

Treatment with *Lactobacillus* Retards the Tumor Growth of Head and Neck Squamous Cell Carcinoma Cells Inoculated in Mice

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Bacteria have been used for more than a century to treat solid tumors. Because solid tumors generate an anaerobic environment, we evaluated the anti-tumor effect of the obligate anaerobe strain KK378, derived from *Lactobacillus casei* (*L. casei*), using mice bearing head and neck cancer. Wild-type *L. casei* is a nonpathogenic bacterium that is commonly used in foods. Moreover, patients with head and neck squamous cell carcinoma often have multiple cancers and cervical lymph node metastasis that can be directly sensed beneath the skin. To establish the animal model bearing head and neck cancer, we inoculated each of human squamous cell carcinoma cell lines, SAS, HSQ89, and HSC2, on the back skin of BALB/cSlc-nu/nu mice. After tumor formation, *L. casei* KK378 was administered directly into the tumor, and tumor size and serum cytokine levels were analyzed. Mice injected with 10^8 cfu of *L. casei* KK378 showed reduction in tumor growth compared with PBS control; especially, the SAS tumor was significantly reduced ($p = 0.008$). Administered *L. casei* KK378 was detected in tumor tissues but not in normal tissues (liver, kidney, and lung) of SAS tumor-bearing mice, which was associated with increased blood cytokines (TNF- α , IFN- γ , IL-5, IL-10, and IL-12). Among these cytokines, the serum levels of IFN- γ and TNF- α were significantly increased ($p < 0.05$). In conclusion, *L. casei* KK378 infection may suppress tumor growth by inducing the host immune response. Direct injection of *Lactobacillus* into the tumor could be a potential strategy to treat head and neck squamous cell carcinoma.

Keywords: cytokine; head and neck squamous cell carcinoma; immuno-modulator; *Lactobacillus casei*; tumor suppression

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Introduction

Bacteria have been used for more than a century to treat solid tumors. Various bacterial strains have been used as treatment strategies for malignant tumors since the 19th century. Coley (1891) described a patient who had recurrent round-celled sarcoma tumors, who was cured by severe erysipelas infection. He attempted to treat patients with malignant tumors by infecting the patient with live *Streptococcus pyogenes*, although he subsequently abandoned the use of live bacteria in favor of isolated preparations of bacteria toxins (Coley 1906).

It was first shown in 1947 that direct injection of spores of *Clostridium histolyticus* into a transplantable mouse sarcoma caused oncolysis and tumor regression

(Parker et al. 1947). However, very few animals survived this treatment because of acute toxic effects resulting in death. Kimura and colleagues implanted Ehrlich ascites tumors in mice and injected a suspension of lyophilized *Bifidobacteria* via the tail vein. The bacteria were highly localized within the tumor, with virtually no bacteria in other organs showing a very high tumor-targeting phenomenon (Kimura et al. 1980).

Salmonella, gram-negative facultative anaerobes, are also known to colonize human tumors (Graham and Coleman 1952; Gill and Holden 1996). Although systemic infection of *Salmonella* induces septic shock and high mortality in humans if not treated soon enough, Bacon et al. (1951) showed *Salmonella* virulence in mice is attenuated in some auxotrophic mutants. In 1997, Pawelek et al.

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(1997) reported that *Salmonella* auxotrophs that were injected into tumor-bearing mice would preferentially replicate within the tumor, indicating this microbe as a tumor-targeting agent.

The use of bacteria for treating tumors clinically causes problems of bacterial pathogenicity and influence on normal tissues. To overcome these problems, we tested the effect of *Lactobacillus casei* (*L. casei*), a nonpathogenic bacterium that is commonly used in foods, on the growth of head and neck squamous cell carcinoma. Wild-type *L. casei* is a facultative anaerobe that can grow under relatively high oxygen concentrations, which would permit growth both in the hypoxic tumor environment as well as in normal tissues (Brown 1999). Instead, we used anaerobic *L. casei* KK378, a derivative strain that was mutated to grow under anaerobic conditions. Importantly, *L. casei* KK378 has low or no growth potential under aerobic conditions, and thus it may not cause severe side effects in normal tissues.

