

# Chapter 2

## Enhancement of Tumor-Targeted Delivery of Bacteria with Nitroglycerin Involving Augmentation of the EPR Effect

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### Abstract

The use of bacteria, about 1  $\mu\text{m}$  in size, is now becoming an attractive strategy for cancer treatment. Solid tumors exhibit the enhanced permeability and retention (EPR) effect for biocompatible macromolecules such as polymer-conjugated anticancer agents, liposomes, and micelles. This phenomenon permits tumor-selective delivery of such macromolecules. We report here that bacteria injected intravenously evidenced a property similar to that can of these macromolecules. Bacteria that can accumulate selectively in tumors may therefore be used in cancer treatment.

Facultative or anaerobic bacteria will grow even under the hypoxic conditions present in solid tumors. We found earlier that nitric oxide (NO) was among the most important factors that facilitated the EPR effect via vasodilatation, opening of endothelial cell junction gaps, and increasing the blood flow of hypovascular tumors. Here, we describe the augmentation of the EPR effect by means of nitroglycerin (NG), a commonly used NO donor, using various macromolecular agents in different tumor models. More importantly, we report that NG significantly enhanced the delivery of *Lactobacillus casei* to tumors after intravenous injection of the bacteria, more than a tenfold increase in bacterial accumulation in tumors after NG treatment. This finding suggests that NG has a potential advantage to enhance bacterial therapy of cancer, and further investigations of this possibility are warranted.

**Key words** EPR effect, Nitroglycerin, Nitric oxide, *Lactobacillus casei*, Macromolecules, Solid tumors

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### 1 Introduction

Extravasation of biocompatible macromolecules including polymer–drug conjugates, micelles, and liposomes as well as bacteria about 1  $\mu\text{m}$  in size is observed in most solid tumor tissues. This phenomenon was named the enhanced permeability and retention (EPR) effect of macromolecules in solid tumors [1]. The EPR effect is becoming a gold standard in the design of macromolecular anticancer drugs and tumor-targeted drug delivery systems [2]. The EPR effect is now known to play a major role in tumor-selective delivery of macromolecular drugs, so-called nanomedicines [2–5].

In experimental tumor models, nanomedicines had 5–100 times greater intra-tumoral drug delivery compared with delivery of the drugs to the blood or normal tissues [4, 5]. Nanomedicines with a molecular weight greater than 50 kDa commonly exhibit the EPR effect, but the effects of molecular weight higher than 800 kDa are poorly understood. Kimura et al. [6] and Hoffman et al. [7], however, reported that some bacteria are preferentially taken up in solid tumors, even bacteria injected intravenously (i.v.).

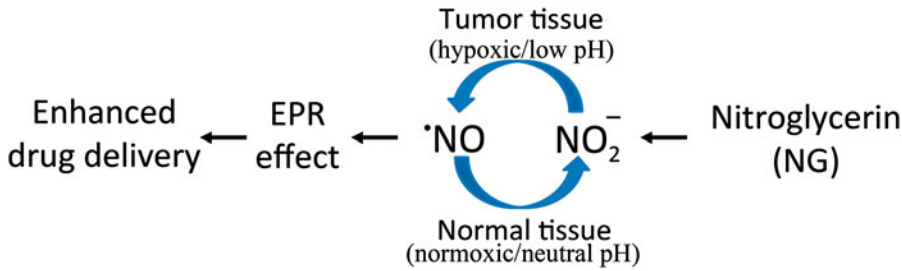
Anaerobic or facultative bacteria have been known for decades to grow selectively in tumors [6–20]. This growth is now attributed to the unique patho-physiological feature found in many tumors, i.e., impaired and abnormal vascular architecture. Consequently, tumor tissues have high vascular permeability (the EPR effect) and hypoxia, or low  $pO_2$ , together with extensive necrosis [5, 7, 18, 20]. In addition, vascular mediators such as nitric oxide (NO) are produced in excess [3–5].

A new anticancer strategy using bacteria was developed and is growing as an attractive option. Hoffman et al. [7, 18] reported that systemic infusion of a modified strain of *Salmonella typhimurium* selectively infected tumor tissues and resulted in significant tumor shrinkage in many tumor (xenograft) models in mice. Taniguchi's group developed tumor-targeted delivery of a prodrug by using genetically engineered *Bifidobacterium longum* expressing cytosine deaminase that would enable the tumor to generate 5-fluorouracil, with remarkable antitumor effects [20]. Both of these methods are now in clinical trials.

Also, Xiang et al. successfully utilized *Escherichia coli* or *Salmonella enterica* serovar typhimurium as a tumor-targeted delivery system to introduce short hairpin RNA into tumor cells that can exhibit RNA interference [19].

In addition, many reports have indicated the anti-tumor therapeutic potential of *Lactobacillus casei*, a non-pathogenic bacterium widely used in dairy products that has enhanced the cellular immunity of the host [21–23]. All these results suggest that bacterial therapy is a promising approach in cancer treatment, and thus, as described in this chapter, the augmentation of bacterial tumor delivery is of great importance.

