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Research paper

Improved stomach selectivity of gene expression following microinstillation of plasmid DNA onto the gastric serosal surface in mice

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Abstract

Stomach-selective gene transfer is a promising approach as a therapeutic strategy for refractory gastric diseases. In this study, we improved the stomach selectivity of gene expression following microinstillation of naked plasmid DNA (pDNA) onto the gastric serosal surface in mice. pDNA encoding firefly luciferase was used as a reporter gene. It was confirmed that the gene expression level in the stomach 6 h after gastric serosal surface microinstillation of pDNA was significantly higher than after intragastric, intraperitoneal and intravenous administration. Regarding selectivity of gene expression, the gene expression level in the stomach after gastric serosal surface microinstillation of 1 μg/1 μL (dose/volume) pDNA was 5.7 times higher than that in the spleen. In our previous study (30 μg/30 μL), the expression level in the stomach was 2.7 times higher than that in the spleen; therefore, the selectivity was 2.1 times higher in this study. When we investigated gene expression at various pDNA solution concentrations, the ratio of the gene expression level in the stomach to that in the spleen was the highest as 1 μg/1 μL of pDNA, which was considered the optimal concentration. Information in this study is useful for further development of target organ-selective gene delivery systems.

Keywords: Gene delivery; Targeting; Stomach; Gene therapy; Plasmid DNA; Mouse; Luciferase

1. Introduction

The stomach is an important organ for digestion and absorption of aliment. People often suffer from stomach disorders such as acute and chronic gastritis, gastric ulcer and gastric cancer. Especially, gastric cancer is one of the most common malignant tumors worldwide. It was the second most common cause of death from cancer (700,000 deaths annually) after lung cancer in 2002 according to the International Agency for Research on Cancer. Moreover, in 2002, the incidence of gastric cancer was estimated to be 934,000 new cases per year worldwide [1]. To treat these refractory diseases, gene therapy is a candidate as a rationalized therapeutic approach. Several studies have been performed to investigate treatment of gastric ulcer [2] and gastric cancer [3]. The *in vivo* gene delivery systems can be categorized as viral [4] and non-viral approaches [5]. Although non-viral vectors generally have a problem in terms of transfection efficiency, non-viral vectors have safety advantages compared with viral vectors. Naked plasmid DNA (pDNA) is the simplest and safest of the non-viral gene delivery systems since it can be used without concerns about cytotoxicity of the gene carrier.

When we use a gene encoding growth factors, gene expression in non-target tissues causes unexpected adverse effects. For example, although it was reported that vascular endothelial growth factor (VEGF) played a role in ulcer healing process [6], it is known as an important factor in tumor angiogenesis [7]. Therefore, target-selective or -specific gene transfer is desirable for maximal therapeutic action and minimal adverse effects in the clinical use of gene therapy. When foreign genes were administered via the vasculature route, they were distributed to the whole body through the bloodstream, leading to inadequate organ-selective or disease site-selective gene delivery, and

rapid degradation by reticuloendothelial cells (liver Kupffer cells, etc.) and nuclease in the blood [8]. It was previously reported that organ-selective gene transfection using naked pDNA was achieved by direct injection [9], electroporation [10], a gene gun [11] and so on. Gene expression in the stomach was observed by direct injection of pDNA into the gastric submucosa in rats [12]. However, there is great concern about safety because these procedures require physical force against organs; consequently, the continuous and repetitive administration of pDNA is limited. However, although pDNA complexes with chitosan [13], N-acetylated chitosan [14] and montmorillonite [15] were studied for oral gene delivery, the oral route has many hampers reducing transfection efficiency such as a low pH, high concentrations of digestive fluid, and rapid turnover of epithelial cells, etc.

We previously developed a method to apply drugs onto the surface of intraperitoneal organs such as the liver [16, 17], kidney [18, 19] and stomach [20-22], and found it to be a useful method for site-selective drug delivery to these organs. Furthermore, we reported on site-selective gene expression following instillation of naked pDNA onto the liver surface [23-26], kidney surface [27] and gastric serosal surface [28] in mice. However, 30 μL of pDNA solution as an instillation volume was too large against the target organ in our previous studies; a large volume causes diffusion of pDNA solution to peripheral tissues. Here, it was postulated that drastically reducing the volume of pDNA solution would prevent diffusion to non-target organs and improve selectivity of gene expression. To elucidate this hypothesis, in this study we performed microinstillation of naked pDNA onto the gastric serosal surface in mice.

2. Materials and methods

2.1. Materials

Sodium pentobarbital was obtained from Abbott Laboratories (Illinois, USA). All chemicals were of the highest purity available (Nacalai Tesque, Inc., Kyoto, Japan).

2.2. Animals

Male ddY mice were purchased from Kyudo Co., Ltd. (Kumamoto, Japan). They were housed in a cage in an air-conditioned room and maintained on a standard laboratory diet (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institutes of Health and the Guidelines for Animal Experimentation of Nagasaki University.