Here we report that direct injection of anaerobic *L. casei* into the tumor site retarded the growth of squamous cell carcinoma cells inoculated in mice, which was associated with the increases in serum cytokine levels.

Materials and Methods

Preparation of *L. casei*

Anaerobic *L. casei* KK378 was developed by chemical mutagenesis of facultative anaerobic *L. casei* with 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG) under anaerobic culture conditions. The obtained *L. casei* KK378 was cultured in MRS broth at 37°C without oxygen.

Cell lines of human squamous cell carcinoma

SAS is a poorly differentiated squamous cell carcinoma cell line derived from human tongue cancer (Takahashi et al. 1989) and was obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Miyagi, Japan. Cells were cultured in RPMI1640 (Sigma R8796, Sigma Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitrogen, San Diego, Ca, USA) at 37°C under atmosphere with 5% CO₂.

HSC2 is a squamous cell carcinoma cell line derived from human oral cancer (Momose et al. 1989), and HSQ89 is a squamous cell carcinoma cell line derived from human maxillary cancer (Wakamatsu et al. 1993). HSC2 and HSQ89 cell lines were obtained from Riken Bio Resource Center, Tsukuba, Ibaraki, Japan. These cells were cultured in DMEM (Sigma D5546) containing 10% fetal bovine serum (Invitrogen) at 37°C under atmosphere with 5% CO₂.

Preparation of tumor-bearing mice

Male five-week-old BALB/cSlc-nu/nu mice (Japan SLC, Inc. Shizuoka Japan) were used in this study. These mice are immunodeficient as they lack the thymus and are unable to produce T-cells. Aliquots of 3.0×10^6 tumor cells suspended in 200 μ l Matrigel (BD Biosciences, Tokyo, Japan) were inoculated on the back skin of the mice. 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 $0.4 \times A \times B^2$. Relative

tumor volume (%) was calculated every day as follows: (tumor volume_{day x})/(tumor volume on the first day of *L. casei* injection_{day 0}). The animal experiments in this study were carried out in accordance with the Guidelines for Animal Experimentation Guidelines of Tohoku University and Iwate Medical University.

Topical injection of *L. casei* KK378 into tumor

Bacterial cell numbers, shown as colony forming unit (cfu), were calculated from the absorbance of the *L. casei* culture fluid. The tumor cells-bearing mice were randomly divided into 4 groups (n = 12 for each cancer cell line). When SAS, HSQ89 and HSC2 tumors reached approximately 200 mm³ (6 mm in diameter), a solution containing either 1.0×10^4 , 1.0×10^6 , or 1.0×10^8 cfu of *L. casei* KK378 was injected in a volume of 100 μ l into the tumor of three groups of mice, and PBS was injected as a control into the tumor of the fourth group. The growing speed of the tumor depended on each cell line. Thus, the day of the *L. casei* injection varied among these tumor bearing mice: about 10th day after SAS inoculation, 9th day after HSQ89 inoculation, and 30th day after HSC2 inoculation. *L. casei* KK378 was administered only once.

Gram staining

Mice were sacrificed under general anesthesia at the indicated day after inoculation of the tumor and their liver, lung, kidney, and tumor were obtained. Two serial 3 μ m-thick sections were made from a representative block of each tissue that had been 10% formalin-fixed and paraffin-embedded. One section was stained with hematoxylin and eosin, and other section was used for Gram staining. Gram staining was done using Hucker's crystal violet as the primary stain, and iodine and 0.25% safranin as the counterstain as described previously (Shiga et al. 2001). All reagents were purchased from Muto Pure Chemicals Co. Inc., Tokyo.

Cytokine assay

Mouse serum samples were collected and analyzed for levels of eight cytokines (IL-2, IL-4, IL-5, IL-10, IL-12p70, GM-CSF, IFN- γ and TNF- α) by Bio-Plex cytokine reagent kit with Bio-Plex mouse cytokine Th1/Th2 Panel in the Bio-Plex 200 system (Bio-Rad, Hercules, CA, USA), as directed by the manufacturer.