That the EPR effect is mediated by NO and many other vascular mediators including bradykinin, prostaglandins, and vascular endothelial growth factor is important, and modulating these factors to enhance the EPR effect and achieve tumor-targeted delivery of drugs or bacteria will be critical [5, 24–26]. Among these factors, NO is one of the most important molecules having a vasodilating effect and facilitates increased blood flow as well as increases vascular permeability where it is generated, particularly in tumor tissues [24–26]. As an NO donor, nitroglycerin (NG) has been used for more than a century as a medication, applied topically or orally, for angina pectoris. That NO is generated more in diseased



**Fig. 1** Hypothetical mechanism of nitric oxide (NO) generation. NO was produced from nitrite, mainly in hypoxic tumor tissues instead of normal tissues. *EPR*, enhanced permeability and retention (from Ref. 30 with permission from the Japanese Cancer Association)

hypoxic tissues, via conversion of nitrite, is of great interest [27–31]. We previously demonstrated that NG was converted to NO in tumor tissues, which exist in a hypoxic state, by mechanisms similar to those operating in infarcted cardiac tissues [28–31]. NG may thus be an ideal NO donor for hypoxic tumors. Figure 1 shows the theoretical mechanism of NO generation from nitrite in tumor tissues and in infarcted cardiac tissues, given that both tissues are similarly hypoxic and acidic.

In view of these data, we previously developed the therapeutic strategy of using topical application of small doses of NG, particularly in combination with macromolecular anti-cancer drugs [30, 31]. More recently, we found that bacteria given by i.v. injection also exhibited enhanced tumor-targeted delivery to a significant extent and with much less delivery of bacteria to normal tissues and organs.

## 2 Materials

NG ointment (Vasolator®, Sanwa Kagaku Kenkyusho, Nagoya, Japan).

Evans blue dye (Wako Pure Chemical Industries, Osaka, Japan).

7,12-Dimethylbenz[*a*]anthracene (DMBA) (Wako Pure Chemical Industries, Osaka, Japan).

Corn oil (Wako Pure Chemical Industries).

Protoporphyrin IX (Sigma-Aldrich, St. Louis, MO, USA).

Succinimidyl derivative of polyethylene glycol (PEG) (NOF Inc., Tokyo, Japan).

*L. casei* strain Shirota (Yakult Honsha Co., Ltd., Tokyo, Japan).

MRS (de Man, Rogosa, Sharpe) medium (Cica; Kanto Chemical Co. Inc., Tokyo, Japan).

Lactulose (4-*O*- $\beta$ -D-galactopyranosyl-D-fructofuranose) (Wako Pure Chemical Industries).

Female Sprague–Dawley rats (5 weeks old) (SLC, Shizuoka, Japan).

Male ddY mice (6 weeks old) (SLC).

Female BALB/c mice (6 weeks old) (SLC).

Male C57BL/6 J mice (6 weeks old) (SLC).

Mouse S-180 sarcoma cells ( $2 \times 10^6$ ).

Mouse fibrosarcoma Meth-A cells ( $2 \times 10^6$ ).

Colon adenocarcinoma C38 cells ( $2 \times 10^6$ ) (Riken Cell Bank, Tsukuba, Japan).

### 3 Methods

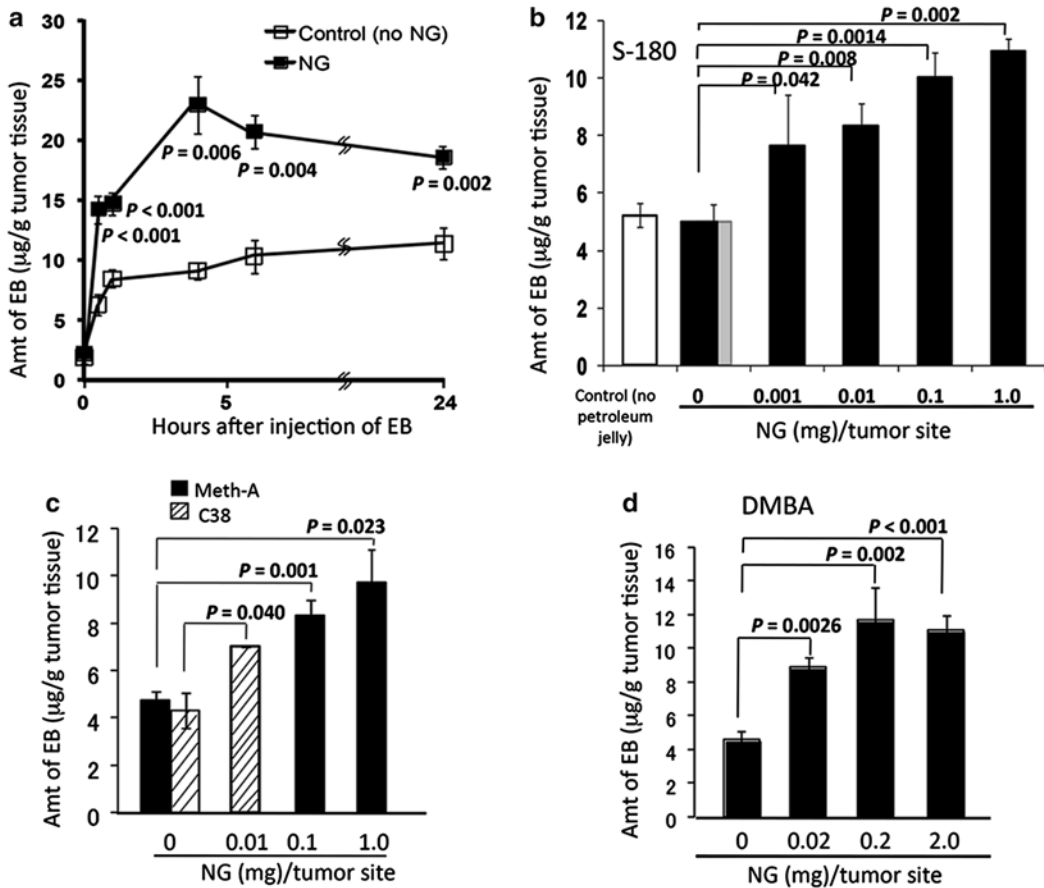
#### **3.1 Delivery of a Putative Macromolecular Drug (Evans Blue/Albumin Complex) in Combination with NG to Rodent Tumors**

