Shinshu Med J, 63(6) : 375～384, 2015

Obligate Anaerobic *Lactobacillus Casei KJ686* Selectively Targets Solid Tumors and Exhibits an Antitumor Effect

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Hypoxia in solid tumors is known to confer treatment resistance and lead to rapid progression and poor prognosis. As a novel cancer drug-delivery candidate, *Lactobacillus casei KJ686 (L.casei KJ686)* is a mutant strain of the facultatively anaerobic *L.casei ATCC393* that was transformed into an obligate anaerobe to suit the hypoxic conditions within solid tumors. In the present study, we intravenously administered *L.casei KJ686* to treat tumor-bearing mice and investigated the in vitro cytotoxicity of *L.casei KJ686* and its culture medium containing bacterial excretion products. We discovered that *L.casei KJ686* localized exclusively in tumor tissues and suppressed tumor growth in our animal experiments. Furthermore, the culture supernatant of *L. casei KJ686* exhibited a strong cytotoxic effect in vitro. The specific cytotoxic substances presented in its culture supernatant may have a pivotal role in direct anti-tumor effect in solid tumors. *Shinshu Med J 63 : 375—384, 2015*

(Received for publication May 19, 2015 ; accepted in revised form August 17, 2015)

Key words : obligate anaerobe, hypoxia, *Lactobacillus casei*, cytotoxicity, culturing-supernatant

Abbreviations : LLC, Lewis lung carcinoma

I Introduction

Cytotoxic chemotherapy is useful for systemic antitumor treatment, but the large doses of drugs that are often needed for effective results may also induce many adverse side effects, such as nausea, vomiting, hair loss, anemia, agranulocytosis, stomatitis, diarrhea, and dehydration. Although many molecular targeted agents have been developed up to the present, they also have many adverse side effects such as hand-foot syndrome and interstitial pneumonitis, because target molecules exist in both tumor and normal tissues.

Accordingly, more specific antitumor effects and/or more selective delivery systems of antitumor drugs to tumor tissues are needed[1)–10)].

Living bacteria have recently been considered as a drug delivery tool in cancer gene therapy ; one such example is lactic acid bacteria (LAB). LAB is a nonpathogenic anaerobic bacteria widely used in food and intestinal health supplements[11)12)]. Numerous reports have documented the safety and functional characteristics of LAB.

Hypoxic conditions are characteristic of solid tumors in both rodent[13)] and many types of human tumors[14)]. The oxygen pressure in human tumors has been reported to be 0–20 mmHg, whereas that in normal tissues shows a range of 20–100 mmHg[4)15)16)]. Tumor hypoxia, mostly stemming from poor perfusion of fresh blood, causes the development of aggressive cell clones and leads to rapid progression and poor prognosis[17)–19)].

Futhermore, hypoxia is known to directly and

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indirectly confer resistance to radiation and some chemotherapies, which often results in treatment failure[20)21)].

Recently, *Bifidobacterium longum (B.longum),* a strain of obligated LAB, has been reported to selectively germinate and grow in the hypoxic regions of solid tumors following intravenous injection and was demonstrated to be a safe and effective drug carrier to solid tumors[1)−4)].

Lactobacillus casei (L.casei), another strain of LAB, is a gram-positive facultatively anaerobic rod-shaped bacterium. *L.casei* is believed to have indirect antitumor effects due to its ability to modulate host immune responses[22)−24)], but no direct antitumor properties have been shown to date[25)]. However, numerous recent reports have noted that the cytoplasmic fractions, proteins, and peptidoglycans of *L.casei* may suppress the proliferation of tumor cell lines[26)−30)].

We directed our attention to the fact that *L.casei* has thin polysaccharides in its wall and may easily excrete proteins synthesized via an expression vector, since *B.longum* with cytosine deaminase has successfully exhibited antitumor effects through intravenous injection in combination with 5-FC[2)−4)].

We examined whether obligated *L.casei* could also be a specific delivery tool to target tumor tissues, and produced *L.casei KJ686,* a novel *L.casei* strain that was transformed to be obligately anaerobic, to germinate and grow in the hypoxic regions of solid tumors after intravenous injection.