Statistical analysis

Statistical analyses were carried out using Students' *t*-test, and all *p* values < 0.05 were considered significant.

Results

Inhibition of tumor growth

Of the 11 cell lines tested for the growth after inoculation on the back skin of nude mice, we found that three cell lines, HSC2, HSQ89 and SAS, had characteristics of rapid growth and tumor formation. We used these cell lines to test whether *L. casei* KK378 can suppress tumor growth after injection into the tumor inoculated on the back skin of nude mice. Three dosages of bacteria, 10^4 , 10^6 , and 10^8 cfu were injected directly into the tumor formed on the mice when tumor volume reached around 200 mm³. Fig. 1 shows the typical result of this experiment. A dose-dependent suppression of tumor growth was seen in SAS tumors infected with *L. casei* KK378, where 10^8 *Lactobacilli*

showed dramatic reduction in tumor size within five days of infection (Fig. 1A). By contrast, only restricted suppression of tumor growth was observed in the HSQ89 tumor (Fig. 1B), despite that 10^8 *Lactobacilli* were injected into the tumor. Approximately 50% reduction of the tumor volume was observed on the fifth day after *Lactobacilli* injection compared with the control group (Fig. 1B), but there was no significant difference. In addition, HSQ89 tumor-bearing mice died during the early days of this experiment, perhaps because HSQ89 tumor cells could induce cachexy in the mouse body. When HSC2 tumor was used, 10^8 *Lactobacilli* showed approximately 50% reduction of the tumor volume by the 12th day after *Lactobacilli* injection compared with the control group (Fig. 1C). However, there was no significant difference between these two groups.

Localization of *L. casei* KK378 in mice tissues

To confirm the localization of *Lactobacilli* in the injected tumor tissues and absence in other tissues, Gram staining was carried out on tissue sections (Fig. 2). We sacrificed *L. casei* KK378-injected SAS tumor-bearing mice 21 days after injection. Bacterial colonies were detected only in tumor tissues, and no bacteria were detected in all other normal tissues analyzed (liver, kidneys, and lung). The tumor tissues in which *Lactobacilli* were injected had necrotic regions inside the tumors and selective localization of *L. casei* at the boundary between the necrotic region and the tumor region.

Serum concentration of cytokines

To examine the influence of *L. casei* on the host immune system, we analyzed the serum concentrations of various cytokines in the SAS tumor-bearing nude mice. Serum concentrations of IL-2, IL-4, IL-5, IL-10, IL-12p70, GM-CSF, IFN- γ , and TNF- α are shown in Fig. 3. Serum levels of IL-5, IL-10, IL-12, TNF- α , and IFN- γ were increased, depending on the number of *L. casei* KK378 administered compared with the control group. Sera of the mice that received 1.0×10^8 cfu of *L. casei* KK378 showed the largest increase in the concentrations of these cytokines. Especially, the IFN- γ and TNF- α levels were significantly increased, compared with those in control mice (Fig. 3).

Discussion

Records of cancer treatment by bacteria have existed for over 100 years. William Coley treated head and neck cancer patients in 1891 using *Streptococcus pyogenes* (Coley 1891). However, these treatments are usually associated with problems of bacterial pathogenicity and poor reproducibility, and therefore the main treatment choice for cancer has been radiotherapy and/or surgery. However, treatment of cancer using bacteria has been used so far, although not in a usual matter (Ishii et al. 1976; Peavy et al. 1979; Yasutake et al. 1984; Matsuzaki et al. 1988a).

Activation of tumor immunity and activation of chemotherapy by lactic acid bacteria, which are nonpathogenic

and indigenous, have been reported and used for the treatment of various cancers (Asano et al. 1986; Matsuzaki et al. 1988b; Kato et al. 1988; Takagi et al. 1999). Recent studies on the relationship between immune checkpoint inhibitor and intestinal bacterial flora have been attracting attention (Sivan et al. 2015; Routy et al. 2018; Gopalakrishnan et al. 2018). Consequently, these findings are also drawing attention to bacteria for cancer treatments.

