部

るとともに

が可能となる．つまり，EPR 効果を利用した DDS 製剤の技術を用いて，光増感剤を腫瘍に集積させ，副作用を軽減させ，治療効果の増大をはかり，癌組織の高感度検出という，全く新しい性質を持つ光線力学的療法剤の開発が可能になる．

2．高分子型光線力学的療法剤について

2．1　亜鉛プロトポルフィリン(ZnPP)

われわれの研究室では以前より，光増感剤として亜鉛プロトポルフィリン(ZnPP)を用いた高分子抗癌剤の研究を行ってきた[2~8]．ZnPP はプロトポルフィリン IX に亜鉛が配位した構造を持つ．ZnPP は光増感剤として機能するのみならず，ヘム酸化酵素(heme oxygenase，HO-1)の阻害剤としても知られる[9]．ヘム酸化酵素は多くの癌細胞において発現亢進が見られる抗酸化酵素であり，HO-1 の機能阻害により，酸化ストレスへの抵抗力を減弱させれば，癌細胞は酸化傷害を受けアポトーシスに至る[10]．つまり，ZnPP は癌[illegible]酸化防御機構を抑制するとともに，光により一重項酸素を発生することで強力に癌細胞に対して酸化傷害を引き起こす

この手法に関する問題点は

ZnPP原体は低分子であり

これに対する問題は，ZnPP の水溶性が[illegible]て低いこと，ならびに他の光増感剤と同様に癌組織への集積性が悪いために，治療薬としては難があった．そこでわれわれは DDS 技術を用いた ZnPP の水溶化と癌組織への集積性の向上を図る必要を認めた．

EPR効果を高分子化により付与し、

研究を行った。

2．2　高分子型亜鉛プロトポルフィリン

ZnPP の問題点に対し，われわれはこれまでにスチレンマレイン酸コポリマー(SMA)またはポリエチレングリコール(PEG)を用い高分子化により，EPR 効果を利用した，腫瘍標的型水溶性 ZnPP 製剤を作製し，その有用性を明らかにしてきた[7,8]．しかし，ZnPP そのものは正常細胞への毒性はほとんどないとはいえ，腫瘍集積性に加え肝臓や脾臓への

し、それ

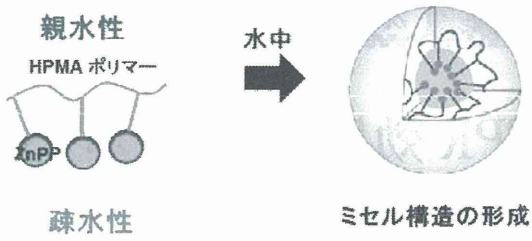

図 1　HPMA-ZnPP の模式図

HPMAポリマー

がやや低すぎるなどの

集積も高く，ZnPP 含有率の低さなどに問題が認められた．その延長線上の研究成果としてわれわれはヒドロキシプロピルメタアクリルアミドポリマー(PHPMA)を用いた高分子 ZnPP 製剤が有用であることを見出した．つまり，PHPMA の側鎖のヒドロキシル基に ZnPP のカルボキシル基を結合した HPMA-ZnPP の作製を行った(図 1)．この HPMA-ZnPP は水中でミセル構造を形成し，高分子化合物として挙動する．さらに ZnPP の含有率を 30%程度まで上げることが可能となり，高い水溶性と腫瘍集積性を合わせ持つ DDS 製剤である．

ポリマー

自分で

易く

ができた.

2．3　光照射による細胞傷害性の獲得

一般に ZnPP などのテトラピロール化合物[illegible]増感作用はよく知られている性質であり，われわれは ZnPP に対して光を照射することで，一重項酸素を発生し，細胞傷害性が大幅に向上することをこれまでに明らかにしてきている．当然のことながら HPMA-ZnPP も光増感作用を有しており，光照射により一重項[illegible]素を発生することを電子スピン共鳴装置[illegible]SR)により確認している(図 2 A，B)．また，一重項酸素の発生は界面活性剤の存在下において顕著に見られ，図 2 B では界面活性剤である Tween 20 存在下において一重項酸素の発生が促進することを示している．さらにわれわれは細胞膜成分であるレシチンも同様の作用を示すことを未発表ではあるが明らかにしており，HPMA-ZnPP が細胞に取り込まれる際に，レシチンの作用により HPMA-ZnPP は一重項酸素を放出できる構造になる

発現する.

