Rubbing Gastric Serosal Surface Enhances Naked Plasmid DNA Transfer in Rats and Mice

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We have developed *in vivo* gene transfer to mesothelial cells on the peritoneal organs, including the stomach. Simple instillation of naked plasmid DNA onto the gastric serosal surface in mice resulted in effective but transient transgene expression. Here, we developed a simple method to improve not only the transfection efficiency but also the duration of transgene expression. Rubbing the gastric serosal surface using a medical spoon immediately after instillation of naked plasmid DNA onto the gastric serosal surface resulted in 59-fold higher transgene expression 24 h after administration in rats. Without rubbing, transgene expression decreased under the detection limit 7 d after administration. On the other hand, rubbing the gastric serosal surface with a medical spoon after instillation of plasmid DNA prolonged transgene expression for one month. Mechanistic study in mice revealed that improved transfection should not be due to stimulation of cell function such as macropinocytosis by rubbing because rubbing before instillation of plasmid DNA did not improve transfection. Plasmid DNA should enter effectively into cells during rubbing. These findings are valuable to develop an effective method of *in vivo* gene transfer into peritoneal organs.

Key words non-viral gene delivery; gene therapy; transfection; naked plasmid DNA

The stomach is a digestive organ that is essential for nutrient intake. Alcohol, drugs, stress, and *Helicobacter pylori* reduce barrier function against acidic pH, subsequently causing gastritis and gastric ulcer. Moreover, gastric cancer is one of the most common malignant tumors in the world. The mortality due to Japanese gastric cancer was over 50000 deaths annually according to the Center for Cancer Control and Information Services, National Cancer Center, Japan. Surgical resection of gastric cancer without metastasis is a first-line therapy and provides relatively good prognosis compared with that of other cancers. However, recurrence, liver metastasis, and/or peritoneal dissemination in gastric cancer are serious problems associated with high mortality. Advanced gastric cancer does not generally respond to conventional chemotherapy or radiotherapy.[1)]

Gene delivery to the stomach is a rational approach to treat gastric ulcer and cancer since various genes are correlated with these diseases.[2)] There are mainly three routes to deliver a transgene to the stomach: mucosal route,[3)] direct injection,[4)] and serosal route.[5—7)] Gene transfer *via* the mucosal route is hardly possible owing to the barrier function of the mucosal layer of the stomach. Direct injection is effective to transfer a transgene; however, the area of transfection is restricted to the injection site(s). The serosal route, which we have developed,[5—7)] is attractive for treatment against serosal invasion of gastric ulcer and cancer. Interestingly, simple instillation of naked plasmid DNA solution onto organ surfaces, including liver,[8)] kidney,[9)] spleen,[10)] and gastric serosal surfaces,[5—7)] resulted in effective transgene expression in mice and rats. We already elucidated that the mechanism of naked plasmid DNA transfer was uptake *via* Rac-mediated macropinocytosis.[11)] However, we were not sure whether the transfection efficiency was sufficient to treat gastric diseases. Moreover, the duration of transgene expression was relatively short. Thus, we considered it necessary to improve transgene expression.

We hypothesized two strategies to improve transgene expression. One is enhancement of Rac signaling pathway controlling macropinocytosis of plasmid DNA. However, protein kinase C enhancer (phorbol 12-myristate 13-acetate, PMA), which is one of the macropinocytosis enhancers, did not affect transgene expression after naked plasmid DNA transfer onto the gastric serosal surface in mice (data not shown). Another strategy is utilization of physical force(s). With regard to physical forces, several methods have been reported, such as electroporation[12)] and use of a gene gun.[13)] However, both methods require specialized devices. Here, we developed a simple method to enhance transgene expression in gastric serosal surface cells by rubbing the surface using a medical spoon after instillation of naked plasmid DNA in rats and mice. This simple rubbing method improved not only transfection efficiency but also duration of transgene expression.

MATERIALS AND METHODS

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

Animals Male Wistar rats and 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 Guidelines for Animal Experimentation of Nagasaki University.