When the diameters of S-180 tumors (*see* **Notes 1** and **2**) reached 5–8 mm, NG (*see* **Note 3**) was applied as an ointment to the skin overlying the tumors at doses of 0.001–1.0 mg/tumor, or to normal skin. Within 5 min, 10 mg/kg Evans blue (*see* **Note 4**) in 0.1 mL PBS was injected i.v. At scheduled times thereafter, mice were killed, tumors and normal tissues were removed, weighed, and immersed in 3 mL of formamide followed by incubation at 60 °C for 48 h in order to extract the dye (Evans blue). The concentration of the dye in each tissue was determined spectrophotometrically at 620 nm. Controls in all experiments consisted of treatment with ointment without NG. Similar experiments were carried out in Meth-A, C26 and C38 tumor models (*see* **Notes 1** and **2**) in mice as well as in the DMBA-induced breast tumor model in rats (*see* **Note 2**).

Results showed a time-dependent increase in accumulation of Evans blue/albumin complex in S-180 solid tumors (Fig. 2a,  $P=0.006$ ). NG induced two- to threefold greater drug delivery to solid tumors at 4 h after drug injection than treatment without NG (Fig. 2a,  $P=0.002$ ). Also, tumors retained the higher drug concentration for at least 24 h after NG treatment. Similar results were observed with other solid tumors (Meth-A, C38, and DMBA-induced breast cancer) (Fig. 2c, d). In addition, all tumor models showed NG dose-dependent increases in drug delivery (the EPR effect) (Fig. 2b–d). The effective NG doses were as low as 0.001 mg/tumor up to 2 mg/tumor.

#### **3.2 Macromolecular Drug Delivery to Solid Tumors as Measured by Radioactivity of $^{65}\text{Zn}$ -labeled PZP**

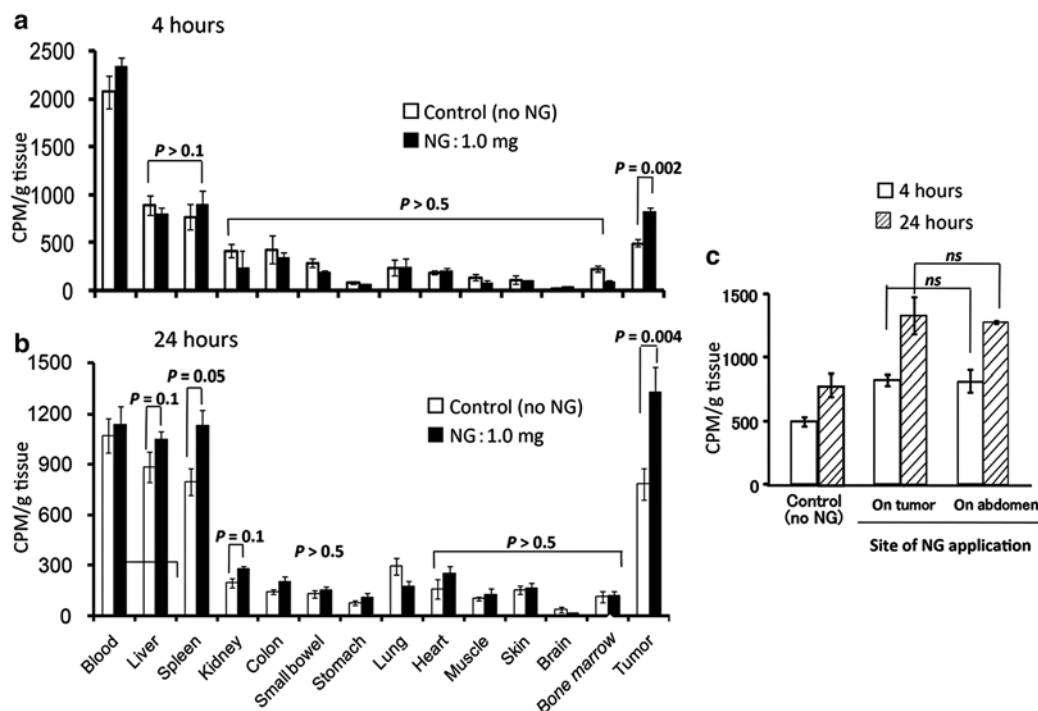
NG ointment at 0.1 mg/tumor was applied to the skin by rubbing over S-180 tumors or to non-tumorous abdominal skin, with the distance to tumors of about 5 cm, after which mice immediately received an i.v. injection of  $^{65}\text{Zn}$ -labeled PZP (*see* **Note 5**) via the tail vein (12,000 cpm/mouse). After 4 and 24 h, mice were killed and the blood was collected from the inferior vena cava. Mice were then perfused with 10 mL saline containing 5 U/mL heparin to



