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Highly stomach-selective gene transfer following gastric serosal surface instillation of naked plasmid DNA in rats

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Short title

Stomach-selective gene transfer in rats

Background. The purpose of this study was to achieve stomach-selective gene transfer in rats by our simple and novel administration method, which is gastric serosal surface instillation of naked plasmid DNA (pDNA). ***Methods.*** Naked pDNA encoding firefly luciferase as a reporter gene was instilled onto the gastric serosal surface in male Wistar rats. As controls, we performed intraperitoneal, intragastric and intravenous administration of naked pDNA. At appropriate time intervals, we measured luciferase activities in the stomach and other tissues. ***Results.*** Gene expression in the stomach 6 h after gastric serosal surface instillation of naked pDNA (5 μg) was significantly higher than that using other administration methods. The present study is the first report on stomach-selective gene transfer following instillation of naked pDNA onto the gastric serosal surface in rats. Also, the gene expression level in the stomach 6 h after gastric serosal surface instillation of naked pDNA was markedly higher than that in other tissues. In a dose-dependent study, the gene expression level was saturated over 5 μg. Gene expression in the stomach was detected 3 h after gastric serosal surface instillation of naked pDNA. The gene expression level was peaked 12-24 h after instillation of naked pDNA, then decreased to similar level to 3 h at 48 h. ***Conclusions.*** Gastric serosal surface instillation of naked pDNA can be a highly stomach-selective gene transfer method in rats.

Key words: gene delivery, targeting, stomach, naked plasmid DNA, rat

Introduction

The stomach is an important target organ for gene delivery due to its capabilities for storing, mixing, digesting and sterilizing foods with gastric acid. 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. The mortality due to Japanese gastric cancer was over 50,000 in 2005 according to the Center for Cancer Control and Information Services, National Cancer Center, Japan. Recently, Matsumoto et al. reported that the activation-induced cytidine deaminase gene was related to *Helicobacter pylori*-associated gastric carcinogenesis.[1] Gene therapy targeted for oncogenes and/or tumor-suppressor genes is a candidate 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 toxicity of the gene carrier. Indeed, clinical investigations have been performed using naked pDNA, which encodes hepatocyte growth factor (HGF), for treatment of peripheral arterial disease in Japan.[6]

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 the ulcer healing process,[7] it is known as an important factor in tumors or pathological retinal angiogenesis.[8] 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 were rapidly degraded by reticuloendothelial cells (liver Kupffer cells, etc.) and nuclease in the blood.[9] It was previously reported that organ-selective gene transfer using naked pDNA was achieved by direct injection,[10] electroporation,[11] gene gun[12] and so on. Gene expression in the stomach was observed by direct injection of pDNA into the gastric submucosa in rats.[13] 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,[14] N-acetylated chitosan[15] and montmorillonite[16] were studied for oral gene delivery, the oral route has many factors reducing transfection efficiency such as gastric contents, low pH, high concentration of digestive fluid, and rapid turnover of epithelial cells, etc. In fact, transgene expression was not observed in the stomach following oral administration with nanoparticles-in-microsphere in rats.[17]

We previously developed a method to apply drugs onto the surface of intraperitoneal organs such as the liver,[18] kidney[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] kidney surface[24] and gastric serosal surface[25] in mice. However, we have not confirmed whether animals other than mice can achieve foreign gene transfer by organ surface administration. As the rat stomach is bigger than that of mice, target-selectivity may be better because the target organ is large compared to instillation volume of

pDNA solution, so the pDNA solution might not easily diffuse from the target organ to peripheral tissues. Furthermore, it was postulated that a lower volume of pDNA solution would not easily diffuse to non-target organs and achieve highly stomach-selective gene expression. Both target size and solution volume may be key factors for target organ-selective gene transfer. To elucidate this hypothesis in the present study we performed instillation of naked pDNA onto the gastric serosal surface in rats.

Materials and methods

Materials

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

Animals

Male Wistar rats 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.

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). In this study, pCMV-luciferase was mainly used except for the experiment of immunohistochemistry. pZsGreen1-N1 encoding reef coral fluorescent protein was obtained from Takara Bio Inc. (Shiga, Japan). Naked pDNA was amplified in the *Escherichia coli* strain DH5α, isolated, and purified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Naked pDNA dissolved in 5% glucose solution was strored at –20°C prior to experiments.