Construction and Preparation of Plasmid DNA Plasmid DNA encoding firefly luciferase (pCMV-luciferase) was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from a pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of a pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). pZsGreen1-N1 encoding reef coral green fluorescent protein was obtained

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from Takara Bio Inc. (Shiga, Japan). Plasmid DNA was amplified in the *Escherichia coli* strain DH5α, isolated, and purified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Plasmid DNA dissolved in 5% glucose solution was stored at −20 °C prior to experiments.

In Vivo* Gene Expression Experiments** Five-week-old male Wistar rats (140—170 g) or ddY mice (23—32 g) were anesthetized with sodium pentobarbital (40—60 mg/kg, intraperitoneal administration). Laparotomy was performed and the stomach was exposed. Naked plasmid DNA solution (5 μg/5 μl) 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 the stomach. Immediately before or after instillation of plasmid DNA, the gastric serosal surface was rubbed with a medical spoon (area of round edge: 0.13 cm^2). Applied force was approximately 0.3 N/cm^2, and duration of rubbing was 30 s. Then, the abdominal wall and the skin were sutured. Subsequently, animals were freed in a cage. At appropriate time intervals after the administration of naked plasmid DNA, animals were killed under anesthesia, and the stomach, liver, kidneys (left and right), spleen, and diaphragm 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 ethylenediaminetetraacetic acid (EDTA).[14] The volume of the lysis buffer that was added was 4 μl/mg of tissue. Homogenates were centrifuged at 15000×g*** for 5 min. Each supernatant was assayed for luciferase activity.

Luciferase Assay Twenty microliters of tissue homogenate supernatant was 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.

Fluorescent Stereomicroscopy for the Stomach Five micrograms of pZsGreen1-N1 (5 μl) were instilled onto the gastric serosal surface with or without rubbing in rats under anesthesia. One or 7 d after administration, transgene expression (ZsGreen1) on the stomach was observed using a fluorescent stereomicroscope (MZ-16F with Plan-apo 1×NA 0.141 objective lens; Leica Microsystems GmbH, Wetzlar, Germany). The camera was DFC300FX (Leica Microsystems GmbH). Acquisition software was Leica Application Suit (Leica Microsystems GmbH).

Statistical Analysis Statistical comparisons were performed by the Mann–Whitney *U* test (Fig. 1) or by Steel's multiple comparison test (Fig. 3).

RESULTS AND DISCUSSION

Figure 1 shows *in vivo* transfection efficiency of instillation of naked plasmid DNA onto a gastric serosal surface with or without rubbing 24 h after administration in rats. Rubbing the gastric serosal surface with a medical spoon after instillation of plasmid DNA significantly enhanced transfection efficiency by 59-fold. In addition, rubbing the gastric serosal surface greatly improved the selectivity of transfection to the gastric serosal surface against that of other organs by 10- to 100-fold (Table 1). Moreover, rubbing the gastric serosal surface notably prolonged the duration of transgene expression (Fig. 2). Transfection efficiency without rubbing decreased under the detection limit 7 d after instillation of plasmid DNA. In contrast, transfection efficiency with rubbing was detectable for at least one month.

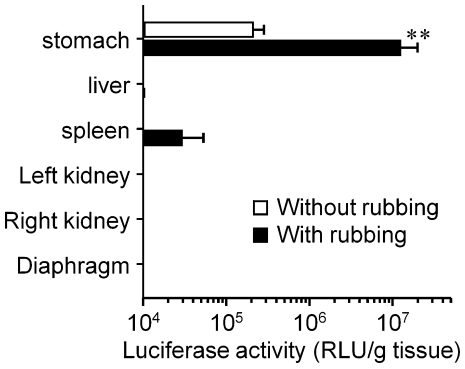

Fig. 1. Transgene Expression with (Filled Bars) or without Rubbing (Open Bars) after Instillation of Naked Plasmid DNA onto Gastric Serosal Surface in Rats

Transgene expression in each tissue was determined 24 h after administration of plasmid DNA. Statistical comparisons were performed by Mann–Whitney *U* test (**$p<0.01$ *vs.* without rubbing group). Each value represents the mean±S.E. of at least 10 experiments.

Table 1. Selectivity of Transgene Expression in the Stomach against That of Other Organs after Instillation of Plasmid DNA onto Gastric Serosal Surface in Rats

Ratio	St/Li	St/Sp	St/LK	St/RK	St/D
Without rubbing	42	41	866	350	55
With rubbing	1658	421	8985	13355	6214

Symbols: St, stomach; Li, liver; Sp, spleen; LK, left kidney; RK, right kidney; D, diaphragm.