**Fig. 2** Time-dependent and nitroglycerin (NG) dose-dependent enhancement of macromolecular drug delivery to solid tumors. **(a)** Time course of enhanced delivery of Evans blue (EB) to S-180 solid tumors by NG ointment (1 mg/tumor) applied to the skin overlying tumors. **(b–d)** Dose-dependent enhancement of delivery of the macromolecular drug to S-180, Meth-A, C38, and 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast tumors, respectively. In all models, at tumor diameters of 5–8 mm, 10 mg/kg Evans blue in 0.1 mL PBS was injected intravenously; 4 h later the Evans blue concentration in tumors was quantified. Error bars indicate 95 % confidence intervals. Differences between control (no NG) and NG groups were compared with the Student's *t*-test. Statistical tests were two sided. (from Ref. 30 with permission from the Japanese Cancer Association)

remove blood components from the blood vessels in various organs and tissues. Tumor tissues and normal organs and tissues, including the liver, spleen, kidney, intestine, stomach, colon, heart, brain, lung, skin, muscle, and bone marrow, were collected and weighed. Radioactivity of the samples was measured by using a gamma counter (1480 Wizard 3"; PerkinElmer Life Sciences, Boston, MA, USA).

Similar to the findings with Evans blue as shown in Fig. 2, NG application resulted in about twice the accumulation of PZP in tumors than did ointment without NG, at both 4 and 24 h after drug injection (Fig. 3a, b). This result was also found when NG was applied to the skin at a distance of 5 cm from the tumor (Fig. 3c).



**Fig. 3** Nitroglycerin (NG)-enhanced delivery of a radioactive polymeric drug to S-180 solid tumors. NG, in an ointment, was applied to tumor-bearing mice at a dose of 1.0 mg/tumor either to the skin overlying tumors (**a, b**) or to non-tumorous dorsal abdominal skin, on the opposite side of the tumor (the distance to the tumor was about 5 cm) (**c**).  $^{65}\text{Zn}$ -labeled PZP (12,000 cpm/mouse) was injected intravenously via the tail vein into these mice. After 4 h (**a**) and 24 h (**b**), mice were killed, after which samples of the blood, tumor, and normal tissues and organs were collected, and the radioactivity of these tissues and organs was measured. Error bars show 95 % confidence intervals. Differences between control (no NG) and NG groups were compared with the Student's *t*-test. Statistical tests were two sided. CPM, counts per minute; *ns*, not significant. (from Ref. 30 with permission from the Japanese Cancer Association)

The enhancement of the EPR effect by NG for delivery of macromolecular drugs to tumors was significant ( $P = 0.002$  and  $0.004$  at 4 and 24 h, respectively), but findings for most normal organs were not significant, except at 24 h in the spleen and liver ( $P = 0.05$  and  $0.1$ ), which evidenced 15–30 % increased delivery (Fig. 3a, b). Another interesting and important finding was that application of NG to abdominal skin—while the tumor was located on the dorsal skin—produced significantly increased delivery of PZP to the tumor (Fig. 3b, c) but no significant changes in most normal tissues, which suggests that production of NO from NG is tumor-specific, probably because of the hypoxic environment.