In the present study, we found that *L.casei KJ686* was a tolerated and selective carrier to solid tumors and exhibited suppressive effects on tumor growth. We also assessed the direct cytotoxic effect of *L. casei KJ686* and/or the substances excreted from this bacterium *in vitro*.

II Materials and Methods

A *In vivo*

1 Growth of *L.casei KJ686*

L.casei KJ686 with the erythromycin resistance gene was developed as a mutant strain of the facultatively anaerobic *L.casei ATCC393* that had been transformed to be obligately anaerobic. The mutant was maintained in MRS broth (OXOID, Hampshire, England) with erythromycin (0.2 μg/ml) at 37 ℃ without O_2. *L.casei KJ686* in the mid-logarithmic growth phase was used for all experiments.

2 Preparation of tumor-bearing mice

Lewis lung carcinoma (LLC) cells were cultured in medium containing DMEM (Sigma, Louis, MO, USA), 10 % fetal bovine serum and streptomycin (10 μg/ml) at 37 ℃ with 5 % CO_2. Male 8-week-old C57BL/6 mice (Japan SLC, Hamamatsu, Japan) of similar size and weight were used and maintained with a standard rodent diet (Oriental Yeast, Tokyo, Japan) at the Shinshu University animal center (Matsumoto, Japan). The animal experiments in this study were carried out in accordance with the Guidelines for Animal Experimentation of Shinshu University School of Medicine. Aliquots of 10^6 tumor cells in 100 μl Hanks' Solution (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) were inoculated under the femoral skin of the mice.

The solid tumors were palpable 5-8 days after inoculation. For each tumor, the maximum diameter (A) and diameter at a right angle to A (B) were measured daily using a sliding caliper. Tumor volume was estimated as $1/2 \times A \times B^2$. All mice survived the testing period.

3 Intravenous injection of *L.casei KJ686* into tumor-bearing mice

L.casei KJ686 was harvested by centrifugation (3000 rpm, 1 min at 4 ℃), washed twice with PBS (−), and then resuspended in PBS (−) at 5×10^8 c.f.u./100 μl. The solid tumor-bearing mice were randomly separated into 2 groups and used for subsequent studies. When tumors reached approximately 65.4 mm^3 (5 mm in diameter), *L.casei KJ686* [5×10^8 c.f.u./100 μl PBS (−)] was injected into a tail vein of one group once a day for 2 days. The other mice underwent no treatment as the control group. The tumor growth ratio was calculated every day as follows:

(tumor volume) / (tumor volume on the first day of intravenous injection).

4 Localization of *L.casei KJ686*

All tumor-bearing mice were sacrified 17 days after the first injection of *L.casei KJ686*. Normal tissue samples from the lung, liver, and tumor were excised and placed into a homogenizer with 9-fold anaerobic diluent. The samples were plated on an MRS agar medium at 37 ℃ without O_2 to assess the bacterial numbers in each tissue. The localization of *L.casei KJ686* in tumor tissue sections was visual ized microscopically with Gram's and Hematoxylin-Eosin staining.

B *In vitro*

1 Preparation of *L.casei KJ686* and culture supernatant of *L.casei KJ686* to examine for cytotoxic effect

LLC cells were maintained at 37 ℃ with 5 % CO_2 and routinely cultured in DMEM supplemented with 10 % fetal bovine serum with or without streptomycin (10 μg/ml). *L.casei KJ686* was harvested by centrifugation (3000 rpm, 1 min at 4 ℃) and washed twice with PBS (−). The bacteria were resuspended in DMEM without streptomycin at 1×10^{10} c.f.u./ml (living *L.casei KJ686*). Heat-killed *L.casei KJ686* was prepared by boiling living *L.casei KJ686* at 95 ℃ for 90 min. After 24 hr of anaerobic culture in DMEM without antibiotics, the culture supernatant of *L.casei KJ686* was harvested by centrifugation (12000 rpm, 10 min at 4 ℃). The supernatant was divided into 6 fractions by molecular weight (over 100 kDa, under 100-50 kDa, under 50-10 kDa, under 10-3 kDa, under 3-1 kDa and under 1 kDa) with Amicon Ultra-0.5 ml Centrifuged Filter devices (100 k, 50 k, 10 k, and 3 k ; Merck Millipore, Darmstadt, Germany), and a Microsep Centrifugal device (1 k ; Pall Life Sciences, Michigan, USA). Each fraction was diluted to its original concentration with DMEM. Furthermore, the under 1 kDa supernatant (1 ml) was vortexed with ethyl acetate (1 ml) and separated into aqueous and organic layers by centrifugation. To remove the ethyl acetate, the organic layer was desiccated in draft for 48 hr. After desiccation, the organic layer powder was vortexed with 990 μl DMEM and 10 μl DMSO. The aqueous layer was threaded into a hydrophobic resin (non-methyl alcohol fraction), and then several concentrations (20-100 %) of methyl alcohol were threaded into the hydrophobic resin and recovered (20-100 % methyl alcohol fraction). Each aqueous supernatant with alcohol was lyophilized in a desiccator and diluted with sterilized water.