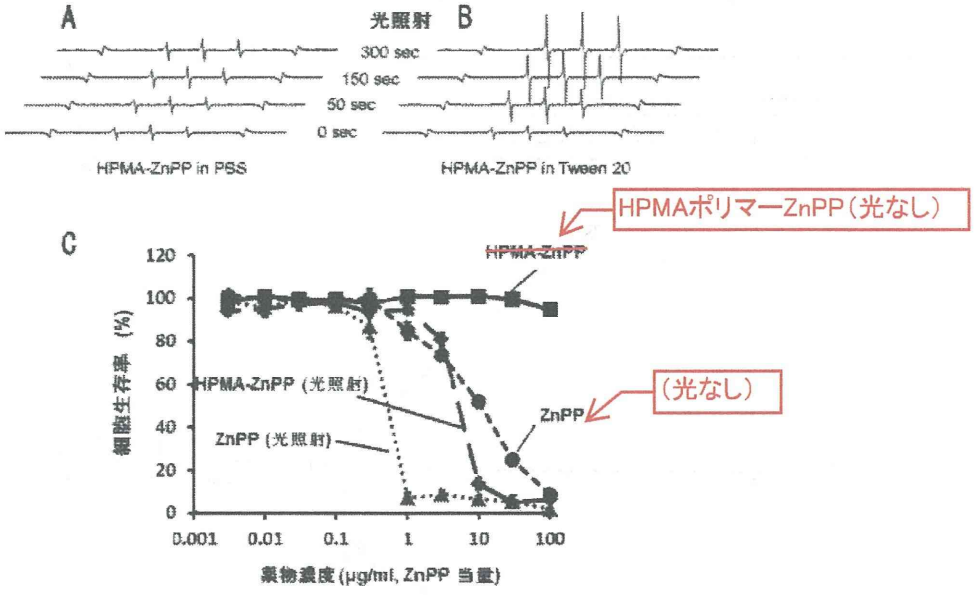

図2　HPMA-ZnPP による一重項酸素の発生と生物活性の増強
光照射による一重項酸素の生成量を PBS (A) または界面活性剤 (B) 中において，電子スピン共鳴 (ESR) 装置を用いて測定した．(C) HeLa 細胞に HPMA-ZnPP または ZnPP を処理し，光を照射した後に細胞生存率を測定した

ことを示唆している．実際に癌細胞に対して HPMA-ZnPP を処理し，光を照射することで，細胞傷害性の大幅な増大が見られ，HPMA-ZnPP が光増感作用を示すことを明らかにした（図 2B）．

ポリマー

2．4　HPMA-ZnPP の薬物動態と光イメージング

HPMA-ZnPP は生体内における薬物動態の改善を目的とした薬剤であり，ZnPP と比較して，大幅な腫瘍集積性の向上と正常組織への分布の抑制が認められる[11]．HPMA-ZnPP が，癌組織に高濃度に集積すること，また光照射により蛍光を発する性質を利用し，癌組織の蛍光イメージングが可能であるかを検討した．S180 担癌マウスに HPMA-ZnPP を投与し，*in vivo* 蛍光イメージング装置（IVIS lumina XR）を用い，蛍光イメージングを行ったところ，癌組織選択的に蛍光が観察された（**図 3A**）．さらに，蛍光フィルターを用いることで，市販のコンパクトカメラでも同様に癌組織の蛍光イメージングが可能であることを明らかにしている（図 3B）．この結果は，

マウスそのままで

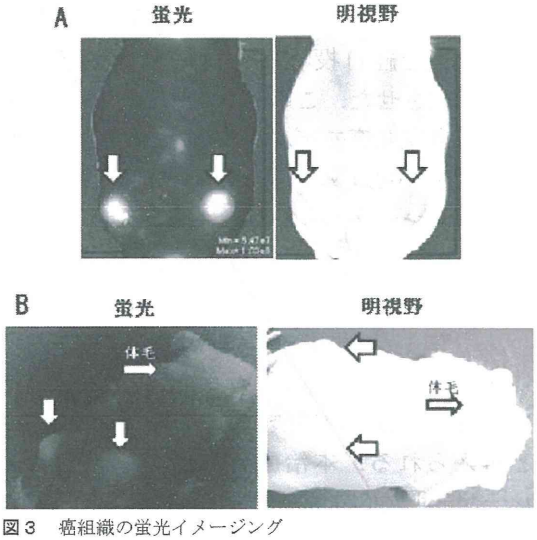

図3　癌組織の蛍光イメージング
S180 担癌マウスに HPMA-ZnPP を投与し，in vivo 蛍光イメージング装置（IVIS Lumina-XR）(A)，または市販のコンパクトカメラ (B) を用いて，腫瘍の画像可視化（蛍光イメージング）を行った。矢印は移植癌を示す．体毛部に非特異的な自家蛍光が認められる