### 3.3 Tumor-Targeted Delivery of *L. casei* and Its Augmentation by NG

#### 3.3.1 Culture and Quantification of Growth of *L. casei*

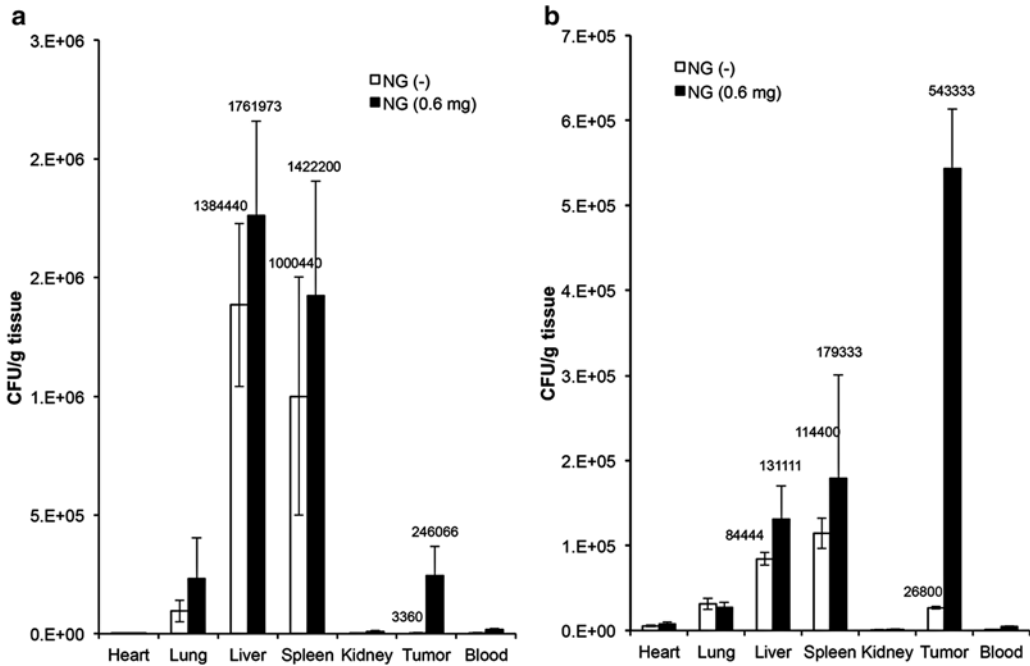
*L. casei* (see Note 6) was first cultured in MRS agar medium in an agar plate—10 cm ( $\Phi$ ) plastic Petri dish. After 24 h culture at 37 °C, one loop of bacteria, taken from a colony on the agar plate, was placed for additional culture into 10 mL of MRS liquid medium in a 50-mL flask incubated in a water bath at 37 °C with shaking (120 rpm). After overnight incubation, 10  $\mu$ L of cultured liquid containing the bacteria was transferred into 100 mL of MRS medium in a 300-mL flask, and cultured under the same conditions (37 °C water bath with shaking). The optical density of the cultured bacteria at 600 nm was measured every 30 min. A 10- $\mu$ L aliquot of the culture was plated on an agar plate followed by incubation at 37 °C to obtain CFU counts after 2 days of incubation. The counts were correlated with optical density.

#### 3.3.2 Pharmacokinetics and Body Distribution of *L. casei* in ddY Mice Bearing S-180 Solid Tumors With and Without NG Treatment

To investigate the bio-distribution of *L. casei*, 0.1-mL samples ( $7 \times 10^6$  CFU) of bacterial culture were injected via the tail vein, followed by i.p. injection of 1 mL of 20 % lactulose [6]. For the NG-treated group, NG ointment at 0.6 mg/tumor was applied by rubbing over the skin just before the injection of bacteria. At scheduled times (1 and 6 h) after the injection of bacteria, mice were killed and the blood was collected from the inferior vena cava, and mice were then perfused with 10 mL 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 1 g of each tissue, 9 mL of cold physiological saline were added, and then tissues were minced and homogenized on ice with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland). Tissue homogenates (50  $\mu$ L) at different dilutions (10–10,000, obtained by using physiological saline) were transferred to 10-cm petri dishes. Then 15 mL of MRS agar medium, kept at 40 °C in a water bath, 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 incubation, *L. casei* colonies were counted. The distribution of bacteria in each tissue was expressed as CFU/g tissue or CFU/mL blood.

The results provided in Fig. 4 were similar to those for the putative macromolecular drug (Evans blue/albumin complex, Fig. 2) and the polymeric drug (PZP, Fig. 3). At 1 h after i.v. injection of bacteria, the distribution was noted mostly in the liver and spleen (Fig. 4a). At 6 h, however, the number of bacteria in the liver and spleen decreased markedly to about 1/10 of those at 1 h, whereas the numbers of bacteria in the tumor increased, approximately 80 -fold (Fig. 4b). These findings suggested that bacterial uptake by 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; see Refs. 4, 31). Moreover, the lower number of bacteria in





**Fig. 4** Body distribution of *L. casei* in ddY mice bearing S-180 solid tumors and its enhancement by nitroglycerin (NG). **(a)** Results at 1 h after i.v. injection of bacteria. **(b)** Results at 6 h after i.v. injection of bacteria. Data are means  $\pm$  SD. See text for details

the liver and spleen may indicate clearance by the lymphatic and reticuloendothelial systems. Another possibility is that under the hypoxic conditions in tumor tissues, facultative and anaerobic bacteria can grow. More important, normal tissues evidenced no significant uptake of bacteria except for the liver and spleen, in which the reticuloendothelial system is responsible for this function.

Furthermore, NG treatment led to a significant increase in the delivery of bacteria to tumor tissues: approximately 70-fold and 20-fold increases were found at 1 h and 6 h, respectively, after NG treatment (Fig. 4). However, normal tissues including the liver and spleen showed no significant increases (Fig. 4), which suggests that NG was converted to NO predominantly in tumor tissues.