Gastric serosal surface instillation

Five-week-old male Wistar rats (140-170 g) were anesthetized with sodium pentobarbital (40-60 mg/kg, intraperitoneal administration). Laparotomy was performed and the stomach was exposed. Naked pDNA solution was quickly instilled onto the gastric serosal surface using a micropipette (PIPETMAN®, GILSON, Inc., Villiers-le-Bel, France). The area of instillation was the middle body of stomach. Rats were kept lying on their back for 1 h, and then the abdominal wall and the skin were sutured. Subsequently rats were freed in the cage. At appropriate time intervals (3, 6, 12, 24 and 48 h) after administration of naked pDNA, a blood sample was taken from the inferior vena cava with a syringe (19 G × 1½" needle, TERUMO, Tokyo, Japan). Immediately, rats were killed under anesthesia, and the stomach, liver, kidneys (left and right), spleen, diaphragm, heart and lung were removed with surgical scissors. 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.[26] 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. Each supernatant was assayed for luciferase activity.

Intraperitoneal, intragastric and intravenous administration

Five micrograms (100 μL) of naked pDNA was administered intragastrically, intraperitoneally and intravenously to anesthetized rats as control experiments. In the intraperitoneal administration study, rats were intraperitoneally administered naked pDNA, kept lying on their back for 1 h and freed in the cage. In the intragastric administration study, rats underwent laparotomy 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, Japan) and the abdominal wall and skin were sutured. Subsequently, rats were freed in the cage. In the intravenous administration study, rats were administered pDNA via the femoral vein and freed in the cage. The following processes were the same as for gastric serosal surface instillation experiments.

Luciferase assay

Twenty microliters of tissue homogenate supernatant and plasma were mixed with 100 μL of luciferase assay substrates (PicaGene®, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) 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.

Immunohistochemistry

Twenty four hours after gastric serosal surface instillation of pZsGreen1-N1 (5 μg/5 μL), rats were killed under anesthesia, and the stomach was removed. Imprints of the gastric serosal surface cells were prepared by modified method which was reported by Foley-Comer et al.[27] Briefly, the stomach was washed twice with saline and dried for 5 min at room temperature. Imprints of the gastric serosal surface cells were obtained on MAS coated micro slide glasses (SUPERFROST® S-9441, Matsunami Glass Ind., Ltd., Osaka, Japan). Imprints were fixed with 4% paraformaldehyde for 10

min and permeabilized for 5 min with phosphate-buffered saline containing 0.2% Triton X-100. Non-specific staining was reduced with Image-iT™ FX signal enhancer (Invitrogen) before incubating the imprints with rat monoclonal antibodies (dilution 1:50; Santa Cruz Biotechnology, Inc., CA, USA) directed against zonula occludens-1 (ZO-1) for 1 h at room temperature in a humidified chamber. Then imprints were incubated with goat anti-rat IgG-Texas red as secondary antibodies (dilution 1:100; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature in a humidified chamber. SlowFade® Gold antifade reagent (Invitrogen) was applied on imprints before mounting.

Microscopy and imaging

Imprints were analyzed by confocal laser scanning microscopy using LSM 510 META (Carl Zeiss Microimaging, Inc., NY, USA).

Statistical analysis

Differences between experimental groups were evaluated statistically using the Steel-Dwass multiple comparison test.

Results

Usefulness of the gastric serosal surface instillation method

We previously demonstrated that the administration of naked pDNA onto the gastric serosal surface in mice resulted in stomach-selective gene expression. [25] Initially, we confirmed whether the instillation of naked pDNA (5 μg/5 μL) onto the gastric serosal surface in rats shows detectable gene expression in the stomach. As controls, we administered 5 μg/100 μL of naked pDNA intragastrically, intraperitoneally and intravenously. Six hours later, luciferase activity in the stomach was determined. Each tissue homogenate without the administration of naked pDNA was mixed with a luciferase assay substrate and showed 10^3 -10^4 RLU/g tissue, which was background luminescence. Therefore, the results represented greater than 10^4 RLU/g tissue in this study, which was considered to be the detection limit of gene expression. The gene expression level in the stomach after gastric serosal surface instillation was significantly higher than the other administration methods (Fig. 1). Moreover, gene expression was not detected in the liver, kidneys, spleen, diaphragm, heart, lung or plasma following intraperitoneal, intragastric and intravenous administration of pDNA (data not shown). These results are consistent with the results in mice. [23, 25]