の

内視鏡や腹腔鏡などの一般的な光学機器を用いた体腔内（腹膜，胸壁，肝皮膜，内膀胱，子宮など）癌組織の蛍光検出に応用可能であることを示唆している．リアルタイムな癌組織の蛍光観察が可能になれば，目視では判断

し難い，微小癌に対する光線力学的療法施術時において光照射部位を決定する際に有用な情報を提供することが可能となる．

2．5　HPMA-ZnPP による抗癌効果

In vitro における HPMA-ZnPP の光増感作用に関しては，前記(図３)の光による細胞毒性(殺細胞作用の増強)は，100 μg/ml で無毒のものが，10 μg/ml 以下で 50％の細胞を殺傷するようになる．また，体内動態の改善も認められ，蛍光イメージングにおいても，容易に HPMA-ZnPP が癌組織へ選択的に集積していることが確認された．これまでに，S180 マウス移植癌モデルに対して HPMA-ZnPP の抗癌効果も確認してきた[11]．さらに，ラット化学発癌モデルに関して HPMA-ZnPP の抗腫瘍効果の検討を行ったところ，同様の結果が得られた．この実験モデルにおいては SD ラットにジメチルベンズアントラセン(DMBA)を経口投与することにより，乳腺癌を形成させた．このモデルは移植癌とは異なり，自家発癌モデルであるため，より本物の癌に近いモデルである．HPMA-ZnPPを投与後に，キセノン光を用いて光照射を行ったところ，HPMA-ZnPP 1 回投与，光照射 2 回で，乳腺癌の消失が確認された(**図４**)．しかし，HPMA-ZnPP 単独(光照射なし)では治療効果が見られなかったことから，治療効果は光照射による一重項酸素生成に起因するものと考えられる．本治療においては，高価なレーザー照射装置などを必要とせず，安価なキセノン光ないしは LED 光で十分な効果が期待できる．また，このモデルにおいても HPMA-ZnPP による蛍光イメージングは可能であった(未発表データ)．

ま と め

手術を必要としない，非侵襲的な癌治療法である光線力学的療法は，癌患者にとって優しい治療法である．しかし，既存の光線力学的療法剤は薬物動態が悪いため，副作用が大きな問題となってきた．われわれの作製した HPMA-ZnPP は，癌組織に選択的に集積するため，副作用の問題は解決できると考えられる．さらに，癌の蛍光イメージングが可能であり，癌組織の場所さらには薬剤が集積しているか否かの判断が非常に容易になっている．そのため，光を照射すべき部位が容易に判断でき，効率的な施術が可能となると考えられる．現在は内視鏡や腹腔鏡を用いた光線力学的治療や，癌組織の検出への応用・導出を目指して検討を行っている．

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Research Article

For J Pharm Sci (*Pharmaceutical Nanotechnology*)

Enhanced bacterial tumor delivery by modulating the EPR effect, and therapeutic potential of *Lactobacillus casei*

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Running title: Enhanced bacterial therapeutic effect by modulating EPR effect

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

Abstract

Bacteria of micrometer size could accumulate in tumor based on 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 24h, 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 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 myleoperoxidase 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.

Keywords: EPR effect, *Lactobacillus casei*, Nitroglycerin, vascular permeability, ACE inhibitor

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 *Sereptococcus 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 et al.[3,4] reported that intravenous 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. successfully developed a tumor-targeted delivery system of short hairpin RNA.[6] 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, i.e., 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 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 angiotensin I converting enzyme (ACE) inhibitor that 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, i.e., 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

NG 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 Co., 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

L. casei strain *Shirota* was kindly from Yakult Honsha Co., Ltd. (Tokyo, Japan) and was cultured in MRS (de Man, Rogosa, Sharpe) medium (Cica; Kanto Chemical Co. 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, CA) 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, Faculty of Pharmaceutical Sciences, 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 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, 48h) 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, 9-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 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 weeks. 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 (MPO) and NO synthase (NOS) activity in tumor after L. casei treatment with/without NG

In S-180 solid tumor model, the 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, CA) and a colorimetric NOS assay kit (Oxford Biomedical Research, Inc.,

Oxford, MI) 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 two 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.

