Enhanced Bacterial Tumor Delivery by Modulating the EPR Effect and Therapeutic Potential of *Lactobacillus casei*

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ABSTRACT: Bacteria of micrometer size could accumulate in tumor based on enhanced permeability and retention (EPR) effect. We report here *Lactobacillus casei* (*L. casei*), a nonpathogenic facultatively anaerobic bacterium, preferentially accumulated in tumor tissues after intravenously (i.v.) injection; at 24 h, live bacteria were found more in the tumor, whereas the bacteria in normal tissues including the liver and spleen were cleared rapidly. The tumor-selective accumulation and growth of *L. casei* is probably due to the EPR effect and the hypoxic tumor environment. Moreover, the bacterial tumor delivery was significantly increased by a nitric oxide (NO) donor nitroglycerin (NG, 10–70 times) and an angiotensin II converting enzyme inhibitor, enalapril (6–18 times). Consequently significant suppression of tumor growth was found in a colon cancer C26 model, and more remarkable antitumor effect was achieved when *L. casei* was combined with NG, probably by modulating the host nonspecific immune responses; tumor necrosis factor-α significantly increased in tumor after the treatment, as well as NO synthase activity and myeloperoxidase activity. These findings suggest the potential of *L. casei* as a candidate for targeted bacterial antitumor therapy, especially in combine with NG or other vascular mediators. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3235–3243, 2014

Keywords: EPR effect; *Lactobacillus casei*; nitroglycerin; vascular permeability; ACE inhibitor; Cancer; Nanotechnology; Permeability; Targeted drug delivery; Distribution

INTRODUCTION

Historical experience using bacteria for the therapeutic purpose against cancer goes back to the end of 19 century pioneered by Wiliam Coley, later called Coley's toxin or Coley's vaccine,[1,2] in which *Streptococcus pyogenes* and *Serratia marcescens* were injected into tumor directly. In the recent decade, bacterial therapy as a new anticancer strategy is gaining more attention than ever through systemic administration of bacteria.

Hoffman and coworkers[3,4] reported that intravenous (i.v.) injection of a modified strain of *Salmonella typhimurium* selectively infected tumor tissues and induced significant tumor shrinkage in many tumor models in mice. Taniguchi's group developed tumor-targeted delivery of genetically engineered *Bifidobacterium longum* expressing cytosine deaminase as a prodrug that would trigger the generation of 5-fluorouracil in tumor, resulting in remarkable antitumor effects.[5] Both of these methods are now in clinical trials. Also, by using *Escherichia coli* or *Salmonella enterica serovar Typhimurium*, Xiang et al.[6] successfully developed a tumor-targeted delivery system of short hairpin RNA. In addition, recently many reports have indicated that *Lactobacillus casei* (*L. casei*), a nonpathogenic bacterium widely used in dairy products, exhibits antitumor therapeutic potential by enhancing the cellular immunity of the host.[7,8] All these results suggest that bacterial therapy is a promising approach in cancer treatment, and thus, it is intriguing to analyze bacterial accumulation and growth in tumor tissues.

Regarding the tumor accumulation of bacteria, anaerobic or facultative bacteria have been known for decades to grow selectively in tumors.[3–5,9–11] This growth is now attributed to the unique pathophysiological features found in many tumors, that is, impaired and abnormal vascular architecture, high vascular permeability and hypoxia, or low pO_2, together with extensive necrosis.[3–5,12] In this context, we have been working on tumor selective drug delivery and found that macromolecules above 40 kDa effectively traverse tumor blood vessels permitting their accumulation in tumor tissues.[13–15] This unique phenomenon of biocompatible macromolecules in solid tumor was coined enhanced permeability and retention (EPR) effect, which is attributed to the defective architecture of neovasculature of tumor, as well as various vascular mediators such as nitric oxide (NO) and bradykinin that facilitates the opening of endothelial cell–cell gaps.[12,15,16] We also found the EPR effect occurs even in macromolecules beyond 10^6 Da or nanoparticles as large as 1000 nm, the size of bacteria.[12] More recently we found that the EPR effect could be further augmented by applying nitroglycerin (NG), which becomes NO in hypoxic milieu of tumor, and by angiotensin I converting enzyme (ACE) inhibitor that

Abbreviations used: EPR, effect, enhanced permeability and retention effect; *L. casei*, *Lactobacillus casei*; NG, nitroglycerin; NO, nitric oxide; ACE, angiotensin II converting enzyme.

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suppresses degradation of bradykinin (kinin) thereby resulting in higher kinin content in tumor.[12,17,18]

In view of these findings, it is strongly indicated that delivery of bacteria to tumor and thus bacterial therapeutic could be further enhanced by modulating vascular mediators, that is, NO and bradykinin. We thus examined, in the present study, whether tumor selective delivery of bacteria can be increased by applying NG and ACE inhibitor (enalapril) both of which are commonly used clinically to cause vascular dilatation or antihypertension.

MATERIALS AND METHODS

Materials

Nitroglycerin ointment (Vasolator®) containing 20 mg of NG/g Vaseline® was from Sanwa Kagaku Kenkyusho (Nagoya, Japan) and was used after 10- or 100-fold dilution with Vaseline®. Enalapril was purchased from Elmed Eisai Company, Ltd. (Tokyo, Japan). NO donor 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7) was from Dojindo Laboratories (Kumamoto, Japan). Other chemicals of reagent grade were from Wako Pure Chemical Industries (Osaka, Japan) and were used without further purification.