Effect of pDNA instillation doses on stomach-selective gene expression

We examined that tissue distribution of gene expression 6 h after gastric serosal surface instillation of several doses of pDNA at a volume of 5 μL (Fig. 2). Although gene expression was not detected in the stomach and other tissues at 1μg (data not shown), evident gene expression was observed in the stomach at 2-10 μg. On the other hand, the gene expression level was negligible in the liver, kidneys,

spleen, diaphragm, heart, lung and plasma following gastric serosal surface instillation of naked pDNA. Regardless of the pDNA dose (2-10 μg), highly stomach-selective gene transfer was ascertained. However, gene expression in the stomach saturated over 5 μg (Fig. 3).

Effect of instillation volume on stomach-selective gene expression

We examined the tissue distribution of gene expression 6 h after gastric serosal surface instillation at several volumes of pDNA solution at a dose of 5 μg (Fig. 4). Evident gene expression was observed in the stomach at 1-50 μL of pDNA solution, while the gene expression levels were negligible in other tissues at 5-50 μL of pDNA solution. At 1 μL, gene expression was detected in the liver and spleen, albeit only slightly. On the other hand, luciferase activities in the stomach tended to gradually decrease up to 10 μL of the instillation volume of pDNA solution (Fig. 5).

Time course of gene expression after gastric serosal surface instillation of pDNA

The time course of gene expression after gastric serosal surface instillation of pDNA (5 μg/5 μL) was examined (Fig. 6). Gene expression in the stomach was observed 3 h after instillation of pDNA and increased until 12 h. The gene expression level in the stomach peaked between 12 and 24 h after instillation of pDNA, and decreased to similar level to 3 h at 48 h. Although gene expression in the spleen was observed 12 and 24 h after instillation of pDNA, the gene expression level was highest in the stomach during the indicated time point.

Microscopy of the gastric serosal surface cells

To identify gene-expressing gastric serosal surface cells, ZO-1 proteins, which is the tight junction-associated protein, were stained after gastric serosal surface instillation of pDNA (pZsGreen1-N1) (Fig. 7). Gastric serosal surface cells expressing ZsGreen1-N1 protein were surrounded by ZO-1 proteins, suggested that these cells were not macrophages. Since ZO-1 is the tight junction-associated protein, these cells might be mesothelial cells.

Discussion

Early stage gastric ulcer and gastric cancer occasionally respond to conventional pharmacological treatment. Also, early stage gastric cancer is treated by endoscopic and/or surgical excision in some cases. However, recurrent and refractory gastric ulcer and advanced gastric cancer do not generally respond to conventional therapy. Thus, stomach-targeted gene transfer is an important method for these refractory gastric diseases. Gene therapy is a rational approach for these severe diseases because a protein defect or deficiency is frequently involved. 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. To overcome these problems, researchers studied strategy by using an adenoviral vector with a β-catenin/T-cell factor-responsive promoter, which is an activated pathway in gastric cancer cells.[28] However, immunogenicity of adenoviruses restricts safety and efficacy with repeated administration. Plus, naked pDNA has advantages in terms of ease and productivity. Thus, we developed the gastric serosal surface instillation of pDNA as a novel, safe and stomach-selective gene delivery method.

The present study was performed to confirm whether stomach-selective gene transfer could be achieved in rats by instillation of naked pDNA onto the gastric serosal surface. We have already reported stomach-selective gene transfer by this administration method in mice.[25] However, gene expression was observed not only in the stomach, but also in peripheral tissues, such as the liver, left kidney and spleen. Although we instilled 30 μL of pDNA solution onto the mouse stomach, 30 μL as an administration volume may be too large for a mouse. Therefore, pDNA solution would diffuse to peripheral tissues after administration onto the gastic serosal surface. Since rats are bigger than mice,

pDNA solution would have difficulty diffusing to peripheral tissues following gastric serosal surface instillation. Additionally, we decreased the volumes of pDNA solution to body weight from 1 μL/g for the mouse to 0.033 μL/g for the rat. Moreover, in view of animal scale-up, it is important to obtain the data in animals other than mice. In the future, information about kinetics and distribution of pDNA and transgene products may be required. Rats are easier to take enough volume of the biological samples than mice, e.g. blood, bile, urine and feces. Considering further studies, the present study is basic and precious step for development of gastric serosal surface instillation as a novel stomach-selective gene transfer method.