## 4 Results

### 4.1 The EPR Effect and Drug Delivery

The EPR effect is considered to be one of the greatest breakthroughs leading to universal targeting to solid tumors in chemotherapy [2, 5, 26, 32]. Matsumura and Maeda first reported the EPR effect in 1986 [1], and Maeda and colleagues continued to perform extensive studies of the effect [3–5, 26]. The EPR effect is based on the fact that most solid tumors have blood vessels with



defective architecture and produce excessive amounts of vascular permeability factors. Such enhanced vascular permeability ensures a sufficient supply of nutrients and oxygen to tumor cells to sustain their rapid growth. This unique anatomical–patho-physiological nature of tumor blood vessels was thus utilized to facilitate delivery of macromolecular drugs to tumor tissues. EPR effect-driven drug delivery does not occur in normal tissues, because their vascular architecture manifests tight endothelial junctions and vessels do not produce excess amount of physiological mediators [3–5, 26]. The EPR effect is thus believed to be a landmark principle in tumor-targeting chemotherapy and is becoming a promising paradigm in anticancer drug development [2].

#### **4.2 Macromolecular Drug Delivery to Tumors Based on the EPR Effect**

The first macromolecular anticancer drug SMANCS (styrene–maleic acid copolymer-conjugated neocarzinostatin) was approved in Japan for use against liver cancer in 1993. Doxil, which is a PEGylated (PEG-coated) liposome-encapsulated formulation of doxorubicin, is now used in clinical settings to treat Kaposi sarcoma and other cancers. Many other polymeric or micellar drugs are undergoing clinical development (phases I and II) [33, 34]. Compared with conventional small-molecular-mass anti-cancer drugs, macromolecular drugs demonstrate superior in vivo pharmacokinetics (e.g., a prolonged plasma half-life) as well as selective tumor-targeting, which result in improved anti-tumor efficacy with fewer adverse side effects [33, 34].

The EPR effect is a molecular size-dependent phenomenon: biocompatible molecules or particles larger than 50 kDa, which is a limit size for renal clearance, had a prolonged circulation time and very slow renal clearance rate. During circulation, they gradually extravasated from tumor blood vessels and were retained in the tumor tissue for a relatively long time (e.g., several days to weeks) [1, 3–5, 26]. The EPR effect was observed with proteins, polymer conjugates, micelles, liposomes, nanoparticles, DNA polyplexes, lipid particles, and bacteria [1, 3–7, 19–21, 26, 33–35].

Using bacteria is now becoming a promising anti-cancer strategy. Here we confirm the advantage of using bacteria for anticancer therapy based on the EPR effect. After achieving tumor delivery of bacteria, a number of effective mechanisms are then proposed effective for bacterial therapy, for example, utilization of genetically-engineered bacterial toxins or prodrug-activating enzymes or activation of innate immunity including natural killer (NK) cells or other immune cells [6, 7, 18–20, 36–39].

The EPR effect involves vascular heterogeneity, i.e., blood vessels in tumors are not usually evenly distributed, which is observed in most large solid tumors having both hyper- and hypo-vascular areas. Some tumors such as pancreatic and prostate cancer, are hypo-vascular. Because the EPR effect is related to vascular

patho-physiology, targeted drug delivery to these hypovascular tumors/areas may be difficult. Methods to augment the EPR effect (blood flow and vascular permeability), especially for hypovascular tumors, are thus important.

### **4.3 Enhanced Delivery of Macromolecular Drugs and Bacteria to Solid Tumors by NG**

As noted above, many vascular mediators are involved in the EPR effect [4, 5, 26]. We have investigated one of these mediators, NO, which is a vital molecule in mammals and has multiple functions such as signal transduction, vasodilatation, increasing the permeability of blood vessels, antioxidant effects, and cell proliferation [5, 39]. In this context, we focused on NG, a well-known NO donor that has been used for more than a century as a medication for angina pectoris. In infarcted-cardiac tissue,  $\text{NO}_2^-$  is first liberated from NG and is then converted to NO by nitrite reductase under hypoxic conditions (Fig. 1) [27–29]. Vasodilatation and increased blood flow can normalize the blood flow of infarcted tissues. The  $\text{pO}_2$  in infarcted-cardiac tissue is low, and the pH is slightly acidic [40, 41], similar to conditions in many tumor tissues. We thus hypothesized that the same situation would occur in tumor tissues as in infarcted-cardiac tissues (Fig. 1; Refs. 26, 31). Application of NG should therefore improve macromolecular drug delivery to tumors having a poor EPR effect, as well as improve the therapeutic efficacy of these drugs.

As expected, we obtained spectacular results in various rodent tumor models, using an Evans blue/albumin complex, polymer conjugates (Figs. 2 and 3; Refs. 26, 29, 31), and bacteria (Fig. 4). These results are similar to those for isosorbide dinitrate [41]. Our results suggested that NG is a useful tool to enhance tumor blood flow, vascular permeability, and the EPR effect to improve tumor-targeted delivery of anti-cancer agents including bacteria.

That NG, in addition to enhancing drug delivery, has a tumor-suppressive effect by itself. NG probably acts by down-regulating the expression of certain critical genes involved in tumor growth [30]. Mitchell et al. [42] and Yasuda et al. [43] also reported that NG increased tumor sensitivity to chemotherapeutic drugs by increasing the blood flow of hypoxic tumors, by suppressing hypoxia-inducible factor-1 $\alpha$ , vascular endothelial growth factor, and P-glycoprotein expression in tumors, all of which play important roles in the resistance of cancer cells to such drugs. Combination therapy with NG may therefore produce a significantly additive chemotherapeutic effect by multiple mechanisms, including enhancement of the EPR effect.

