremoris* の抗腫瘍活性

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抗腫瘍活性を示したスカンジナビアの粘質酸乳“ヴィリー”中の活性成分を検索する目的の一環として、ヴィリーより莢膜性粘質物産生 *Lactococcus lactis* subsp. *cremoris* (*L. cremoris* KVS 20) を分離し、その菌体の抗腫瘍活性を Sarcoma-180 (S-180) 腹水および固型腫瘍系で検討した。その結果、腫瘍移植後連日 9 日間の腹腔内投与で両腫瘍系において腫瘍増殖抑制効果が認められた。50 mg/kg 投与では、腫瘍増殖が有意に抑制され、平均生存日数 (WMST) が増加した。一方、*L. cremoris* KVS 20 は *in vitro* において、S-180 腫瘍細胞に対して直接細胞障害活性を示さなかった。これらの結果より、*L. cremoris* KVS 20 の腫瘍増殖抑制効果は宿主免疫の増強を介して誘導されるものと考えられた。

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