We compared the gene expression levels in the stomach 6 h after gastric serosal surface instillation, intraperitoneal, intragastric and intravenous administration. Gastric serosal surface instillation of pDNA showed evident gene expression in the stomach, while intraperitoneal, intragastric and intravenous administration resulted in no detectable gene expression there (Fig. 1). For intraperitoneal administration, pDNA was mainly instilled onto the small intestine and could not sufficiently contact the stomach. As for intragastric and intravenous administration, naked pDNA would be degraded by digestive fluid in the stomach, or reticuloendothelial cells and nuclease in the blood, respectively. These results suggested that the gastric serosal surface is a candidate as a novel gene transfer route.

The tissue distribution of gene expression 6 h after gastric serosal surface instillation of pDNA at 2-10 μg (5 μL) was examined (Fig. 2). Although gene expression in the stomach could be detected at least 2 μg, other tissues showed no or little luciferase activities at 2-10 μg. Since 5 μL was a very small volume for the rat stomach, diffusion and contact of pDNA solution to peripheral tissues

would have been limited. To evaluate the effect of instillation doses on transfection efficiency, a conversion into a pDNA dose-dependent curve of luciferase activity in the stomach is shown (Fig. 3). Despite an increasing pDNA dose, the gene expression level was not proportional to the pDNA dose, and luciferase activity peaked at 5 μg. 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.

The tissue distribution of gene expression 6 h after gastric serosal surface instillation of pDNA at 1-50 μL (5 μg) was examined (Fig. 4). When the volume of pDNA solution increases, it is possible that gene expression in the non-target tissues will be observed. Although a large volume of solution diffuses easily to peripheral tissues after gastric serosal surface instillation, the pDNA concentration is diluted. Then, despite an increasing volume of pDNA solution, gene expression would be negligible in peripheral tissues above 5 μL. To evaluate the effect of instillation volumes of pDNA solution on gene expression, a conversion into a pDNA solution volume-dependent curve of luciferase activity in the stomach is shown (Fig. 5). At volumes up to 10 μL of instilled pDNA solution, the gene expression level in the stomach gradually decreased, while further increases in instillation volume (50 μL) maintained the same gene expression level in the stomach as at 10 μL.

The result of time course experiments suggested that gene expression was transient after gastric serosal surface instillation of pDNA, and the stomach selectivity of gene expression was high from 3-48 h after instillation (Fig. 6). Although the pDNA dose to body weight in rats was lower than that in mice, the effective term of gene expression after gastric serosal surface instillation of pDNA in rats was almost the same as that in mice.[25]

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.[24, 25] 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. For clinical use, however, diffusion cell may be troublesome and impractical for repeated administration in terms of ease and biocompatibility. Here, we demonstrated that highly stomach-selective gene transfer could be achieved by gastric serosal surface instillation of pDNA in rats. Recently, medical instruments, such as endoscopes, laparoscopes and so on, have been developed. So laparotomy is not always necessary for the gastric serosal surface instillation method. Indeed, we have already reported that liver- and lobe-selective gene transfer could be achieved by instillation of pDNA using a catheter onto the liver surface in mice.[29]

Although we evaluated the stomach selectivity of gene expression in the present study, the spatial distribution of pDNA and gene expression after gastric serosal surface instillation is important information for future clinical use. Taking the size of pDNA into consideration, pDNA may hardly penetrate to mucosal side of the stomach. To check this point, we are considering the further study for spatial distribution using fluorescent-labeled pDNA, pDNA encoding β-galactosidase or fluorescent protein.

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. [7] This process involves many genes encoding growth factors, including epidermal growth factor, VEGF, keratinocyte growth factor, HGF, 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, 30-32] Moreover, gene therapy has been tried for gastric cancer *in vitro* and *in vivo* with various strategies, such as transfer of suicide genes, [33] the p51A gene, [34] dominant negative insulin-like growth factor I receptor gene, [35] and RhoA and RhoC short interfering RNA. [36] Efficient and target-selective gene delivery systems are important factors determining whether or not gene therapy succeeds. Gastric serosal surface instillation of viral or non-viral vectors, as well as naked pDNA, could potentially succeed in stomach-selective gene transfer.