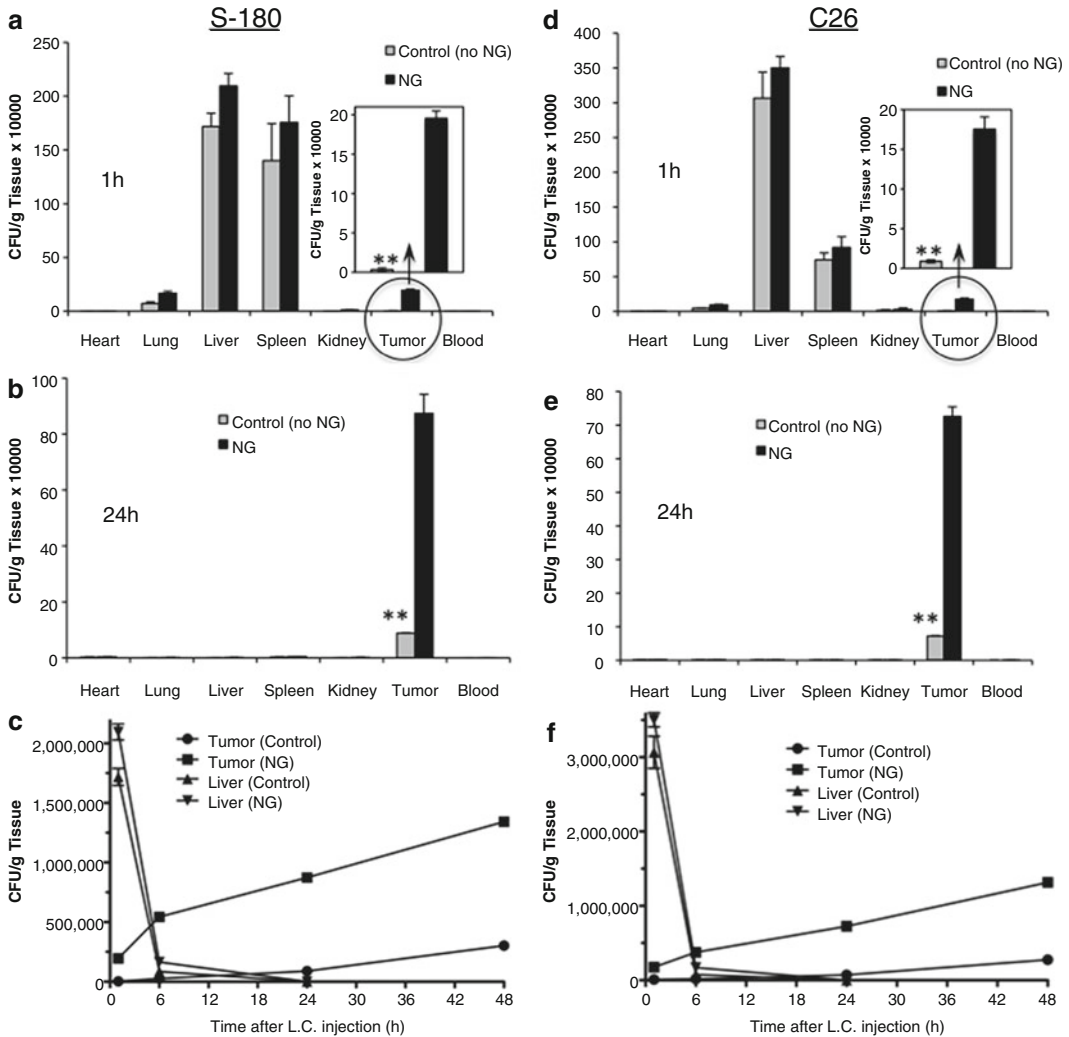
Factors and methods other than NG and NO have been investigated to enhance the EPR effect. These methods included angiotensin II-induced hypertension during arterial infusion of SMANCS in cancer patients [4, 5, 44], and the use of angiotensin-converting enzyme inhibitor [26] and the prostacyclin PGI<sub>2</sub> agonist beraprost [45]. We recently found that carbon monoxide (CO), a gas molecule with physiological functions similar to those of NO, also enhanced the EPR effect [46]. Administration of a CO-releasing molecule or its polymer micelles significantly increased extravasation and the accumulation of macromolecular agents in tumors [46].

#### **4.4 *L. casei*: A Promising Candidate for Bacterial Therapy**

*L. casei* is a nonpathogenic facultatively-anaerobic bacterium that is also a component of the normal bacterial flora in the human intestinal tract and reproductive system. *L. casei* is widely used in various dairy and food supplements, as a so-called probiotic. More important, this bacterium also showed antitumor activity by stimulating nonspecific immune responses, such as macrophage and NK cell activation [36–39], and is considered useful as a medication to prevent recurrence of bladder cancer [21, 22, 47, 48], with mechanisms similar to those of Bacille Calmette-Guérin (BCG) [49]. *L. casei* is thus suitable for bacterial therapy either as a drug vector to carry or deliver genes or as a nonspecific immunostimulant.