Enzyme-linked immunosorbent assay (ELISA) for interleukin-6 (IL-6) and tumor necrosis factor (TNFα) 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 IL-6 and TNFα were performed. Namely, serum and tumor tissues from S-180 tumor bearing mice with each treatment were subjected to ELISA (ELISA kits, R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Effect of sodium nitrite and NOC7 on the growth of L. casei

To investigate the potential effect of nitrite and NO on the growth of *L. casei*, in vitro experiments were carried out using cultured *L. casei*. Namely, *L. casei* was first cultured in MRS agar medium. After 24 h of culture at 37°C, a speck of bacteria was taken from a colony was put into 10 ml of MRS liquid medium and cultured at 37°C with shaking (120 rpm). After overnight incubation, 10 μl of cultured bacteria was transferred into 50 ml of MRS medium, in which different concentrations of sodium nitrite or NO donor NOC7 were added, and the bacteria were cultured continued under the same conditions. The bacterial growth was measured by absorbance at 600 nm every 30 min.

Statistical analysis

All data are expressed as means ± SD. Data were analyzed by one-way analysis of variance followed by the Bonferronit-test. Some studies with two experiments were analyzed by Mann–Whitney U-test, and a Fisher's exact test was used to analyze the data of survival rate. A difference was considered statistically significant when $P < 0.05$.

RESULTS

Body distribution of L. casei in tumor-bearing mice after i.v. injection

At 1h after i.v. injection of *L. casei* in S-180 tumor-bearing mice, most bacteria were found in liver and spleen (Fig. 1A). However, bacterial counts in normal tissues (e.g., liver and spleen) at 6h after injection significantly decreased to about 1/10 of those at 1 h, whereas the numbers of bacteria in the tumor significantly increased; it increased about 80 times (Fig. 1B). And at 24h, almost no living bacteria could be detected in normal tissues including liver and spleen (Fig. 1C), whereas, the bacteria in tumor have increased gradually with time; namely at 24h the bacteria in tumor were significantly higher (more than 50 times) than those in normal

tissues (Fig. 1B, C, Fig. 2C). These findings suggested that accumulation of bacteria in tumor tissues was due to an EPR effect that was a time-dependent phenomenon requiring more than several hours (e.g., >4 h in mice; Ref 15). Similar results were obtained when C26 solid tumor model was used (Figs. 1D-F, Fig. 2F). These data are consistent with previous report using *Bifidobacterium bifidum*.[11]

Enhancement of bacteria tumor delivery by NG

We then investigated the potential effect of NG on bacteria tumor delivery. NG was applied to the tumor-bearing mice just before i.v. injection of *L. casei*. As seen in Fig. 2A-C, NG significantly augmented the delivery of bacteria to tumor tissues: approximately 70-fold, 20-fold and 10-fold increases were found at 1h, 6h and 24h, respectively, after NG treatment. However, no apparent increase of bacteria accumulation was found in normal tissues including the liver and spleen (Fig. 2), which suggests that NG was converted to NO predominantly in tumor tissues. Similar results were also found in C26 tumor-bearing mice (Figs. 2D-F).

Because NO shows effects on cell proliferation and apoptosis,[19] we further investigated whether the accumulation and growth of bacteria in tumor is partly due to the effect of NO from NG, the effect of NO on *L. casei* growth was examined in an in vitro bacteria culture system using sodium nitrite as well as NO donor NOC-7. The results showed that both sodium nitrite (0.1-100 μg/ml) and NOC-7 (0.23-230 μM) did not affect the growth of *L. casei* (supplemental data Fig. S1), suggesting the accumulation/growth of bacteria in tumor is mostly due to the EPR effect and tumor microenvironments.

Enhancement of bacteria tumor delivery by enalapril

Similarly, the effect of ACE inhibitor enalapril on the bacterial accumulation in tumor was evaluated. According to the clinical pharmacokinetic profile of enalapril, it was administered 4h before i.v. injection of *L. casei*. As shown in Fig. 3A-C, remarkable increase of tumor delivery of bacteria was also observed by enalapril: approximately 18-fold, 9-fold and 6-fold increases were found at 1h, 6h and 24h after enalapril treatment, respectively. Similar results were also found in C26 tumor-bearing mice (Figs. 3D-F)

Bacterial antitumor effect and survival benefit of L. casei with NG

The therapeutic effect of i.v. administration of *L. casei* in the presence or absence of NG was investigated in C26 solid tumor model. As Fig. 4 demonstrates, i.v. injection of *L. casei* showed a dose-dependent antitumor effect; at the dose of 2×10^7 CFU weekly for 3 times a significant delay of tumor growth was achieved (Fig. 4A). NG at the dose of 0.6 mg did not significantly inhibited the tumor growth, however a more remarkable antitumor effect was obtained when *L. casei* was combined with NG (Fig. 4A). During this experiment, a slight

but not significant decrease of body weight was found at the beginning of treatment in *L. casei* (2×10^7 CFU) treatment and *L. casei*/NG combination group, the body weight recovered later and continued to grow, at the end of the experiment, the body weights of mice in these groups were not apparently different to the untreated control group judging from the weight of tumor in each group (Fig. 4B), suggesting no severe side effects occurred in these treatment protocols.