2.3. Construction and preparation of pDNA

pCMV-luciferase was constructed by subcloning the *Hin*d III/Xba I firefly luciferase cDNA fragment from a pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of a pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the *Escherichia coli* (*E. coli*) strain DH5α, isolated and purified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). pDNA dissolved in 5% glucose solution was strored at –20°C prior to experiments.

2.4. In vivo gene expression experiments

2.4.1. Gastric serosal surface microinstillation of naked pDNA

Five-week-old male ddY mice (24-35 g) were anesthetized with sodium pentobarbital (40-60 mg/kg, intraperitoneal injection). Laparotomy was performed and the stomach was exposed. pDNA was instilled onto the gastric serosal surface using a micropipette (PIPETMAN®, GILSON, Inc., Villiers-le-Bel, France). Mice were kept lying on their back for 1 h, and then the peritoneum was sutured. Subsequently mice were freed in the cage. Six hours after pDNA instillation, a blood sample was taken from inferior vena cava. Immediately, the mice were killed under anesthesia, and the stomach, liver, kidneys (left and right), spleen, diaphragm, heart and lung were removed. The tissues were washed twice with saline and homogenized with a lysis buffer which consisted of 0.1 M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA [29]. The volumes of the lysis buffer added were 4 μL/mg of tissue. Blood samples and homogenates were centrifuged at 15,000 × ***g*** for 5 min. Twenty microliters of supernatant were mixed with 100 μL of luciferase assay substrates (PicaGene®, Toyo Ink Mfg. Co., Ltd., Tokyo) and the light produced was immediately measured using a luminometer (MiniLumat LB9506, BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany). The luciferase activity is indicated as the relative light units (RLU) per gram of tissue.

2.4.2. Intragastric, intraperitoneal and intravenous administration of naked pDNA

Two micrograms of pDNA, the maximum dose in this study, was administered intragastrically, intraperitoneally and intravenously to anesthetized mice as control experiments. In the intragastric administration study, laparotomy was performed and the stomach was exposed. Subsequently, a 26

G × 1/2” needle (Nipro Co., Ltd., Osaka, Japan) was inserted via the duodenum and pDNA was injected into the stomach. Then, the pinhole was sealed with a thin film of surgical adhesive (Aron Alpha, Sankyo Co., Ltd., Tokyo). The following processes were the same as for gastric serosal surface microinstillation experiments.

2.5. Statistical Analysis

Statistical comparisons were performed by Steel-Dwass multiple comparison tests.

3. Results and discussion

Although gastric ulcer and gastric cancer in the early stage are treatable by conventional medicine and/or surgery, recurrent and refractory gastric ulcer and advanced gastric cancer do not generally respond to conventional therapy. Gene therapy is a promising approach for these severe diseases because a protein defect or deficiency is frequently involved in these diseases. In most cases, a viral vector has been used as the gene delivery method to the stomach, but selectivity and safety concerns have been raised about viral vectors. There have been attempts to improve these problems by using an adenoviral vector with a cancer specific promoter [30] and a fiber-modified adenoviral vector targeted to the carcinoembryonic antigen expressed on the surface of gastric cancer cells *in vitro* and *in vivo* [31]. However, immunogenicity of adenoviruses restricts safety and efficacy in repeated administration. Thus, we developed a novel, safe and stomach-selective gene delivery method instilling naked pDNA onto the gastric serosal surface.

We previously demonstrated that the administration of naked pDNA onto the gastric serosal surface in mice resulted in stomach-selective gene expression [28]. The present study was performed to improve the stomach selectivity of gene expression following the microinstillation of naked pDNA. At the beginning, we confirmed whether the microinstillation of naked pDNA (2 μg/1 μL) onto the gastric serosal surface in mice showed detectable gene expression in the stomach. As control experiments, we administered 2 μg/100 μL of naked pDNA intragastrically, intraperitoneally and intravenously (Fig. 1). Six hours later, luciferase activity in the stomach was determined (Fig. 2). Each tissue homogenate without the administration of naked pDNA was mixed with a luciferase assay substrate and showed approximately 3×10^3 RLU/g tissues, which was background

luminescence. Gastric serosal surface microinstillation of naked pDNA showed evident gene expression in the stomach, while intragastric, intraperitoneal and intravenous administration resulted in negligible gene expression in the stomach. Moreover, gene expression was negligible in the liver, kidneys, spleen, diaphragm, heart, lung and plasma following the intragastric, intraperitoneal and intravenous administration of pDNA (data not shown). These results are consistent with the results in our previous reports [23, 24]. In intragastric and intravenous administration, naked pDNA would be degraded by digestive fluid in the stomach and nuclease in the blood. As for intraperitoneal administration, pDNA solution was mainly instilled onto the small intestine, and the volume of pDNA solution would be too small to sufficiently contact with the stomach and other tissues.