This is the first report demonstrating that direct injection of *Lactobacillus* into the human squamous cell carcinoma established on backs of nude mice suppressed tumor growth. The tumor inhibiting effect was most effective in the group of mice injected with 1.0×10^8 cfu *L. casei* in all three groups of mice bearing SAS, HSQ89, and HSC2. No tumor inhibiting effect was observed in the group of mice injected with PBS, and little tumor inhibiting effect was observed in 1.0×10^4 or 1.0×10^6 cfu of *L. casei* compared with PBS. In addition, the tumor suppressing effect was not efficient in HSQ89 and HSC2 tumor-bearing mice by injection of 10^8 *L. casei*. Our results indicated that tumor suppressing effect of *L. casei* was dose-dependent and at least 1.0×10^8 cfu of bacteria were required to cause tumor suppression in SAS tumor-bearing mice. These results could reflect the characteristic features of the cell lines. Yazawa et al. (2001) have reported the tumor inhibiting effects of intravenously injected *Bifidobacterium longum* at $5\text{--}6 \times 10^6$ cfu in mice. These studies suggested that a certain number of bacteria is necessary to obtain noticeable effect of tumor suppression. It is the most interesting and crucial matter that the tumor suppressing effects among these three cell lines were different. However, it is another problem to clarify by independent research with phenotype analyses and molecular biological analyses.

We investigated serum cytokines in the SAS tumor-bearing mice that received 1.0×10^4 cfu and 1.0×10^8 cfu of *L. casei* KK378. IL-5, IL-10, IL-12, IFN- γ and TNF- α showed elevation in the sera of *Lactobacillus*-injected mice in dose-dependent manner. In particular, serum levels of TNF- α and IFN- γ in mice that received 1.0×10^8 cfu *L. casei* KK378 were significantly higher than those in the PBS injected group (TNF- α : $p = 0.047$; IFN- γ : $p = 0.039$). These results indicate that tumor inhibition by *L. casei* KK378 could be mediated by activation of the host immune response and that *L. casei* KK378 influenced this response both locally and systematically. Several studies demonstrated that *L. casei* is phagocytosed and can induce cytokine production (Miettinen et al. 1998; Shida et al. 2006). It is suggested that tumor suppression develops when bacteria accumulate in the tumor. Therefore, route of injection is important in order to accumulate bacteria locally within the tumor.

In the present study, we injected bacteria directly into the tumor. Patients with head and neck squamous cell carcinoma, which we target, often have multiple cancers and cervical lymph node metastasis. Subcutaneous cervical lymph node metastasis or tumor invasion can often be