2 Gram's stain

For both living and heat-killed *L.casei KJ686,* the neo B & M (Wako Pure Chemical Industries, Ltd., Osaka, Japan) method was performed according to the manufacturer's instructions.

3 Cell viability assay

The Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan) is based on the cleavage of the tetrazolium salt WST-1 by cells to form a red formazan dye. Cell viability was measured with this assay according to the manufacturer's instructions. LLC cells (1×10^4 in 90 μl/well) on a 96-well microplate were incubated for 24 hr at 37 ℃ with 5 % CO_2 with various concentrations of *L.casei KJ686* culture supernatants ($1\times10^6-1\times10^8$ in 10 μl/well of living or heat-killed *L.casei KJ686*). WST-1 reagent (1 :10) was then added. After 60 min of incubation at 37 ℃ with 5 % CO_2, the absorbance of the colored formazan was determined using a Vmax automated microplate reader (Wako, Osaka, Japan) at 450 nm and 650 nm wavelengths. The mean absorbance of control wells (cells without supernatant) represented 100 % cell viability.

4 Colony-forming assay

LLC cells (500/well) on a 6-well plate were incubated for 24 hr at 37 ℃ with 5 % CO_2. Various conditions of *L.casei KJ686* culture supernatants were then added to the LLC cells, which were then incubated further. After 8 days, the supernatants were removed from the wells and the cells were fixed and stained with 10 % formalin (Wako, Osaka, Japan) and crystal violet solution (Wako, Osaka, Japan) according to the manufacturer's instructions. The toxic activity of supernatants was estimated by the viability of LLC cells.

C Statistical analysis

Experimental data are expressed as the mean± SE of the mean. Statistical analysis was performed using the Mann-Whitney *U*-test. A $p<0.05$ was