Moreover, the survival rate of tumor-bearing mice was significantly improved after each treatment, especially in the *L. casei*/NG treatment group in which the survival times of animals were prolonged almost double (median survival time: 40 days of untreated control vs 79 days of *L. casei*/NG group) (Fig. 4C).

Induction of TNFα and NOS activity and other inflammatory factors in tumor by L. casei/NG treatment

To elucidate the antitumor mechanisms of *L. casei*/NG treatment, various cytokines or factors which may possibly involved in the host defense and inflammation and antitumor responses, i.e., TNFα, MPO, NOS, IL-6 and MCP-1 were examined after each treatment. As seen in Fig. 5, no change of IL-6 and MCP-1 was found under each treatment both in serum and in tumor tissues (Figs. 5D-F). For TNFα, its levels in serum of tumor-bearing mice were below the detection limit in all groups. In contrast, TNFα was increased in tumor in all *L. casei* treated groups, and a significant difference was seen for *L. casei*/NG group vs untreated control (Fig. 5A). Significant increase of NOS activity in tumors was also seen in all the treated groups, especially *L. casei*/NG group (Fig. 5B). MPO activities in tumors did not change in *L. casei* alone and NG alone group respectively, but a tendency of increase of MPO activity was seen in *L. casei*/NG group (Fig. 5C).

DISCUSSION

In the present study, we showed data that indicating a new antitumor strategy by systemic administration of *L. casei*, i.e., bacterial therapy. *L. casei* is a nonpathogenic facultatively anaerobic bacterium that is also a component of the normal bacterial flora in human intestinal tract and reproductive system. *L. casei* is now widely used as a probiotic in various dairy and food supplements. More important, it has been known for decades that it could stimulate nonspecific immune responses, such as macrophage and NK cell activation to fulfill antitumor activity,[20-23] and thus it is considered useful as a medication to prevent recurrence of bladder cancer,[7,8,24,25] like Bacille Calmette-Guérin (BCG) as an immune modulator.[26]

Most previous studies using *L. casei* to challenge cancer were through oral route or i.p. injection. These administration routes ensure the immune stimulation of whole body of the

host, however, no tumor selectivity could be achieved. Because *L. casei* is a nonpathogenic bacterium, systemic i.v. application is possible with very little adverse effects such as sepsis. We thus hypothesized that *L. casei* can be delivered selectively in tumor after i.v. injection according to the EPR effect, consequently achieve tumor-targeted therapeutic effect. As expected, we found tumor-selective accumulation and growth of the bacteria after i.v. injection, without accumulation of bacteria in normal tissues (Fig. 1). Although *L. casei* accumulated mostly in the liver and spleen, both are rich in reticuloendothelial system, at 1 h after i.v. injection, these amounts decreased dramatically after 6 h; and at 24 h, the numbers of living bacteria in tumor tissue were far greater (i.e., 50 times) than those in the liver and spleen (Fig. 1), indicating *L. casei* could not survive and grow in normoxic normal tissues even it is uptaken. These results are similar with previous studies using different bacteria,[3-5, 9-11] and we believe this tumor-targeted delivery and enhanced growth is mostly due to the EPR effect and the hypoxic microenvironment of tumors that is seen in most solid tumors.[12] Namely, EPR effect drives the accumulation/delivery of bacteria into tumor, and then the favorable tumor environment ensures the growth of bacteria.

EPR effect is a unique phenomenon caused by the anatomical and pathophysiological features of tumor blood vessels, and many vascular mediator such as NO and bradykinin are involved in EPR effect as noted above.[12,15,16] We thus developed approaches to enhance the EPR effect by focusing and utilizing these mediators. One successful and interesting example is the use of NG, a well-known NO donor that has been used for more than a century as a medication for angina pectoris. NG selectively liberates NO_2^- first which is then converted to NO by nitrite reductase under hypoxic conditions in cardiac infarcted tissue.[27,28] The low pO_2 and slightly acidic pH in cardiac infarcted tissue are similar to conditions in many tumor tissues.[29] Thus NG is an ideal agent to enhance the EPR-driven drug tumor delivery. Our previous studies clarified the enhancement of EPR effect by NG with remarkable results in various rodent tumor models, by using polymer conjugates and putative macromolecular agents.[17] Along this line, the NG-enhanced EPR effect was also seen in the present study using bacteria; the numbers of live bacteria delivered into tumor were markedly increased, e.g., 70 folds, after NG treatment, whereas no significant changes of bacteria in normal tissues including the liver and spleen (Fig. 2). Because NO seemed no effects on bacteria proliferation (Supplement data Fig. S1), the effect of NG/NO on bacterial tumor accumulation is believed to be mostly due to the enhanced EPR effect. In addition, ACE inhibitor enalapril has been known for long time to induce tumor vascular permeability.[30] This notion was also supported in this study, more than 10-time of bacterial tumor accumulation was achieved with the use of enalapril (Fig. 3). These data suggested NG and

enalapril, both are clinically used safe drugs, are potentially useful as enhancer of macromolecular anticancer drug delivery as well as bacterial therapy.