We examined the tissue distribution of gene expression 6 h after gastric serosal surface microinstillation of several doses of pDNA at a volume of 1 μL. One microliter was one thirtieth of the instillation volume in our previous study [28]. Gene expression 6 h after gastric serosal surface instillation of pDNA (30 μg/30 μL) was stomach-selective; however, the gene expression level in the stomach was 2.7 times higher than that in the spleen [28]. Figure 3 shows gene expression in the stomach, liver, kidneys (left and right), spleen, diaphragm, heart, lung and plasma after microinstillation onto the gastric serosal surface. At 1 μg, the gene expression level in the stomach was 5.7 times higher than that in the spleen. The ratio of the gene expression level in the stomach to that in the spleen was the highest at a dose of 1 μg. These results suggested that there was an optimal pDNA concentration for successful stomach-selective gene transfer (Table 1). To confirm the advantage of the microinstillation method, we calculated luciferase activities per pDNA dose about the microinstillation (1 μg/1 μL) and previous (30 μg/30 μL) methods. These were approximately

125,000 (microinstillation method) and 65,000 (previous method) RLU/g tissue/μg pDNA. These values suggested that microinstillation method could improve stomach selectivity of gene expression without decreasing transfection efficiency per pDNA dose.

To evaluate the effect of instillation doses on gene expression, luciferase activity was replotted against a scale of instillation doses of pDNA (Fig. 4). The gene expression levels in the stomach and liver were saturated over 1 μg and 1.5 μg. Because 1 μL as the instillation volume was a very small drop, the contact area of pDNA solution with the organ surface would be limited. pDNA above a certain amount might not be taken up and/or some of the subsequent processes leading to gene expression might be saturated after uptake of pDNA. However, gene expression in the spleen and diaphragm were not saturated.

We examined the tissue distribution of gene expression 6 h after gastric serosal surface microinstillation at several volumes of pDNA (1 μg) solution (Fig. 5). Stomach selectivity of gene expression was the highest at 1 μL. In our previous report [28], gene expression was slightly observed not only in the stomach, liver and spleen, but also in the left kidney and lung. However, gene expression after gastric serosal surface microinstillation of naked pDNA was negligible in the kidneys and lung in each condition.

To evaluate the effect of instillation volumes of pDNA solution on gene expression, luciferase activity was replotted against a scale of instillation volumes (Fig. 6). At volumes up to 1 μL of instilled pDNA solution, the gene expression level in the stomach was enhanced, while further increases in instillation volume reduced the gene expression levels. These results might be due to the following reasons: (1) There is an optimal pDNA concentration for efficient gene expression; (2) The

contact area with the stomach at very low instillation volumes was limited.

Although the ratio of the gene expression level in the stomach to that in the spleen was low at a dose of 2 μg, it improved to 5.7 with a decrease to 1 μg of the instillation dose (Table 1). In our previous reports, *in situ* gene expression following the administration of pDNA in a cylindrical diffusion cell attached onto the organ surface was found only in the applied tissues [25, 27, 28]. These results suggested that diffusion of pDNA to peripheral tissues resulted in gene expression in non-administered tissues. Contact of pDNA with non-target tissues is an important factor in target-specific gene transfer. In addition, site selectivity of gene expression in the stomach is an important issue. We have already achieved stomach site-specific gene expression by use of a cylindrical diffusion cell [28]. However, a cylindrical diffusion cell is unfavorable for clinical application. Microinstillation improved stomach selectivity, but gene expression was not stomach-specific. Thus further innovations such as continuous microinstillation of naked pDNA using an infusion pump and/or a formulation using viscous additives are required for organ- and site-specific gene transfer.

The stomach-selective or -specific gene transfection methods are expected to be safe and effective treatments against refractory gastric ulcer and gastric cancer. Gastric ulcer and gastric cancer are generated in the gastric mucosal side and then invade the gastric serosal side. Gastric serosal surface instillation of pDNA encoding therapeutic genes is thought to help resistance primarily against the invasion of gastric ulcer or gastric cancer to the serosal side. The ulcer healing process comprises many steps: cell migration, proliferation, re-epithelialization, angiogenesis, and matrix deposition [6]. This process involves many genes encoding growth factors, including

epidermal growth factor, VEGF, keratinocyte growth factor, hepatocyte growth factor, platelet-derived growth factor, basic fibroblast growth factor, and angiopoietins. It has been reported that genes encoding these growth factors have an ulcer healing effect *in vivo* [2, 32-34]. Moreover, gene therapy has been tried for gastric cancer *in vitro* and *in vivo* with various therapeutic genes, namely p53 [35], FHIT [36], NK4 [37, 38] and the Fas ligand [39]. In addition, adenovirus-mediated suicide gene therapy by *E. coli* cytosine deaminase/5-fluorocytosine [30] or *E. coli* uracil phosphoribosyltransferase/5-fluorouracil have been reported [31]. Efficient and target-selective gene delivery systems are essential for successful gene therapy. Gastric serosal surface microinstillation of other vectors, as well as naked pDNA, could potentially succeed in stomach-specific gene transfer.

In summary, we demonstrated the improved stomach selectivity of gene expression following the microinstillation of naked pDNA onto the gastric serosal surface in mice. The transgene expression levels in the tissues were stomach-selective, but not yet stomach-specific. Further developments in administration methods and/or formulations of pDNA solution are required for stomach-specific gene transfer.

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Figure legends

Fig. 1. Scheme of the pDNA administration procedures in mice.