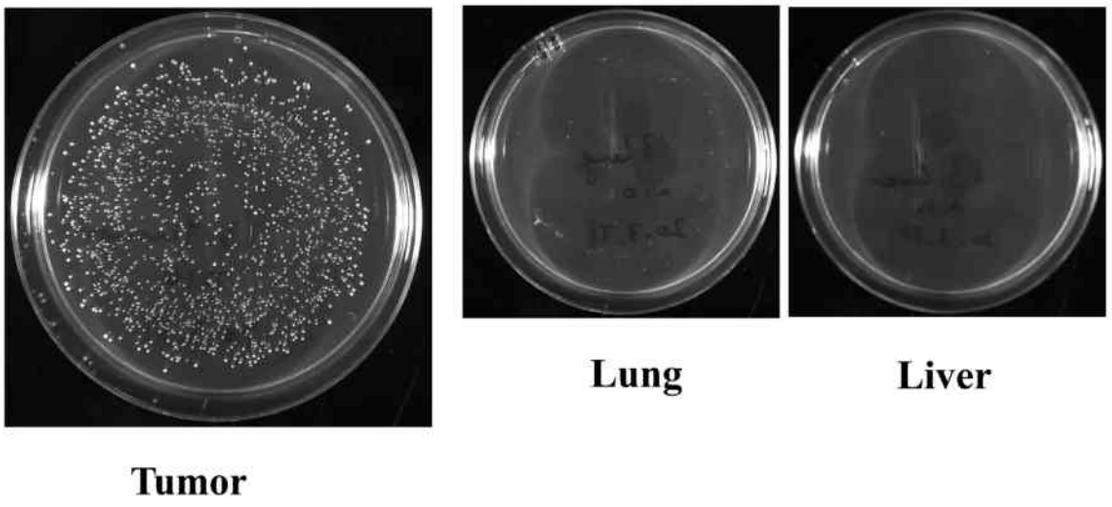

Fig. 1 Germination of *L.casei KJ686*. Seventeen days after intravenous inoculation of *L.casei KJ686,* organs and tumors were homogenized with a 9-fold anaerobic diluent. Samples of 100 μl of homogenized tissues were cultured on an MRS agar medium at 37 ℃ without O_2 for 3 days.

considered to be statistically significant.

Ⅲ Results

A *In vivo*

1 Selective localization of *L.casei KJ686* in tumors

We examined the germination of *L.casei KJ686* in the lungs, livers and tumors of *KJ686*-injected mice.

Another obligate anaerobic bacterium, *B.longum* was not at a detectable level in any normal tissues after seven days[1]. So we sacrified *KJ686*-injected mice nine days after intravenous injection, *L.casei KJ686* was selectively detected in tumors, with no bacterial growth in either the lungs or livers (**Fig. 1**). Each tumor had necrotic areas in its center (**Fig. 2**). Gram's staining of tumor tissues showed that *L. casei KJ686* was focally scattered in the border areas between necrotic and viable regions (**Fig. 2**). Neutrophils existed in necrotic and viable regions. The number of *L.casei KJ686* in solid tumors estimated by tissue homogenation appeared to reflect the bacterial number in border areas.

2 Inhibition of tumor growth with *L.casei KJ686*

Fig. 3 shows the tumor growth ratio after intravenous injection of *L.casei KJ686* (n=25) and in controls (n=36). After administration of *L.casei KJ686,* neither suppression of activity nor death were observed in the test group. From day 5 after the first injection, the tumor growth ratio of the *KJ686* group was significantly less than that of the control group. We next estimated RNA expression of IL-2, IL-4, IL-12p, IL-1β, and IFN-γ by real time RT-PCR of the border areas of tissue samples, but no significant differences were noted (data not shown). Cytokine levels in the serum of *KJ686*-injected mice were also estimated on days 3 and 9, but no significant findings were seen except for IL-1β on day 3 (data not shown). Although IL-1β was transiently increased, enhancement of host immunological defenses by *L.casei KJ686,* such as an increase in the number of NK cells in tumors, was not observed (data not shown).

B *In vitro*

1 Cytotoxic effect of *L.casei KJ686* on LLC cells

The direct cytotoxic effect of *L.casei KJ686* on LLC cells was assessed by the WST-1 method. The morphology of *L.casei KJ686* was not visibly altered by heat treatment (**Fig. 4**), which indicated that the bacterial body was not ruptured. The viability of LLC cells with living *L.casei KJ686* decreased in a concentration-dependent manner, while LLC incubated with heat-killed *L.casei KJ686* exhibited much the same viability as controls (**Fig. 5**).

2 Cytotoxic effect of *L.casei KJ686* culture supernatant on LLC cells

Conditioned media in which *L.casei KJ686* had been cultured for 24 hr and split into 6 fractions by