In summary, we demonstrated highly stomach-selective gene transfer following gastric serosal surface instillation of naked pDNA in rats. However, it is necessary to improve the gene transfection efficiency for clinical application. Additional studies are needed to elucidate the mechanism of gene transfer after gastic serosal surface administration of naked pDNA.

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Figure legends

Fig. 1. Gene expression in the stomach 6 h after gastric serosal surface instillation, intraperitoneal (i.p.), intragastric (i.g.) and intravenous (i.v.) administration of pDNA at a dose of 5 μg in rats. Each bar represents the mean + S.E. of at least 7 experiments. Significantly different from other methods of administration (**p<0.01).

Fig. 2. Gene expression in the stomach, liver, kidneys (left and right), spleen, diaphragm, heart, lung and plasma 6 h after gastric serosal surface instillation of pDNA at doses of 2-10 μg (5 μL) in rats. Each bar represents the mean + S.E. of at least 13 experiments.

Fig. 3. Effect of instillation doses on gene expression in the stomach 6 h after gastric serosal surface instillation of pDNA at a volume of 5 μL in rats. Each value represents the mean ± S.E. of at least 13 experiments. Significantly different from the 1 μg group (**p<0.01).

Fig. 4. Gene expression in the stomach, liver, kidneys (left and right), spleen, diaphragm, heart, lung and plasma 6 h after gastric serosal surface instillation of pDNA at a dose of 5 μg (1-50 μL) in rats. Each bar represents the mean + S.E. of at least 14 experiments.

Fig. 5. Effect of instillation volumes on gene expression in the stomach 6 h after gastric serosal surface instillation of pDNA at a dose of 5 μg in rats. Each value represents the mean ± S.E. of at least 14 experiments.

Fig. 6. Time course of gene expression in the stomach (●), liver (▲), spleen (▼) and diaphragm (■) after gastric serosal surface instillation of pDNA at a dose of 5 μg (5 μL) in rats.

Fig. 7. Immunohistochemical localization of a foreign gene product (ZsGreen1-N1 protein) in the gastric serosal surface cells.

Green, ZsGreen1-N1 protein. Red, ZO-1 protein.

Fig.1

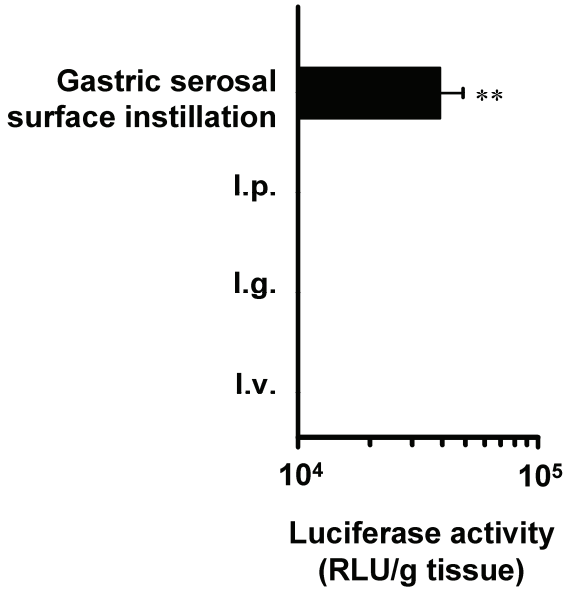
Gastric serosal surface instillation
**
I.p.
I.g.
I.v.
10^4
10^5
Luciferase activity (RLU/g tissue)

Fig.2

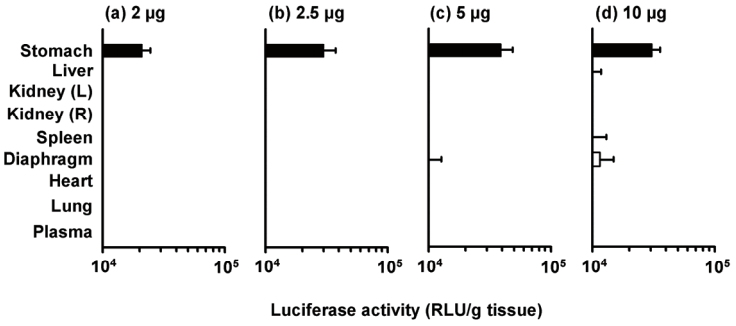
(a) 2 μg
(b) 2.5 μg
(c) 5 μg
(d) 10 μg
Stomach
Liver
Kidney (L)
Kidney (R)
Spleen
Diaphragm
Heart
Lung
Plasma
10⁴
10⁵
Luciferase activity (RLU/g tissue)