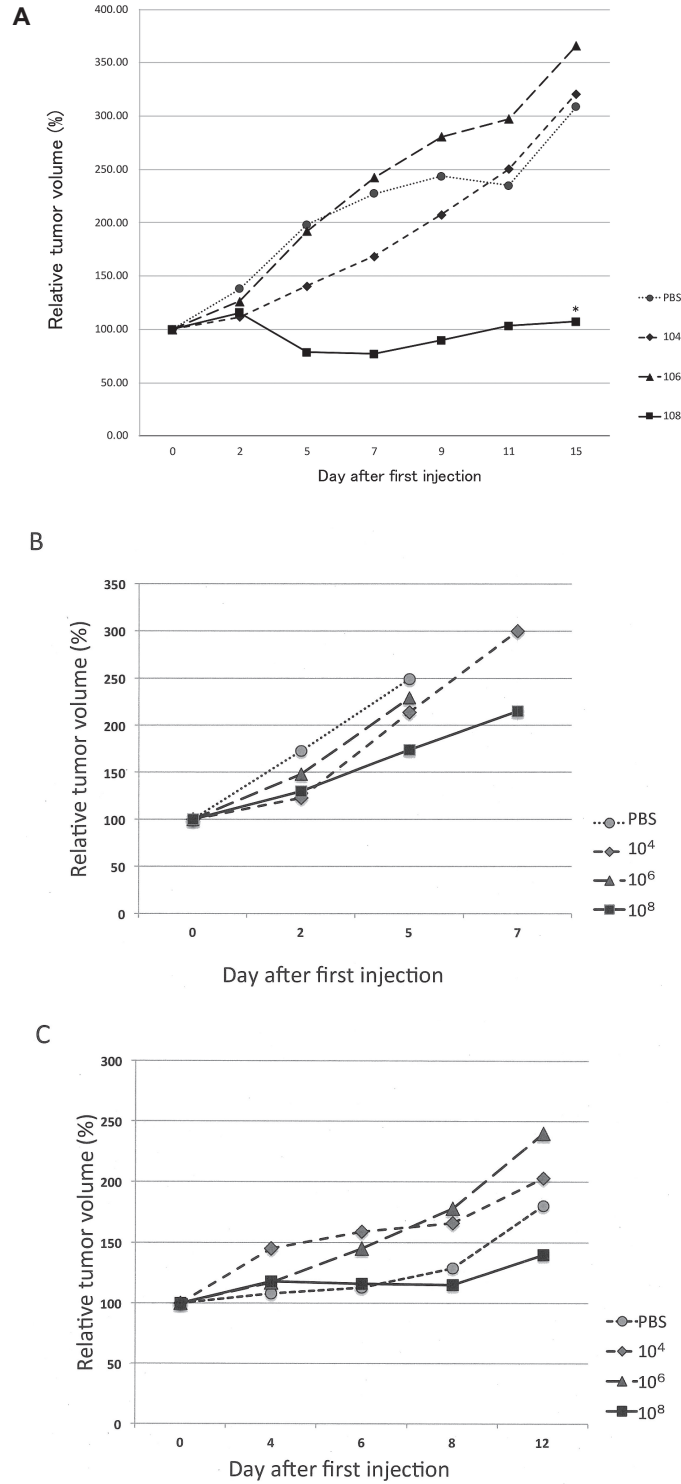


Fig. 1. Tumor growth curves.

Nude mice were inoculated SAS (A), HSQ89 (B), and HSC2 (C) human squamous cell carcinoma cells, and tumors were established on their backs (n = 12). PBS, 10⁴, 10⁶, or 10⁸ cfu *L. casei* KK378 were injected in a volume of 100 μl on day 10 after SAS inoculation, on day 9 after HSQ89 inoculation, and on day 30 after HSC2 inoculation when the tumor volume was reached around 200 mm³. Tumor growth curves were calculated and scored by the average of three tumors for each case. Tumor volumes are expressed as 100% on day 0 of *L. casei* injection. When 10⁸ cfu *L. casei* KK378 were injected to the SAS tumor, their growth was significantly reduced compared with PBS (p = 0.008) and 10⁶ cfu *L. casei* KK378 (p = 0.003) injected tumors. Astarisk shows the statistically significant difference (A). When 10⁸ cfu *L. casei* KK378 were injected to the HSQ89 tumor, their growth was reduced approximately 50%, but no significant reduction was observed compared with PBS (p = 0.21) (B). Similarly, when 10⁸ cfu *L. casei* KK378 were injected to the HSC2 tumor, their growth was reduced 50%, but no significant reduction was observed compared with PBS (p = 0.41) (C).