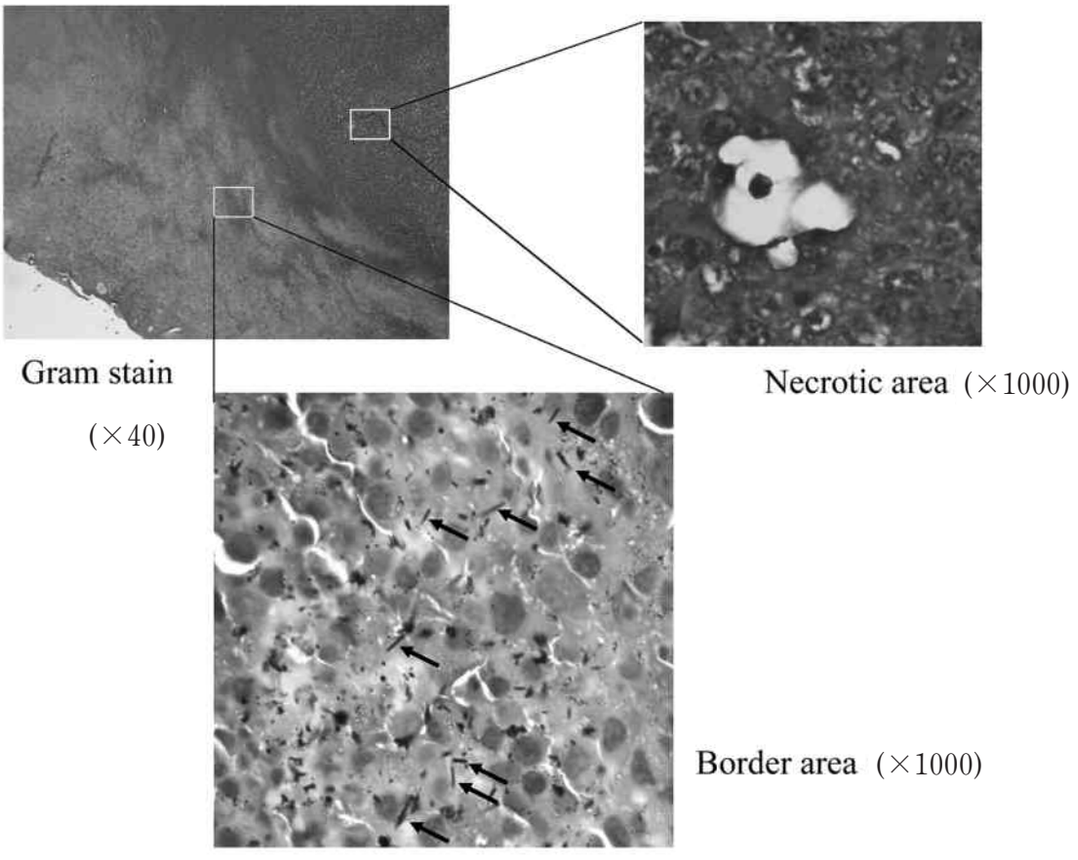

Fig. 2 Localization of *L.casei KJ686* in tumors. On day 9, Gram's staining of histological sections indicated that *L.casei KJ686* was scattered in the border areas between necrotic and viable regions.

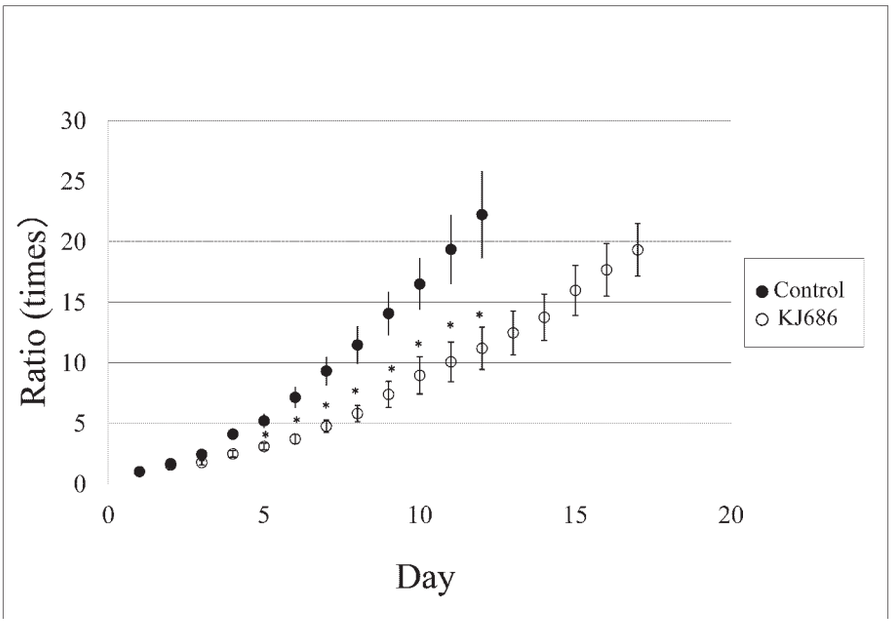

Fig. 3 *In vivo* tumor growth ratio. Controls were not given any treatment after tumor inoculation, while the test group was intravenously injected with *L.casei KJ686*. The tumor growth ratio became significantly different from day 5 (*$p<0.01$).