Fig.3

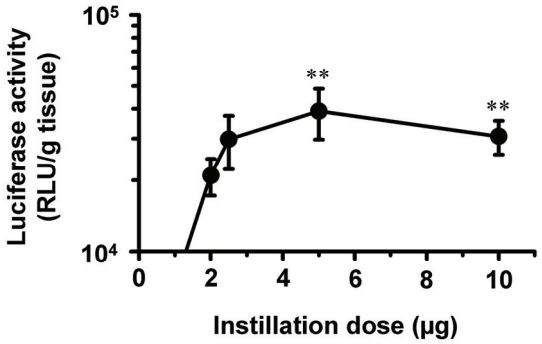
Luciferase activity
(RLU/g tissue)
10⁵
10⁴
**
**
0
2
4
6
8
10
Instillation dose (μg)

Fig.4

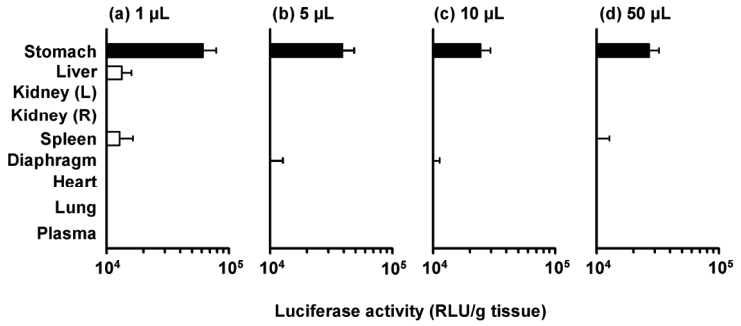
(a) 1 μL
(b) 5 μL
(c) 10 μL
(d) 50 μL
Stomach
Liver
Kidney (L)
Kidney (R)
Spleen
Diaphragm
Heart
Lung
Plasma
10⁴
10⁵
Luciferase activity (RLU/g tissue)

Fig.5

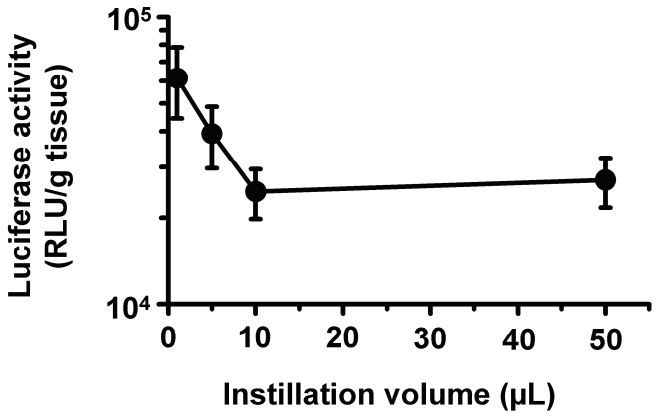
Luciferase activity
(RLU/g tissue)
10^5
10^4
0
10
20
30
40
50
Instillation volume (μL)

Fig.6

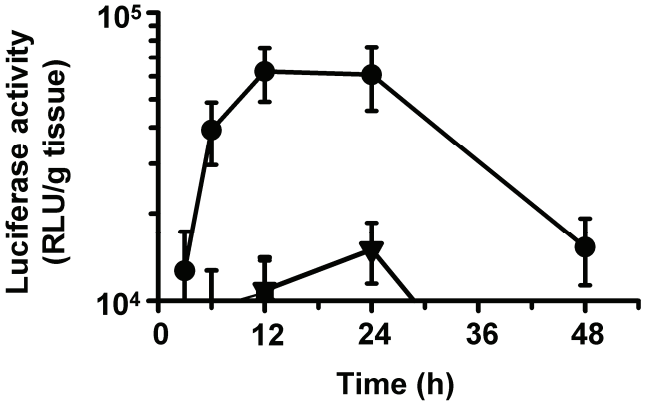

Fig.7