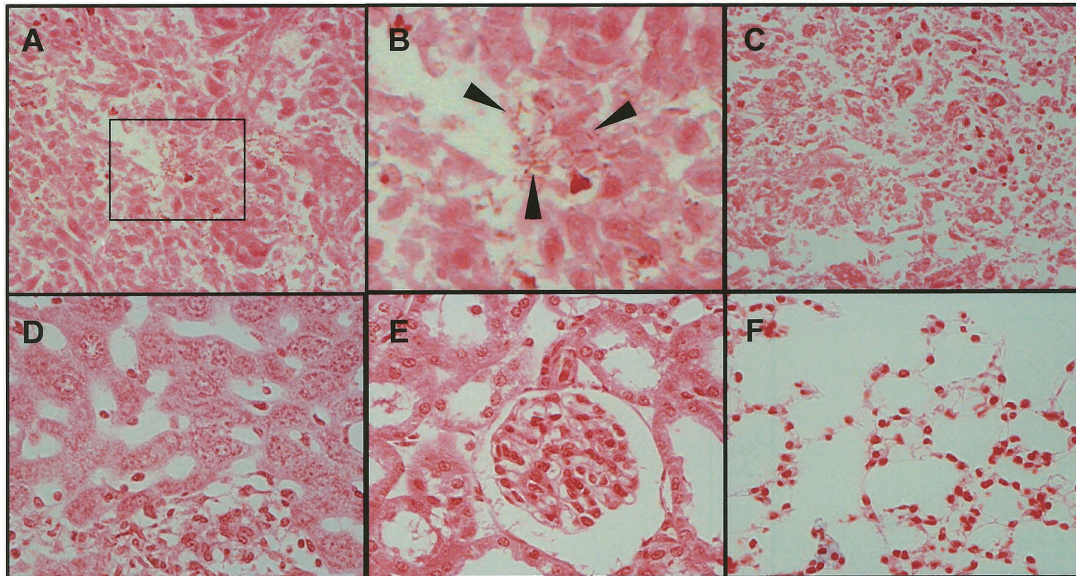


Fig. 2. Gram staining of tissues of the *L. casei* KK378-injected mice.

Nude mice were inoculated SAS cells and one tumor was grown on each back. PBS or 10^8 cfu *L. casei* were injected when the tumor volume was reached around 200 mm^3 . Mice were sacrificed under general anesthesia and their organs and tumors were obtained as pathological examined specimens. Gram staining was performed each organs and tumor tissues. (A) tumor tissue injected with 10^8 cfu *L. casei*, (B) magnified view of A, the enlarged area is indicated by black-lined rectangle in (A), (C) tumor tissue injected with PBS; (D) liver; (E) kidney; (F) lung. There are irregular shaped tumor cells located in the tumor tissue (A) and *Lactobacilli* are clearly visible in B. Arrowheads indicate *Lactobacilli*.

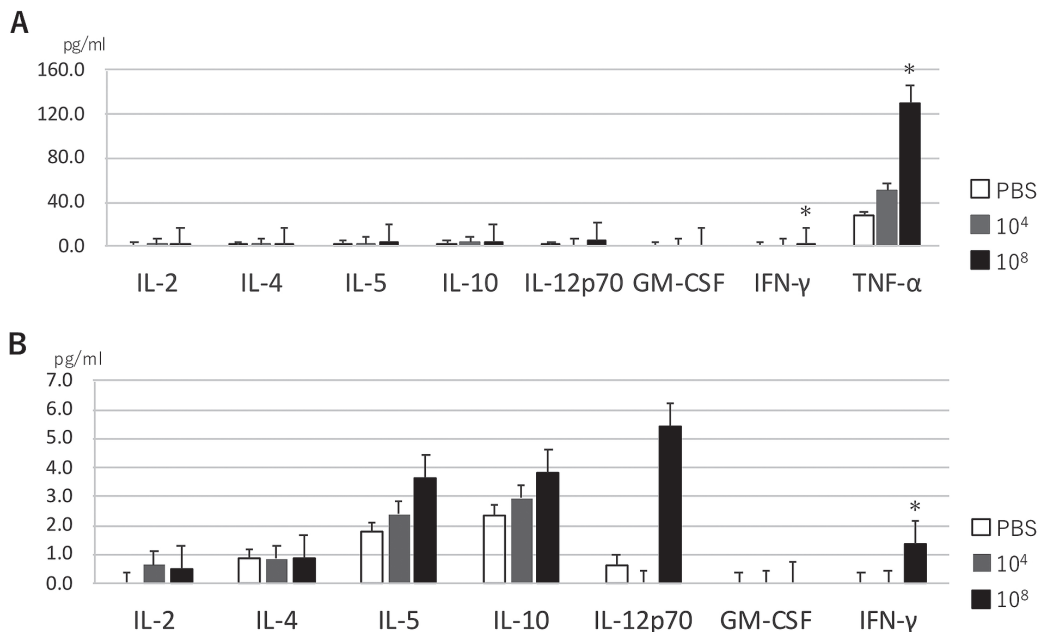


Fig. 3. Serum cytokine concentration in *L. casei* KK378-injected mice.