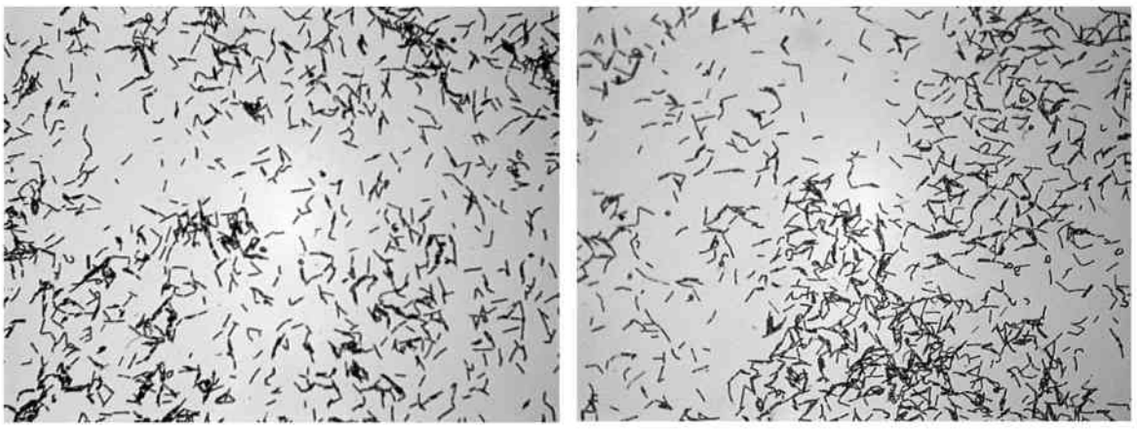

Fig. 4 Gram's staining by the neo B & M method. The morphology of living and heat-killed *L.casei KJ686* was not apparently altered.

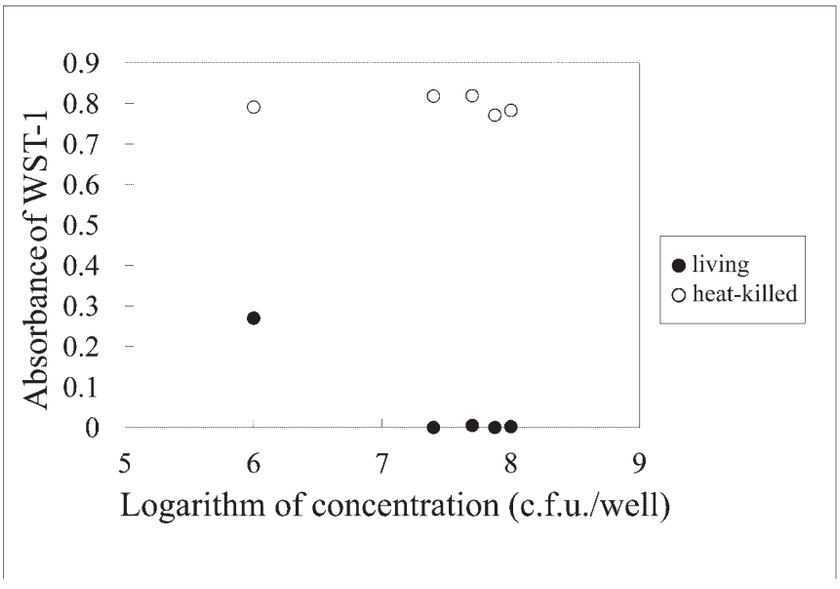

Fig. 5 Cytotoxic effect of *L.casei KJ686* on LLC cells. The survival of cells treated with living *L.casei KJ686* (○) was significantly less than those treated with heat-killed *L.casei KJ686* (□). Untreated LLC cells exhibited much the same viability as heat-killed *L.casei KJ686* cells.