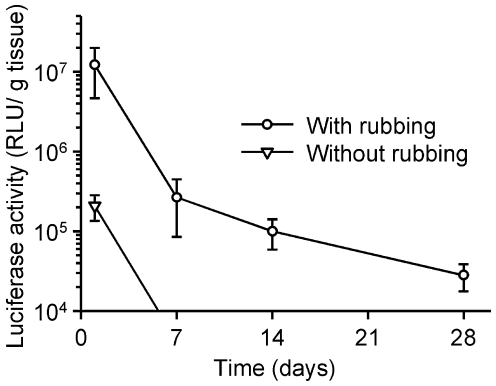

Fig. 2. Time Course of Transgene Expression with (Circle) or without Rubbing (Inverted Triangle) after Instillation of Naked Plasmid DNA onto Gastric Serosal Surface in Rats

Each value represents the mean±S.E. of at least 10 experiments.

We have tried to improve the transfection efficiency after instillation of naked plasmid DNA onto peritoneal organs since the first report about the liver surface.[15] The composition of the solution of plasmid DNA was shown to be an important factor when glass-made cylindrical diffusion cell was applied to limit the application area.[16] Hypotonic solution enhanced the transfection efficiency of naked plasmid DNA administered into the diffusion cell, while hypertonic solu-

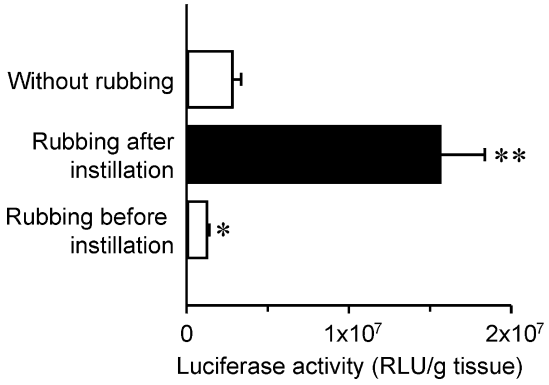

Fig. 3. Timing of Rubbing Affects Transfection Efficiency after Administration of Naked Plasmid DNA onto Gastric Serosal Surface in Mice

Transgene expression in the stomach was determined 6 h after administration of plasmid DNA. Statistical comparisons were performed by Steel's multiple comparison test ($*p<0.05$, $**p<0.01$ *vs.* without rubbing group). Each value represents the mean± S.E. of 12 experiments.

tion inhibited transfection efficiency. However, when we simply instilled several solution compositions of naked plasmid DNA, the transfection efficiency did not change. To obtain information for improvement of the transfection efficiency after instillation of naked plasmid DNA, we analyzed the detailed transfection mechanism of naked plasmid DNA transfer into gastric mesothelial cells.[11] The endocytic route for naked plasmid DNA was neither clathrin- nor caveolae-mediated endocytosis, but rather macropinocytosis. Rac-mediated cell signaling pathway controlled macropinocytosis of naked plasmid DNA in gastric mesothelial cells. In accordance with this information, we tried to improve the transfection efficiency using macropinocytosis enhancer PMA; however, PMA did not improve the transfection efficiency. Then, we focused on the improvement mechanism of rubbing method. Using mice, we changed the timing of rubbing (Fig. 3). Rubbing before instillation of plasmid DNA onto the gastric serosal surface did not change the transfection efficiency, while rubbing after instillation significantly enhanced transfection efficiency. Thus, improved transfection by rubbing should not be due to stimulation of cell function such as macropinocytosis. Plasmid DNA should enter into cells effectively during rubbing.