Fig. 2. Gene expression in the stomach 6 h after gastric serosal surface microinstillation, intragastric (i.g.), intraperitoneal (i.p.) and intravenous (i.v.) administration of pDNA at a dose of 2 μg in mice. Each bar represents the mean + S.E. of at least 4 experiments. Significantly different from i.g. administration (**p<0.01), from i.p. and i.v. administration (†p<0.05).

Fig. 3. Gene expression in the stomach, liver, kidneys (left and right), spleen, diaphragm, heart, lung and plasma 6 h after gastric serosal surface microinstillation of pDNA at doses of 0.25-2 μg (1 μL) in mice. Each bar represents the mean + S.E. of at least 16 experiments. Significantly different from the spleen (*p<0.05), kidneys, heart and lung (††p<0.01), and from plasma (‡p<0.05, ‡‡p<0.01).

Fig. 4. Effect of instillation doses on gene expression in the stomach, liver, spleen and diaphragm 6 h after gastric serosal surface microinstillation of pDNA at a volume of 1 μL in mice. Each value represents the mean ± S.E. of at least 16 experiments.

Fig. 5. Gene expression in the stomach, liver, kidneys (left and right), spleen, diaphragm, heart, lung and plasma 6 h after gastric serosal surface microinstillation of pDNA at volumes of 0.25-5 μL (1 μg) in mice. Each bar represents the mean + S.E. of at least 15 experiments. Significantly different

from the spleen (*p<0.05), kidneys, heart and lung (††p<0.01), and from plasma (‡p<0.05, ‡‡p<0.01).

Fig. 6. Effect of instillation volumes on gene expression in the stomach, liver, spleen and diaphragm 6 h after gastric serosal surface microinstillation of pDNA at a dose of 1 μg in mice. Each value represents the mean ± S.E. of at least 15 experiments.

Table 1

Table 1

Ratio of the gene expression level in the stomach to that in the spleen

	Instillation dose of pDNA (μg)				
	0.25	0.5	1	1.5	2
Ratio	2.6	3.8	5.7	2.6	1.3

The volume of pDNA solution was 1 μL.

Fig.1

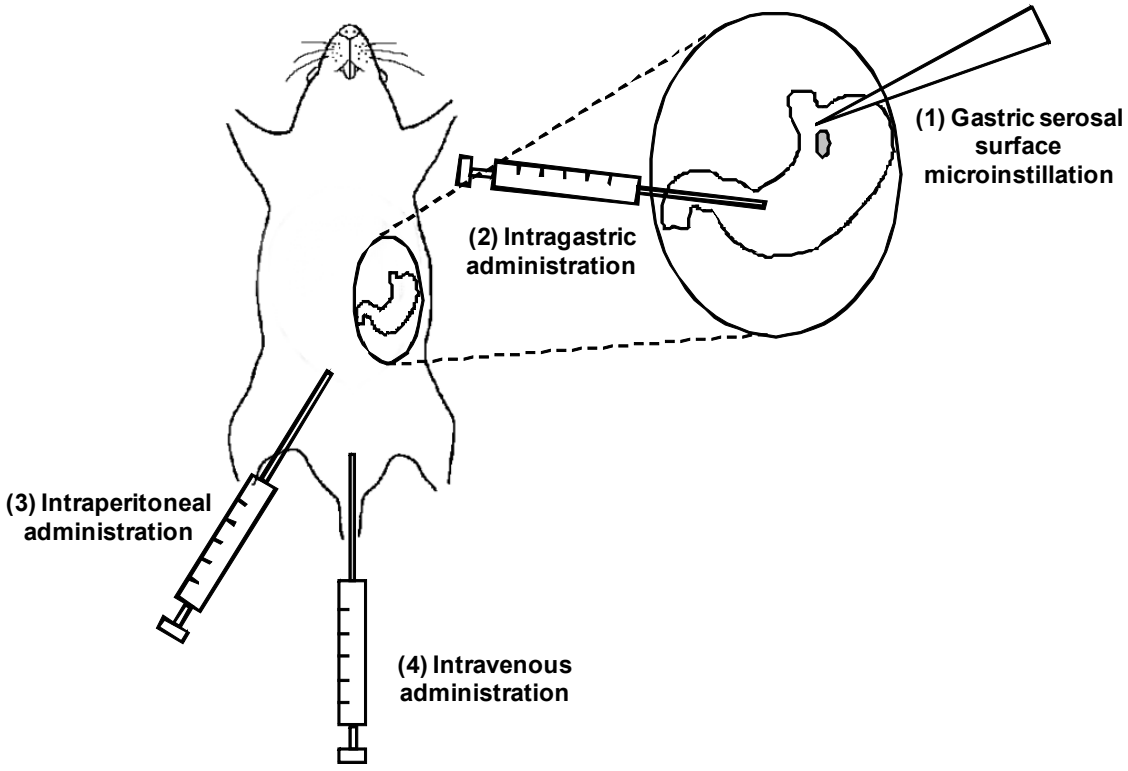

(1) Gastric serosal surface microinstillation
(2) Intragastric administration
(3) Intraperitoneal administration
(4) Intravenous administration