molecular weight were evaluated for cytotoxic effect. The fraction with a molecular weight of less than 10 kDa exhibited a comparable cytotoxic effect as the original conditioned medium, while the other fractions did not (**Fig. 6**). We then compared the aqueous layer with the organic layer of the supernatant under 1 kDa, and found that only the aqueous layer had a significant cytotoxic effect (**Fig. 7**). However, no fraction of the under 1 kDa supernatant further separated by a hydrophobic resin column exhibited cytotoxicity (data not shown). When conditioned medium and cytotoxic fractions were boiled at 100 ℃ for one minute, cytotoxicity showed only a slight decrease (data not shown). To test for pH-mediated cytotoxicity, we added a comparatively acidic pH4.0 lactic acid solution instead of the supernatant, but no cytotoxicity was observed (data not shown).

Ⅳ Discussion

Hypoxia in solid tumors is known to confer resistance to radiation and some chemotherapies, which

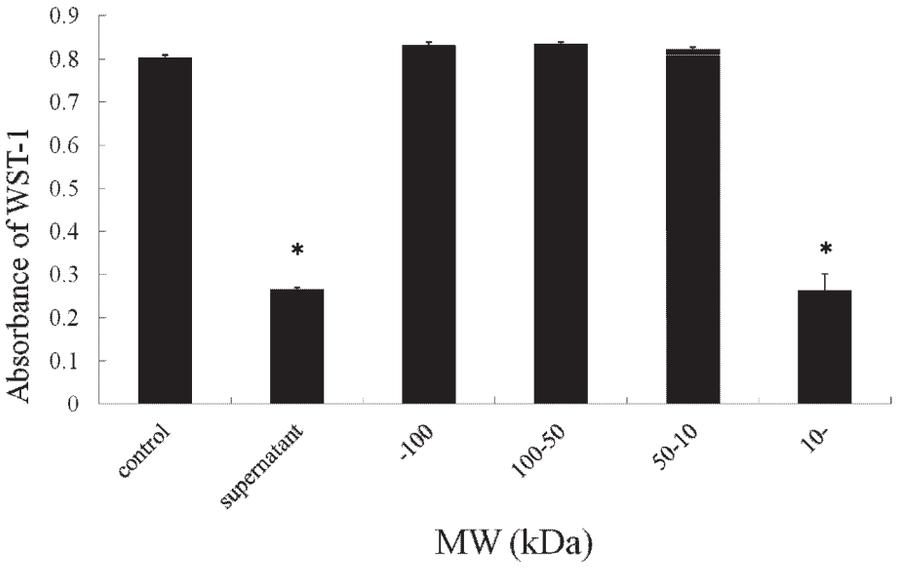

Fig. 6 Cytotoxic effect of the conditioned medium of *L.casei KJ686* on LLC cells. The concentration of each fraction was adjusted to that of the original supernatant with DMEM (*$p < 0.01$ vs. control).

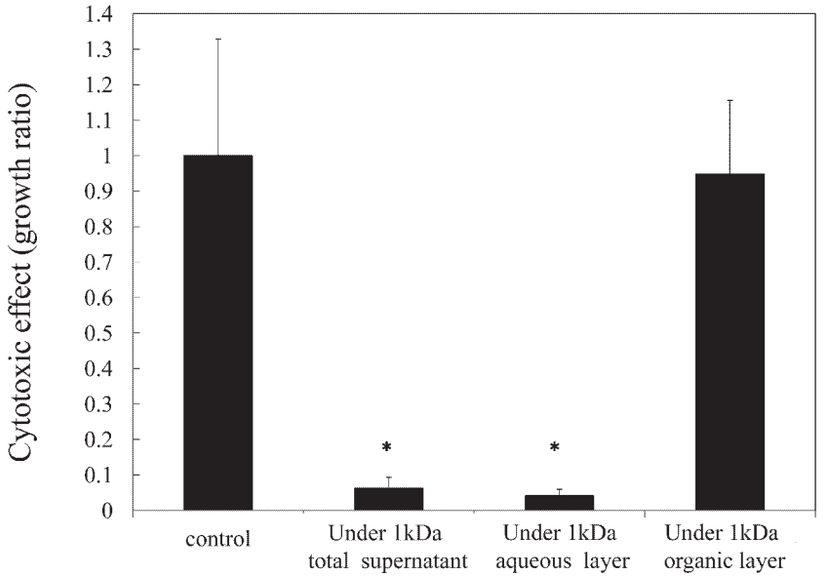

Fig. 7 Comparison of the cytotoxic effect of aqueous and organic layers of the conditioned medium fraction under 1 kDa. The concentration of each fraction was adjusted to that of the original supernatant with sterilized water (*$p < 0.05$ vs. control).