According to the EPR-based tumor-targeted bacterial delivery, we hypothesized *L casei* selectively accumulated in tumor will induce tumor-specific immune activation and thereby have an antitumor effect, which is realized by systemic i.v. injection, but not topical application like BCG for bladder cancer. The results clearly supported our hypothesis; significant suppression of tumor growth by i.v. administration of *L. casei* in C26 colon tumor model, with significantly increased survival rate of the mice (Figs. 4A, C). Moreover, this antitumor effect was further significantly augmented by combining with NG (Figs. 4A, C). As NO is also known to show tumor-suppressive effect probably acting by down-regulating the expression of certain critical genes involved in tumor growth,[17,31,32] the effect of NG treatment per se was also examined, and we did not find significant delay of tumor growth under this treatment regimen with the dose of 0.6 mg/tumor (Fig. 4A). We thus believed the enhanced antitumor effect of *L. casei*/NG is mostly the consequence of enhanced bacterial tumor drug delivery. More important, because of the high tumor selective delivery of *L. casei* by i.v. injection, this administration would cause very little side effects in normal tissues and organs, as seen in Fig. 4B. When we examined the inflammatory and antitumor cytokines after these treatments, we found no effects on serum levels of inflammatory cytokines including IL-6, MCP-1 and TNFα, whereas significant increases of immune responses such as TNFα and NOS activity were found in tumor tissues (Fig. 5). These profiles further suggested the targeted tumor suppressing effect, potential applicability and safety for clinical setting of this bacterial therapy using *L. casei.*

Regarding the antitumor mechanisms of *L. casei*, the common notion is immune modulation triggered by *L. casei* as described above. Namely, significant increases of TNFα and NOS activity in tumor were seen after L. casei/NG treatment (Figs. 5A, B), as well as the increase of MPO activity though no significant difference was shown (Fig. 5C). These findings suggested the present bacterial therapy mostly rely on the activation of antitumor nonspecific or innate immune responses, i.e., upregulation of TNFα, macrophage and NK cell activation and the subsequently generated NO as well as other oxidative free radicals such as superoxide and peroxynitrite.

In addition, we previously reported bacterial proteases such as serratial proteases exhibited potent antitumor effect when administered into the tumor.[33-35] Bacterial proteases have no effective inhibitor in the plasma of mammals, however, they from a transitory complex with α_2 macroglobulin in the plasma which is effectively transported into the tumor cells via α_2 macroglobulin receptor that is highly expresses in tumor.[34] In this context, *L.*

casei may exhibit antitumor effect through secretion of bacterial proteases, which need further investigations.

In conclusions, we reported here that i.v. injection of *L. casei* resulted in selective accumulation/growth of bacteria benefiting from the EPR effect as well as specific tumor environments. The tumor delivery of bacteria could also be further significantly improved by NO donor NG and ACE inhibitor enalapril (bradykinin potentiator) that affect vascular tone involved in the EPR effect. Consequently, remarkable antitumor effect was achieved through the antitumor host responses induced by *L. casei* accumulated in tumor, especially when *L. casei* was combined with NG, with no apparent adverse effects. These data suggested the potential of *L. casei* as a candidate for bacterial antitumor therapy, moreover, it could also be a useful drug delivery system to carry or deliver genes or antitumor nanoparticles.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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Figure legends

Figure 1. Body distribution of live *L. casei* in sarcoma S-180 (A, B, C) and colon cancer C26 (D, E, F) tumor-bearing mice after i.v. injection. To S-180 or C26 solid tumor-bearing mice, *L. casei* (7×10^6 CFU) were injected i.v.. After indicated times, the mice were killed and plasma and each tissue were collected. The live bacteria in each tissue were measured by culturing the tissue homogenates and counting the colonies formed in the medium. Data are given as means ± SD; $n = 6$. $^{***}P < 0.005$ vs normal tissues. See text for details.