With regard to delivery of *L. casei* to tumors, we found tumor-selective accumulation and/or growth of the bacteria after i.v. injection (Fig. 4), which is a result similar to that found in other experiments [6, 7, 18–20]. We now believe that this tumor-preferred accumulation is based on the EPR effect, as discussed above. Moreover, systemic (i.v.) application of the bacteria did not lead to sustained accumulation of bacteria in normal tissues. Although bacteria accumulated mostly in the liver and spleen at 1 h after i.v. injection, the number of *L. casei* decreased dramatically after 6 h (Fig. 4); and at 24 h, almost no bacteria could be found in liver and spleen (Fig. 5). However, the number of bacteria in tumor tissue was far greater than in the liver and spleen (Fig. 5). EPR-based tumor selectivity can induce tumor-specific immune activation and have an anti-tumor effect, with i.v. injection of the bacteria, but not with topical application, such as how BCG for bladder cancer is used. Also, high tumor selectivity will ensure fewer side effects in normal tissues and organs.

More important, as with other macromolecular drugs, NG enhanced the accumulation of bacteria in tumors, i.e., a 70-fold increase at 1 h, 20-fold increase at 6 h and 10-fold increase at 24 h



**Fig. 5** Enhancement of tumor delivery of *L. casei* by NG. Panels **a–c** show the results in the S-180 tumor model, and **d–f** shows the C26 tumor model. In (**a**) and (**d**), the *insets* show enlarged scales for tumor. In (**c**) and (**f**), time course of accumulation of *L. casei* in tumor and liver with/without NG treatment is shown. Data are means  $\pm$  SD;  $n=6$ .  $**P<0.01$  (no NG control vs. NG treatment group) (from Ref. [50] with permission)

after i.v. injection of *L. casei* (Figs. 4 and 5). Bacterial therapy may thus be improved by combination with NG and/or other EPR-enhancing agents, and this possibility warrants additional investigation.

## 5 Notes

- 1. Tumor models:** Female Sprague–Dawley rats (5 weeks old), male ddY mice (6 weeks old), female BALB/c mice (6 weeks old), and male C57BL/6 J mice (6 weeks old) were purchased from SLC (Shizuoka, Japan). Rats were housed three per cage,

and mice were housed four or five per cage. For all animals, conditions were maintained at  $22 \pm 1$  °C and  $55 \pm 5$  % relative humidity with a 12-h light/dark cycle. All experiments were carried out according to the Laboratory Protocol for Animal Handling of Sojo University.

2. **Tumors:** Mouse S-180 sarcoma cells, from ascites maintained by weekly passage, were implanted subcutaneously (s.c.) in the dorsal skin of ddY mice, to obtain the S-180 tumor model. Mouse fibrosarcoma Meth-A cells ( $2 \times 10^6$ ) were maintained by intraperitoneal (i.p.) passage and then implanted s.c. in BALB/c mice. Colon adenocarcinoma C38 cells ( $2 \times 10^6$ ), purchased from the Riken Cell Bank (Tsukuba, Japan), were implanted s.c. in C57BL/6 mice. In addition, rat breast cancer was induced by oral administration of 10 mg of DMBA (a carcinogen) in 1 mL of corn oil. Bacterial distribution studies were started when the tumor diameters were 5–10 mm.
3. **Preparation of NG:** NG ointment contained 20 mg/g Vaseline® and was used after 10- or 100-fold dilution with Vaseline®.
4. **Evans blue:** When injected into circulation, it binds to albumin to form a complex of about 69 kDa and is thus considered a putative macromolecular drug.
5. **PEG-conjugated zinc protoporphyrin IX (ZnPP) (PZP):** The synthesis, purification, and characterization of PZP were previously described [51]. PZP consists of two chains of PEG, each about 2,500 Da, conjugated to ZnPP to form micelles of about 180 nm in diameter, and a mean molecular mass of about 110 kDa determined by size-exclusion chromatography. Radiolabeled PZP was obtained by utilizing  $^{65}\text{Zn}$  during the zinc insertion step of PZP synthesis [52].
6. ***Lactobacillus* bacteria:** *L. casei* strain Shirota was cultured in MRS (de Man, Rogosa, Sharpe) medium. Lactulose (4-*O*- $\beta$ -D-galactopyranosyl-D-fructofuranose), was used during in vivo experiments with *L. casei* [6].

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## Acknowledgment

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Culture, Sports and Technology of Japan (No. 08011717), a Cancer Speciality grant from the Ministry of Health, Welfare and Labour (H23-Third Term Comprehensive Control Research, General-001), and research funds of the Faculty of Pharmaceutical Sciences at Sojo University.

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Bacterial Therapy of Cancer

Methods and Protocols

Hoffman, R. (Ed.)

2016, XVI, 186 p. 43 illus., 33 illus. in color., Hardcover

ISBN: 978-1-4939-3513-0

A product of Humana Press