The spatial distribution of transgene expression with or without rubbing after gastric serosal surface instillation of plasmid DNA is important information for future clinical use. Taking the size of plasmid DNA into consideration, plasmid DNA may hardly penetrate into the mucosal side of the stomach. To check this, we evaluated the distribution of transgene expression using plasmid DNA encoding green fluorescent protein ZsGreen1 (Fig. 4). Rubbing the gastric serosal surface clearly increased the number of transgene-positive cells (Fig. 4C) compared with that in the no rubbing group (Fig. 4A) 1 d after instillation of plasmid DNA. In accordance with luciferase activity, transgene-positive cells could be detected 7 d after instillation of plasmid DNA (Fig. 4D). Furthermore, the shapes of transgene-positive cells were not only mesothelial-like (Fig. 4E) but also muscle-like (Fig. 4F) upon rubbing the gastric serosal surface. About the spatial structure of the stomach, stomach can divide 4 layers; *i.e.* mesothelial layer (serosal side), muscular layer, submucosal layer and epithelial layer (mucosal side). This result should

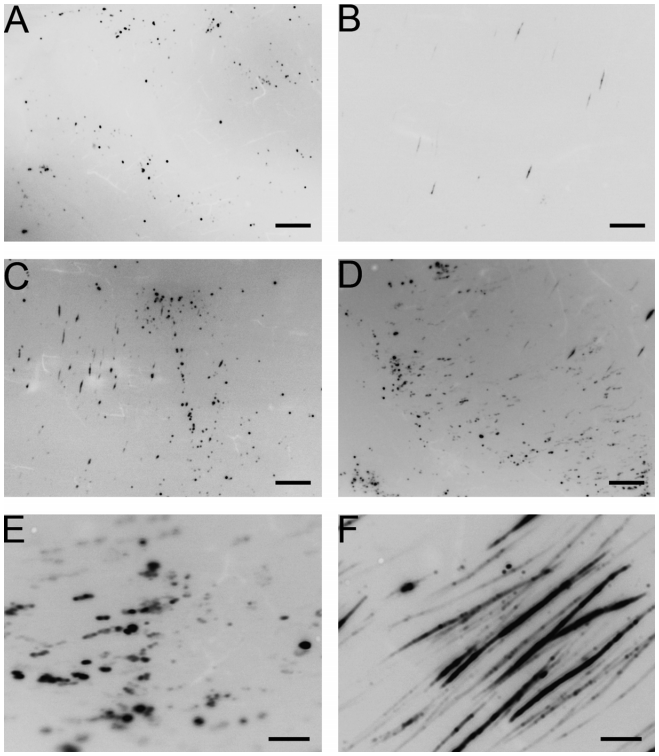

Fig. 4. Detection of Transgene-Positive Cells with (C—F) or without Rubbing (A, B) after Instillation of Naked Plasmid DNA onto Gastric Serosal Surface in Rats

Transgene-positive cells on the stomach were detected 1 (A, C) or 7 d (B, D—F) after administration of plasmid DNA. Color images were converted into grayscale; subsequently, white and black colors were reversed to check transgene-positive cells easily (green fluorescence of ZsGreen1 was converted to a black color). Scale bar, 0.5 mm (A—D), 100 μm (E, F).

indicate that rubbing operation may improve the depth of penetration of plasmid DNA.

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 plasmid DNA 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.[17] This process involves many genes encoding growth factors, including epidermal growth factor, vascular endothelial growth factor, 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*.[18—21] Moreover, gene therapy has been tried for gastric cancer *in vitro* and *in vivo* with various strategies, such as transfer of suicide genes,[22] the p51A gene,[23] dominant negative insulin-like growth factor I receptor gene,[24] and RhoA and RhoC short interfering RNA.[25] Efficient and target-selective gene delivery systems are important factors determining whether gene therapy succeeds.

In this study, we focused on stomach-selective enhancement of transgene expression. On the other hand, we also deliver naked plasmid DNA onto the liver,[8] kidney,[9] spleen

surface.[10] Here, rubbing organ surface might be applicable to these organs. Exactly, transgene positive cells on the liver surface 7 d after instillation of plasmid DNA and subsequent rubbing was detectable at a similar level with those on the stomach (data not shown). Gene transfers to the liver or kidney are theoretically useful against hepatoma or renal cancer. Moreover, gene transfer to the spleen might be applicable as DNA vaccine as discussed previously.[10]

In summary, we demonstrated that rubbing the gastric serosal surface with a medical spoon enhanced transgene expression following gastric serosal surface instillation of naked plasmid DNA in rats and mice. This information is useful to develop an effective method of *in vivo* gene transfer into peritoneal organs.

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