Fig.2

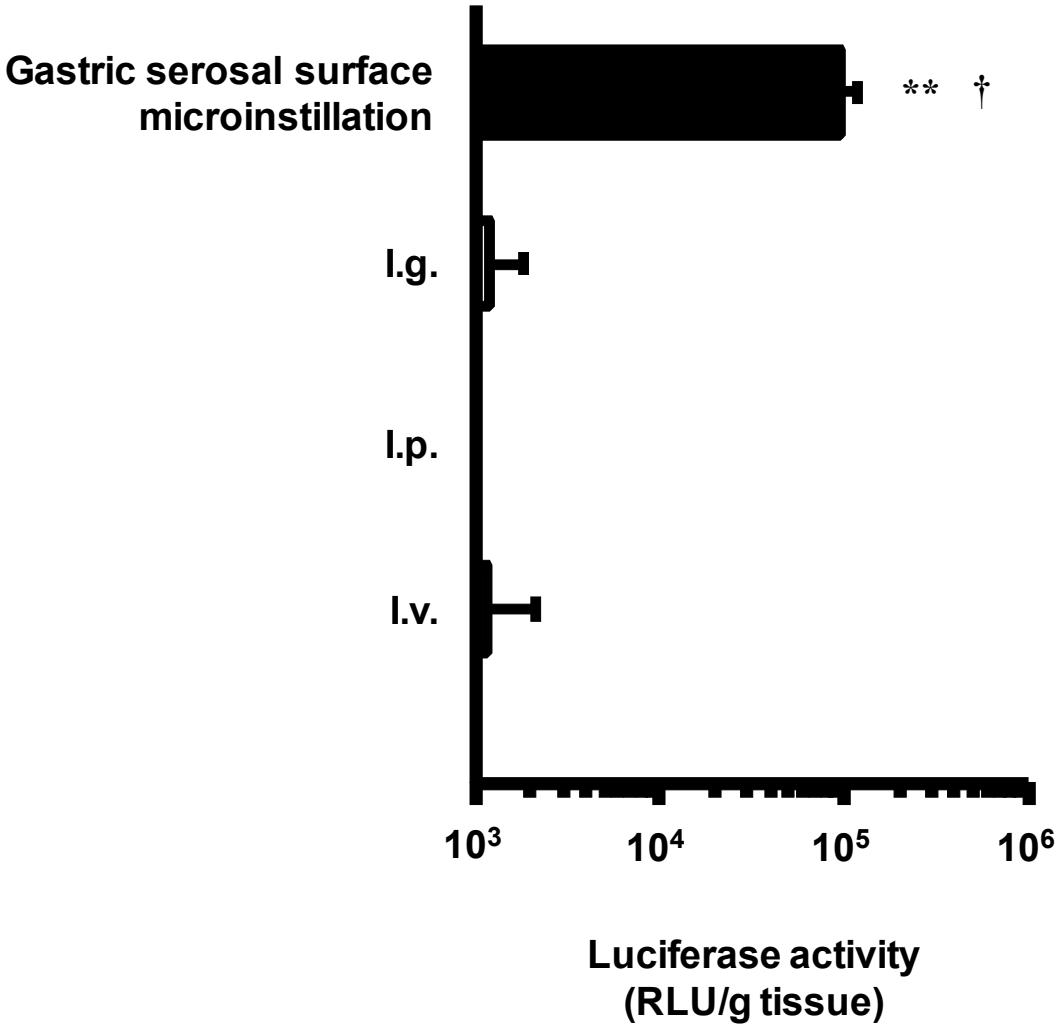

Gastric serosal surface microinstillation
**
†
i.g.
i.p.
i.v.
10^3
10^4
10^5
10^6
Luciferase activity (RLU/g tissue)