Bacteria and Cells

Lactobacillus casei strain *Shirota* was kindly from Yakult Honsha Company, Ltd. (Tokyo, Japan) and was cultured in MRS (de Man, Rogosa, Sharpe) medium (Cica; Kanto Chemical Company Inc., Tokyo, Japan). Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose; Wako Pure Chemical Industries) was used during *in vivo* experiments with *L. casei*.

Mouse S-180 sarcoma cells were maintained in ascites of ddY mouse by weekly passage. Colon cancer C26 cells were kindly gift from Dr. Ishima of Kumamoto University (Japan), and were maintained and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, California) at 37°C in an atmosphere of 5% CO_2/95% air

Animal Solid Tumor Models

Male ddY mice of 6 weeks old and female BALB/c mice (6 weeks old) were obtained from Kyudo Inc. (Saga, Japan). All animals were maintained under standard conditions: a 12-h dark/light cycle and a temperature of 23 ± 1°C. Mice were fed water and murine chow *ad libitum*. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Sojo University.

Mouse S-180 sarcoma cells (2×10^6) were implanted subcutaneously (s.c.) in the dorsal skin of ddY mice, to obtain the S-180 tumor model in mice, and cultured colon adenocarcinoma C26 cells were implanted s.c. in the dorsal skin of BALB/c mice as C26 solid tumor model. At 10–15 days after implantation of tumor cells, when the tumors became 8–10 mm in diameter, the following studies were carried out.

Body Distribution of *L. casei* in Murine Solid Tumor with and without NG or Enalapril Treatment

To investigate the biodistribution of *L. casei*, bacteria (7×10^6 CFU, in 0.1 mL culture medium) were injected i.v. via the tail vein in S-180 or C26 mouse solid tumor model, followed by intraperitoneal (i.p.) injection of 1 mL of 20% lactulose. For the NG-treated group, NG ointment (at NG dose of 0.6 mg/tumor) was applied to the skin over the tumors 5 min before the injection of bacteria. For enalapril-treated group, enalapril (10 mg/kg) was given orally 4 h before the injection of bacteria.

At scheduled times (i.e., 1, 6, 24, and 48 h) after the injection of bacteria, mice were killed and blood was collected from the inferior vena cava, and mice were then subjected to reperfusion with 10 mL of physiological saline containing 5 U/mL heparin to remove blood components from the blood vessels of various organs and tissues. Tumor tissues and normal organs and tissues, including the liver, spleen, kidney, heart, and lung, were collected and weighed. To each tissue, nine-time volume of cold physiological saline was added, and then tissues were minced and homogenized on ice with Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland). Tissue homogenates (50 μL) at different dilutions were transferred to 10 cm Petri dishes, and then 15 mL of MRS agar medium kept at 40°C was added and thoroughly mixed. The dishes were then placed at room temperature to solidify the agar medium, after which they were placed in an incubator at 37°C. After 2 days of incubation, *L. casei* colonies were counted. The distribution of bacteria in each tissue was expressed as CFU/g tissue or CFU/mL blood. All experiments were performed duplicate and under sterilized conditions.

In Vivo Therapeutic Effect of *L. casei* by i.v. Injection and its Enhancement by NG

The therapeutic effect of *L. casei* was investigated in the C26 solid tumor model. Ten days after injection of C26 tumor cells in BALB/c mice, when tumor diameters became 5–8 mm, *L. casei* (7×10^6 CFU or 2×10^7 CFU) was injected i.v.; in some experiments, NG ointment (at an NG dose of 0.6 mg/tumor) was rubbed on the skin overlying the tumors just before administration of bacteria. This therapeutic protocol was carried out once a week for 3 times. During the period of experiments, 1 mL of 20% lactulose was i.p. injected daily till 2 days after the last injection of *L. casei*. Tumor volume and body weight of animals were measured, and tumor volume was estimated by measuring longitudinal cross-section (L) and transverse section (W) and applying the formula $V = (L \times W^2)/2$.

Measurement of Myeloperoxidase and NO Synthase Activity in Tumor after *L. casei* Treatment with/without NG

In S-180 solid tumor model, the myeloperoxidase (MPO) activity and iNOS activity in tumor after *L. casei* with/without NG were measured by using the colorimetric MPO activity assay kit (BioVision Inc., Milpitas, California) and a colorimetric NO synthase (NOS) assay kit (Oxford Biomedical Research, Inc., Oxford, Michigan) respectively, according to the manufacturers instructions. In this experiment, NG and/or *L. casei* (2×10^7 CFU) were administered by the protocol as described above but applied once every 2 days, and 1 mL of 20% lactulose was given i.p. daily. Four days after the last injection of bacteria, mice were killed and tumor tissues were collected for the assays.

ELISA for Interleukin-6 and Tumor Necrosis Factor in Serum and Tumor of S-180 Tumor Bearing Mice after *L. casei* Treatment with/without NG

By the same protocol for measurement of MPO and NOS activity, quantifications of cytokines interleukin-6 (IL-6) and tumor necrosis factor (TNFα) were performed. Namely, serum and tumor tissues from S-180 tumor bearing mice with each