Nude mice were inoculated with SAS cells to establish one tumor on each back. These mice were classified into 3 groups and PBS, 10^4 or 10^8 cfu *L. casei* KK378 were injected when the tumor volume was reached around 200 mm^3 . Mice were sacrificed under general anesthesia and their blood was obtained. Serum cytokine concentrations were analyzed by EIA. Cytokine concentrations are indicated as pg/ml at vertical axis. Serum TNF- α level was significantly higher in mice injected with 10^8 cfu *L. casei* than that in PBS injected mice ($p = 0.047$). Also, serum IFN- γ levels were significantly higher in 10^8 cfu *L. casei*-injected mice compared with those in PBS-injected mice ($p = 0.039$). Apparently, serum levels of IL-2, IL-5, IL-10 and IL-12p70 in mice injected 10^8 cfu *L. casei* were higher than those of PBS-injected mice, but there were no significant differences. (A) All eight cytokine levels are shown. (B) Levels of all cytokines but TNF- α are shown to clearly depict the cytokine concentration, with the exception of TNF- α that showed much higher concentrations than those of the other cytokines. Asterisks show the statistically significant difference compared with the PBS-injected control.

directly sensed beneath the skin. Local injection is simpler in these cases compared to other solid tumors, as the tumor can be reached easily, especially to cervical lymph nodes.

We think that the control injection must be PBS or saline because some immunoreactive effect was expected using killed or inactivated *Lactobacilli* (Lee et al. 2015). However, it is an interesting research to use killed or inactivated *Lactobacilli* so that the experiment will be prepared in the future.

Hypoxia within the tumor is useful for accumulating anaerobic bacteria in the tumor. The system for delivering drugs into tumors has been devised to counter hypoxia in solid tumors (Taniguchi et al. 2010; Fang et al. 2014). Direct injection of anaerobic *L. casei* KK378 into the tumor could ensure their proliferation under the hypoxic environment. The antitumor effect of *Lactobacillus* can be expected when injected directly where the treatment is necessary. If anaerobic *L. casei* shows a significant therapeutic effect in the hypoxic environment within the tumor, new treatments for solid tumors will be possible. This treatment can be used in combination with existing chemotherapy and radiotherapy. Hypoxia in solid tumors has been reported to inhibit radiation therapy and chemotherapy (Ishii et al. 1976; Wan et al. 2012). If bacterial treatment can be combined with chemotherapy or radiotherapy, it is not only possible to reduce side effects but also to increase the effectiveness of antitumor treatment. In our study, although microbes were observed in the tumor tissues, no microbes were detected in the normal tissues and tumor suppression was observed. Compared to previous studies that show antitumor effects by intravenous administration, our local injection method is less prone to bacteremia and thus may be a more effective and safe treatment for patients with head and neck squamous cell carcinoma. However, there have been reports of abscess formation and bacteremia in infections with lactic acid bacteria (Cannon et al. 2005).

Even if *L. casei* is nonpathogenic and injected directly into the tumor site, there is a risk of live bacteria leaking out of the tumor tissue, proliferating in normal tissues, resulting in side effects in normal tissues. Patients with head and neck cancer, particularly those in advanced stages, easily become immunosuppressed by the tumor itself or by chemotherapy and/or radiotherapy. In these cases, viral or bacterial infection can be a life-threatening matter for the patients. Therefore, injection of dead cells of *L. casei* or injection of supernatant fluid into the tumor seems to be a preferable method, if these methods show the similar tumor suppressing effects as live bacteria. However, the efficacy of these treatments should be examined in the future studies.

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Conflict of Interest

The authors declare no conflict of interest.

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