Fig.3

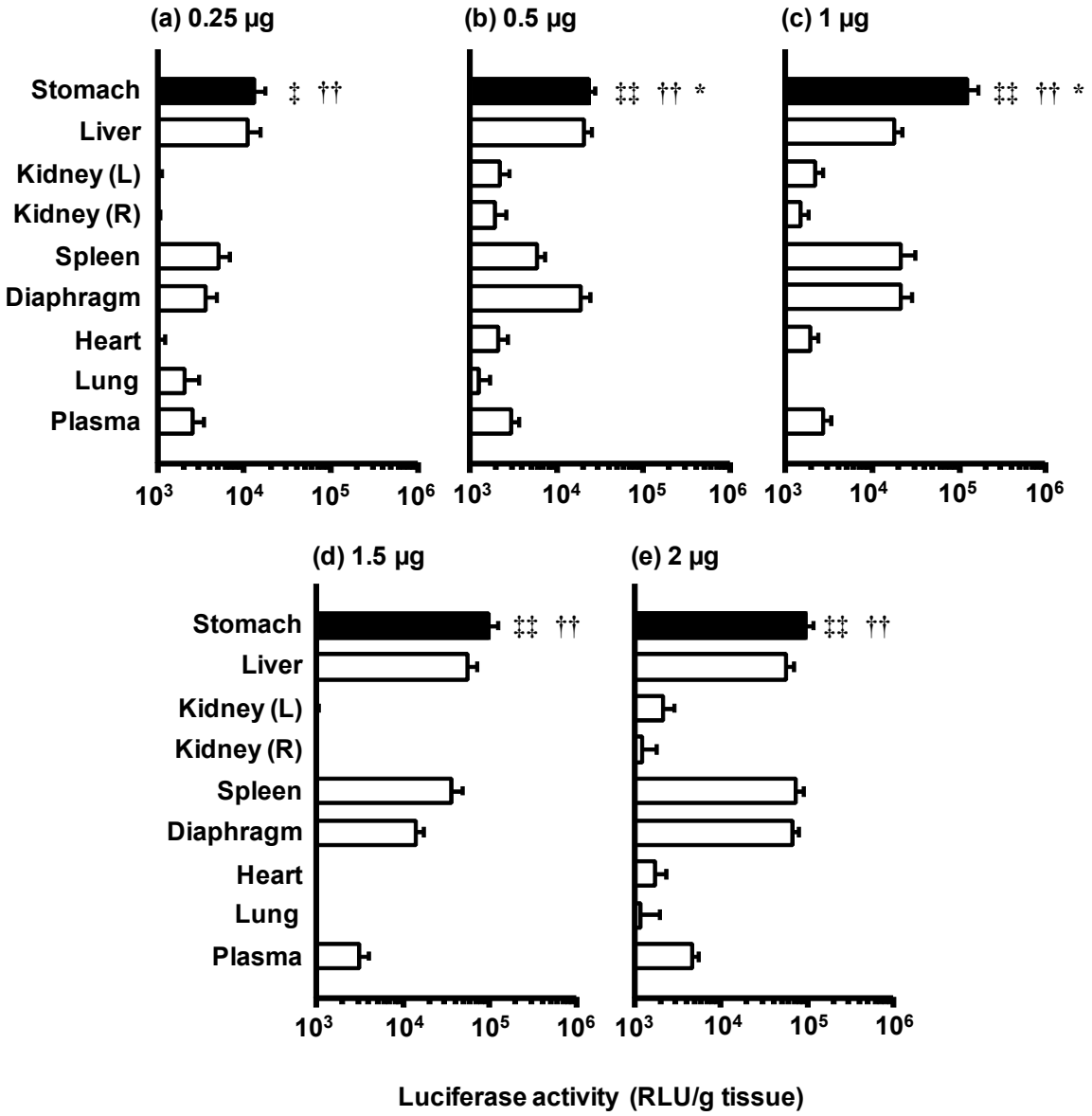

(a) 0.25 μg
(b) 0.5 μg
(c) 1 μg
(d) 1.5 μg
(e) 2 μg
Stomach
Liver
Kidney (L)
Kidney (R)
Spleen
Diaphragm
Heart
Lung
Plasma
‡
††
‡‡
*
10³
10⁴
10⁵
10⁶
Luciferase activity (RLU/g tissue)