Figure 2. Enhancement of tumor delivery of *L. casei* by NG. A, B and C show the results in S-180 tumor model, and D, E, F shows those from C26 tumor model. Administration of *L. casei* was carried out as same protocol as that shown in Fig. 1, and NG was applied into the skin over the tumor at the dose of 0.6 mg/mouse, 5 min before i.v. injection of *L. casei*. In A and D, the insets show the enlarged scales for tumor. In C and F, time courses of accumulation of *L. casei* in tumor and liver with/without NG treatment were shown. Data are as means ± SD; $n = 6$. $^{**}P < 0.01$ (no NG control vs NG treatment group). See text for details.

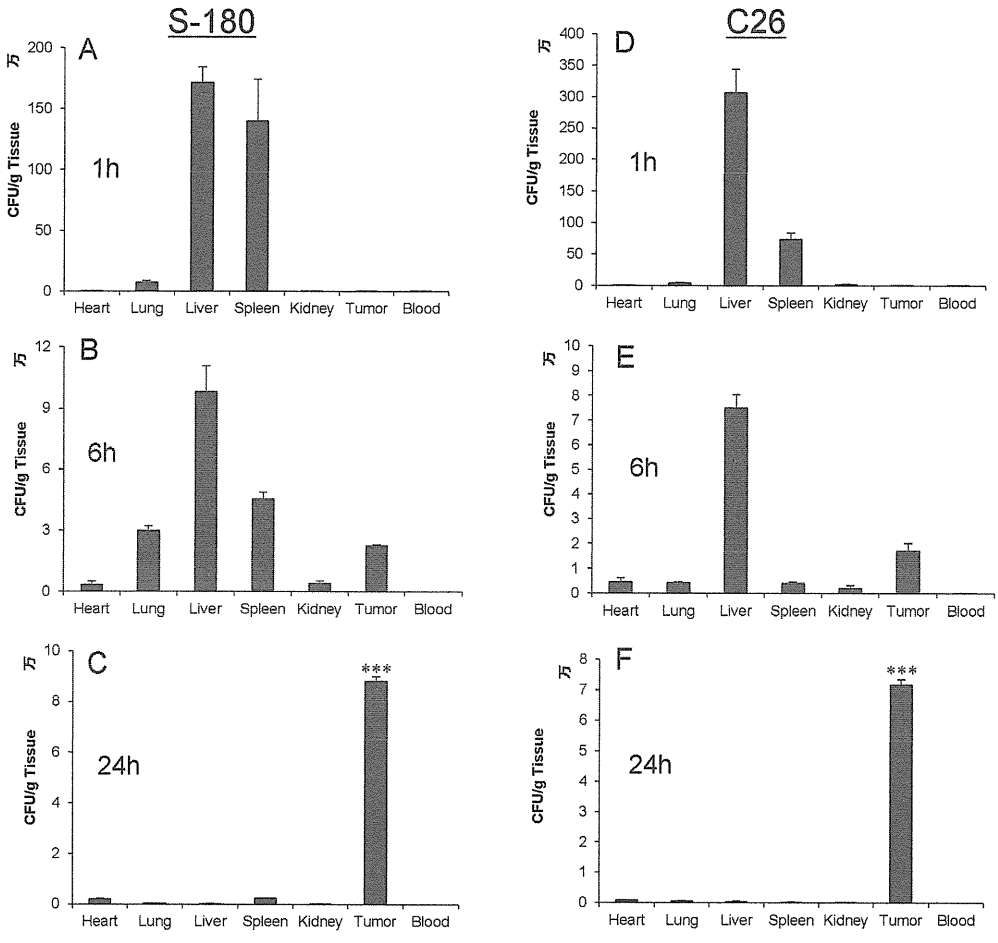
Figure 3. Enhancement of tumor delivery of *L. casei* by enalapril. A, B and C show the results in S-180 tumor model, and D, E, F shows those from C26 tumor model. Administration of *L. casei* was carried out as same protocol as that shown in Fig. 1, and enalapril (10 mg/kg) was given orally 4h before i.v. injection of *L. casei*. In A and D, the inlets show the enlarged results in tumor. In C and F, time courses of accumulation of *L. casei* in tumor and liver with/without enalapril treatment were shown. Data are as means ± SD; $n = 6$. $^{**}P < 0.01$ (no enalapril control vs enalapril treatment group). See text for details.

Figure 4. *In vivo* antitumor effect of *L. casei* after i.v. injection with/without NG in 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* 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. Arrows indicate the injection of *L. casei*. A, antitumor effect of each L. casei with/without NG; B, body weight changes of tumor-bearing mice after each treatment; C, survival rate of tumor-bearing mice after each treatment. L.C (L), treatment with 7×10^6 CFU of bacteria; L.C (H), treatment with 2×10^7 CFU of bacteria. Data are given as means ± SD; $n = 6$-12. $^{*}P < 0.05$, $^{**}P < 0.01$; in C, $P < 0.05$: NG alone vs control,

L.C (H) alone vs control; $P < 0.01$: L.C (H) + NG vs control. See text for details.