often results in treatment failure[20)21)]. To overcome this impasse, several new means of delivering agents to solid tumors are being explored[1)–10)].

Intravenous injection of living bacteria is generally understood to induce bacteremia and sepsis. This was surprisingly not the case for *B.longum*[1)–4)], nor was it for *L.casei KJ686,* which accumulated specifically in tumor tissues without any adverse symptoms in the mice. This fact demonstrates that *L.casei KJ686* would be safely used for systemic treatment. Interestingly, a significant difference between *L.casei KJ686* and *B.longum* is that the former has the ability to inhibit tumor growth by itself. We believe that this inhibition is due to the local effects of *L.casei KJ686* and not immunomodulation. In previous studies, inoculation of a large dose of heat-killed bacteria had no direct anti-tumor effect, indicating that the property was not

attributed to host immunoresponses[25)29)]. Thus, we next investigated the direct anti-tumor effect of *L. casei KJ686 in vitro*. Following the addition of living *L.casei KJ686,* the growth of LLC cells was strongly inhibited, but this was not the case for heat-killed *L.casei KJ686*. Both living and heat-killed *L. casei KJ686* exhibited the same morphology by Gram's stain, implying that heat treatment did not cause destruction of the cell wall. These results suggested that living *L.casei KJ686* itself and/or substances excreted from *L.casei KJ686* imparted a cytotoxic effect on LLC cells. We further investigated which fractions of the *L.casei KJ686* culture supernatant had this effect. Manjunath et al. reported that a 40-fold concentrated bacterial culture supernatant exhibited cytotoxicity *in vitro*[30)]. Although we used a culture supernatant that was not concentrated, we nonetheless witnessed that very low molecular weight (<1 kDa) excreted products of *L.casei KJ686* had an inhibitory effect on the growth of LLC cells. As the inhibitory properties of the fraction were partially stable against both heat and lactic acid treatment, it can be said that *L.casei KJ686* had a direct suppressive effect, possibly through several factors, on LLC cells rather than indirect stimulation of host immunoresponses.

In terms of safety, *L.casei* may have immunological tolerance because it can be found in the human intestinal tract just after birth[31)]. Although it was reported that *L.casei* sometimes induced bacteremia in patients with cancer, diabetes, broad spectrum antibiotic therapy, and transplantation, there have been no reported cases of patient death if adequate treatment was performed[32)]. In conclusion, it appears that specific cytotoxic substances present in the culture supernatant of *L.casei KJ686* and their mode of action may be pivotal as direct anti-tumor agents once they have been identified.

Ⅴ Acknowledgements

Our deep appreciation goes to Prof. Shun'ichiro Taniguchi of the Department of Molecular Oncology, Institute of Pathogenesis and Disease Prevention, Shinshu University Graduate School of Medicine, and Prof. Minoru Fujimori of the Department of Breast Surgery, Tokyo Medical University Ibaraki Medical Center, who provided carefully considered feedback and valuable comments. We also owe a very important obligation to Prof. Tomoyuki Fujita of the Department of Sciences of Functional Foods, Graduate School of Agriculture, Shinshu University, and Akihiro Shou of the Department of Molecular Oncology, Institute of Pathogenesis and Disease Prevention, Shinshu University Graduate School of Medicine, whose chemical analysis and technical assistance helped us greatly. Finally, we thank Dr. Shizunobu Igimi and Akinobu Kajikawa of the National Institute of Health Sciences for provision of the original stock culture of *Lactobacillus casei KJ686* that was used in this study.

This study was supported in part by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (No. 21591678)

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(2015. 5. 19 received ; 2015. 8. 17 accepted)