Fig.4

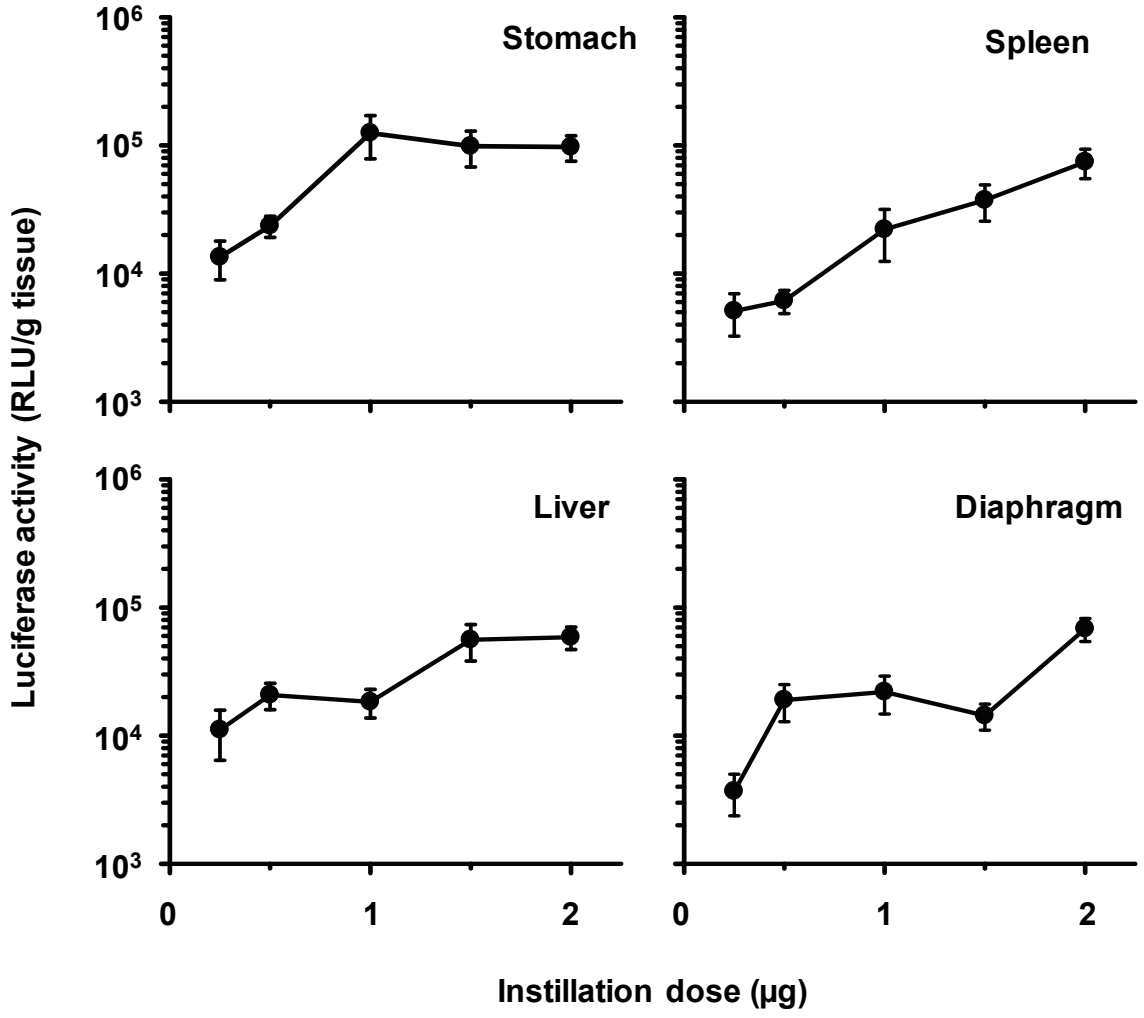

Stomach
Spleen
Liver
Diaphragm
Luciferase activity (RLU/g tissue)
10^6
10^5
10^4
10^3
0
1
2
Instillation dose (μg)