Figure 5. Effect of *L. casei* and/or NG on MPO and NOS activity in tumor and the production of antitumor, anti-inflammatory cytokines. In S-180 solid tumor model, NG (0.6 mg/mouse) and/or *L. casei* (2×10^7 CFU) were administered similarly to the treatment protocol described in Fig. 4, but applied once every two days. Ninety-six hours after the last injection, mice were killed and plasma and tumor tissue were collected for each assay. A, B, and C show the TNFα levels, NOS activity and MPO activity in tumor respectively; D and E show the IL-6 levels in plasma and tumor after each treatment respectively; F and G show the MCP-1 levels in plasma and tumor after each treatment respectively. Data are given as means ± SD; n = 4-5. $^{**}P < 0.01$, $^{***}P < 0.005$. See text for details.

Fig. 1


S-180
C26
A
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1h
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Heart
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Kidney
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Blood
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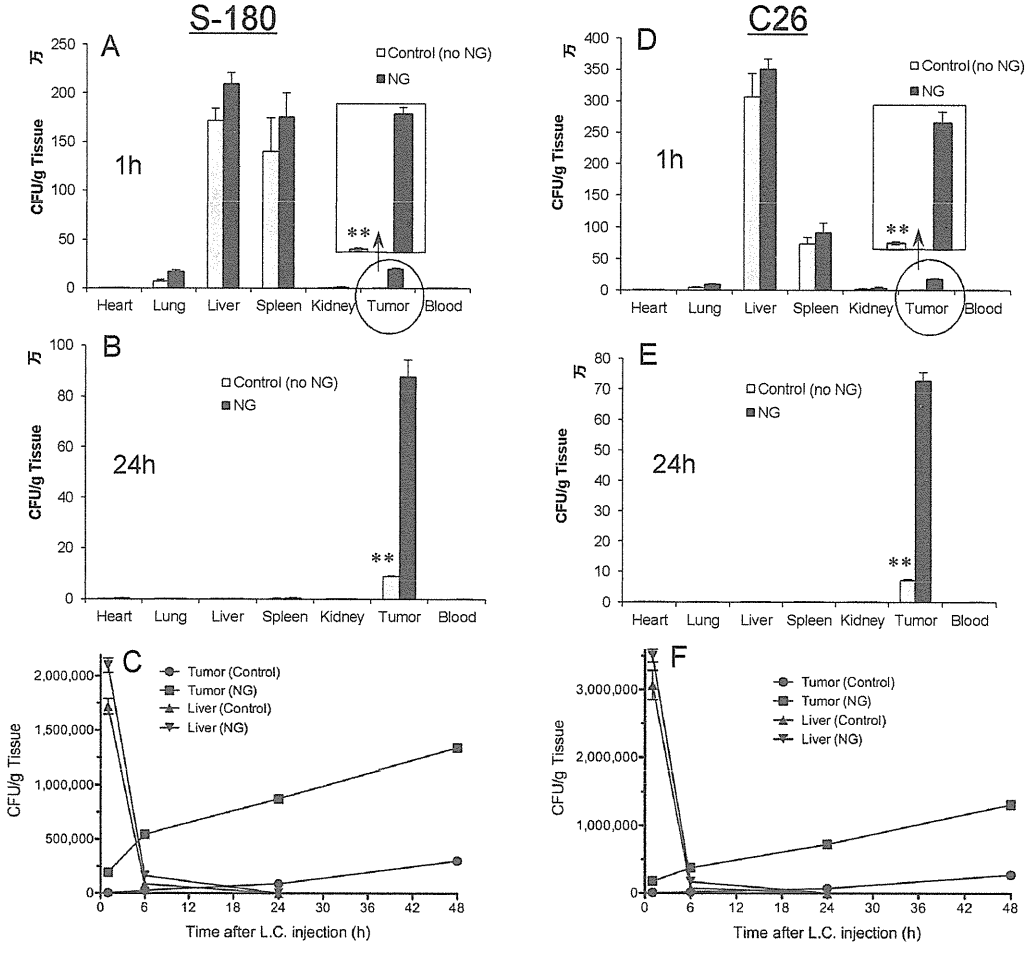
Fig. 2


S-180
C26
A
B
C
D
E
F
1h
24h
Control (no NG)
NG
**
Tumor (Control)
Tumor (NG)
Liver (Control)
Liver (NG)
CFU/g Tissue
Time after L.C. injection (h)