Fig.5

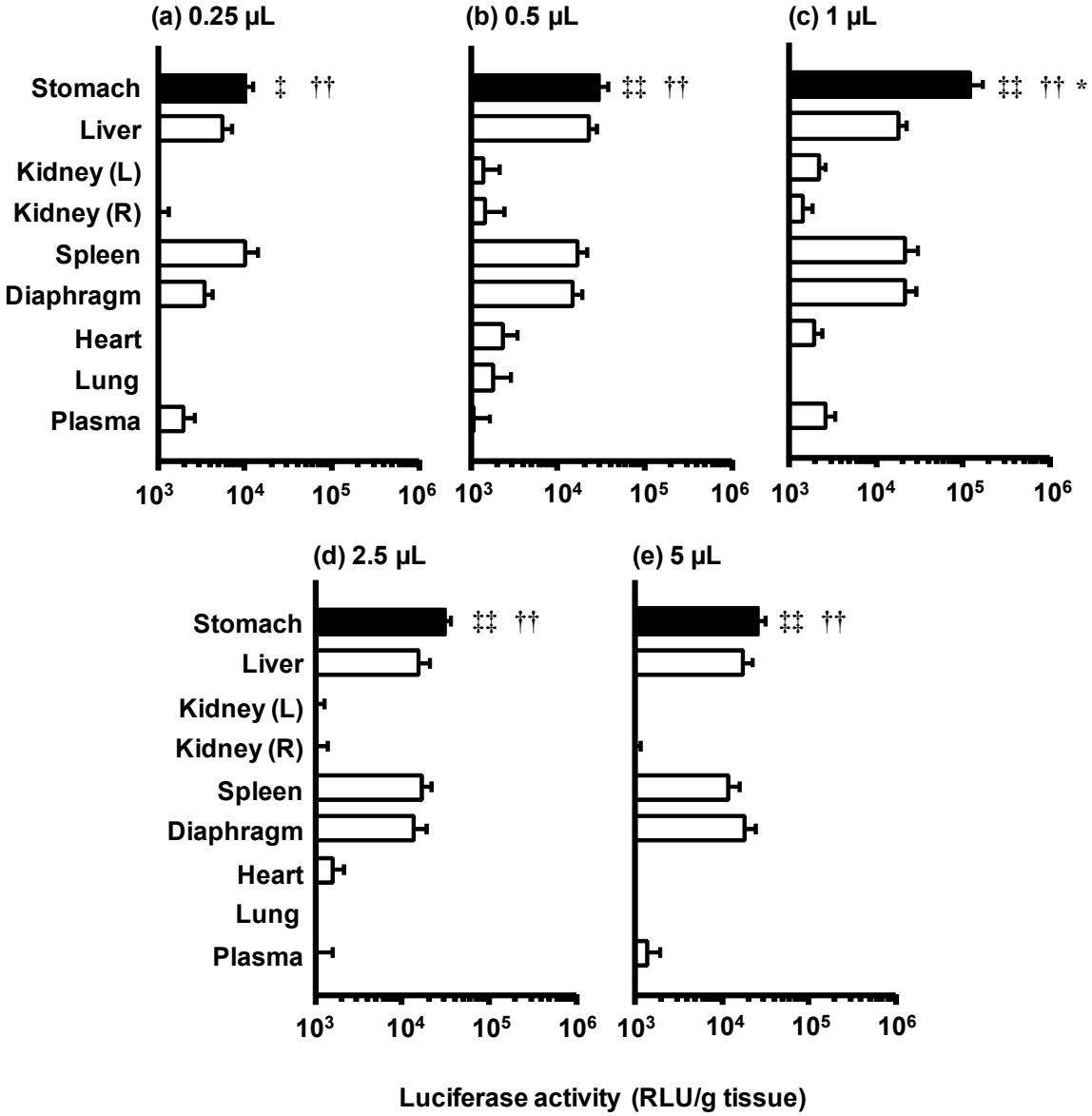
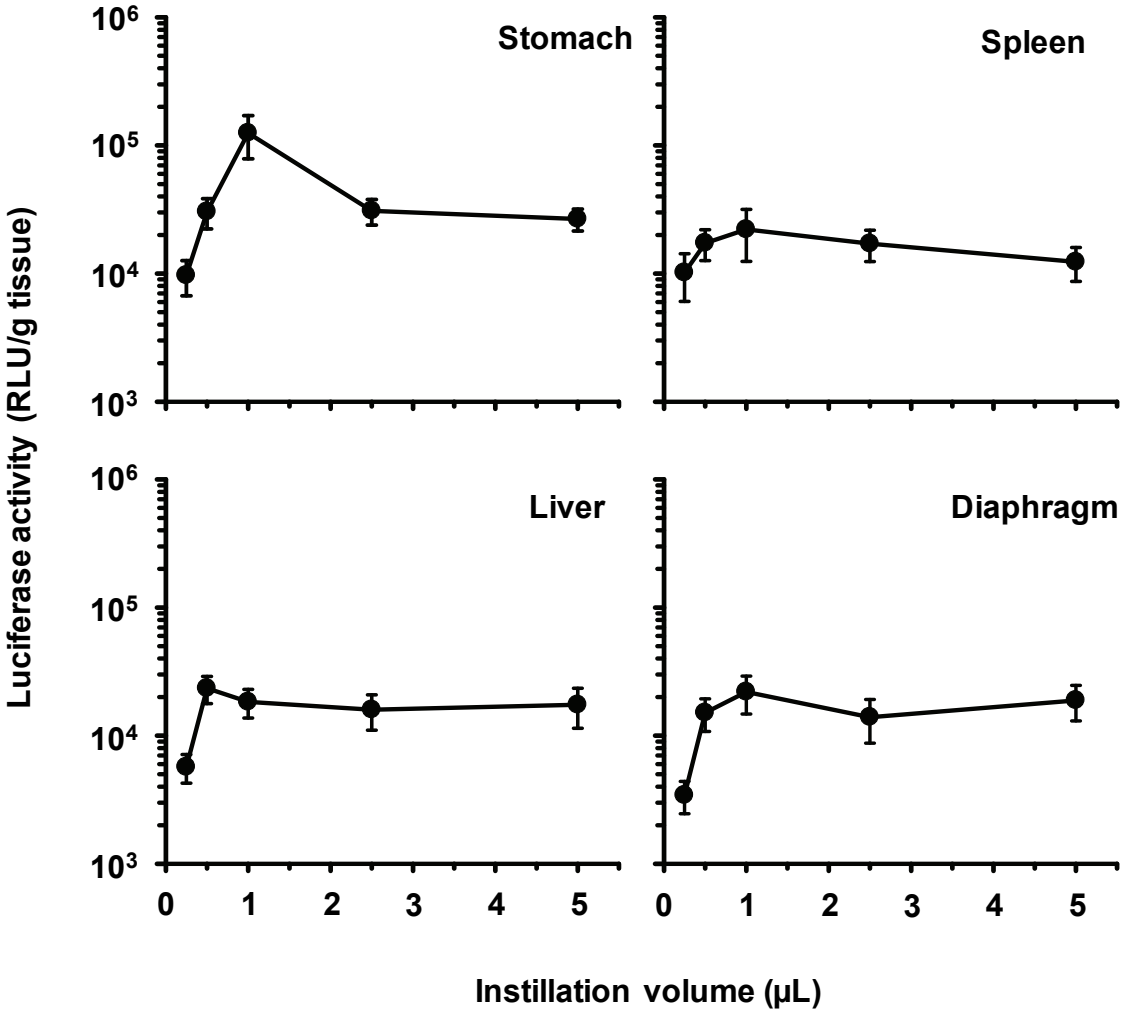

(a) 0.25 µL
(b) 0.5 µL
(c) 1 µL
(d) 2.5 µL
(e) 5 µL
Stomach
Liver
Kidney (L)
Kidney (R)
Spleen
Diaphragm
Heart
Lung
Plasma
‡
††
‡‡
*
10³
10⁴
10⁵
10⁶
Luciferase activity (RLU/g tissue)

Fig.6


Stomach
Spleen
Liver
Diaphragm
Luciferase activity (RLU/g tissue)
Instillation volume (μL)
10^6
10^5
10^4
10^3
0
1
2
3
4
5