Pretreatment with Epidermal Growth Factor Enhances Naked Plasmid DNA Transfer onto Gastric Serosal Surface in Mice

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We have developed a simple administration method, which is gastric serosal surface instillation of naked plasmid DNA (pDNA) in experimental animals. The purpose of this study was to improve gastric gene transfer efficiency by pre-treatment with a macropinocytosis enhancer, such as fetuin or epidermal growth factor (EGF), in mice. A series of concentrations of fetuin were instilled onto gastric serosal surface prior to instillation of naked pDNA in mice; however, fetuin did not improve transgene expression in the stomach 6 h after administration of pDNA. EGF also did not affect transgene expression in the stomach when pDNA was instilled immediately after EGF instillation. On the other hand, when pDNA was instilled onto gastric serosal surface 24 h after EGF treatment, transgene expression in the stomach was significantly improved by 2.6-fold. In addition, transgene-positive cells were increased 5.3-fold by EGF pre-treatment. High transgene expression in the stomach lasted for 48 h in the EGF pre-treatment group in comparison with that in the no pre-treatment group. These findings are valuable to develop an effective method of *in vivo* gene transfer to the stomach.

Key words gene therapy; non-viral gene delivery; epidermal growth factor; stomach; naked plasmid DNA

The stomach is an important target organ for gene delivery owing to its capabilities for storing, mixing, digesting and sterilizing foods with gastric acid. Many people suffer from stomach disorders such as acute and chronic gastritis, gastric ulcer and gastric cancer. In particular, gastric cancer is one of the most common malignant tumors worldwide. The mortality due to gastric cancer in Japanese was over 50000 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 problems in terms of transfection efficiency, they 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 the toxicity of the gene carrier. Indeed, clinical investigations have been performed using naked pDNA that encodes hepatocyte growth factor (HGF) for treatment of peripheral arterial disease in Japan.[6] 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.[7] It was previously reported that organ-selective gene transfer using naked pDNA was achieved by direct injection,[8] electroporation,[9] gene gun[10] and so on. Transgene expression in the stomach was observed by direct injection of pDNA into the gastric submucosa in rats.[11] However, there is great concern about safety because these procedures require physical force against organs; consequently, the possibility of continuous and repetitive administration of pDNA is limited. However, although pDNA complexes with chitosan,[12] *N*-acetylated chitosan[13] and montmorillonite[14] were studied for oral gene delivery, the oral route has many factors that reduce transfection efficiency, such as gastric contents, low pH, high concentration of digestive fluid and rapid turnover of epithelial cells. In fact, transgene expression was not observed in the stomach following oral administration with nanoparticles in microspheres in rats.[15]

We previously developed a method to apply drugs onto the surface of intraperitoneal organs such as the liver,[16] kidney[17] and stomach,[18–20] 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,[21] kidney surface[22] and gastric serosal surface[23,24] in mice. Recently, we found that stomach selectivity of transgene expression was sufficiently high when animal scale-up was performed using rats.[25] Next, we elucidated the mechanism of naked pDNA transfer into gastric mesothelial cells to obtain useful information for rationalized strategies to improve transgene expression. The uptake route of naked pDNA for efficient transgene expression was neither clathrin- nor caveolae-mediated endocytosis, but rather macropinocytosis.[26] Thus, in this study, we tested two macropinocytosis enhancers, fetuin and epidermal growth factor (EGF), to improve efficiency of naked pDNA transfer in mice.

MATERIALS AND METHODS

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

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

The authors declare no conflict of interest.

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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 U.S. National Institutes of Health and the Guidelines for Animal Experimentation of Nagasaki University.

Construction and Preparation of pDNA Plasmid DNA encoding firefly luciferase driven by cytomegalovirus promoter (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 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 stored at −20°C prior to experiments.

Gastric Serosal Surface Instillation Five-week-old male ddY mice (20–37 g) were anesthetized with sodium pentobarbital (40–60 mg/kg, intraperitoneal administration). Laparotomy was performed and the stomach was exposed. Naked pDNA 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 stomach. Then, the abdominal wall and the skin were sutured. Subsequently, mice were freed in the cage. At appropriate time intervals (6, 24, 48, 96 h) after administration of naked pDNA, mice were killed under anesthesia, and the stomach was 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).[27] The volumes of the lysis buffer added were 4 μL/mg of tissue. Blood samples and 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 (the relative light units/g tissue) was converted into the amount of luciferase (pg/g tissue) using a standard curve.

Fluorescent Stereomicroscopy for the Stomach Five micrograms of pZsGreen1-N1 (5 μL) was instilled onto the gastric serosal surface in mice under anesthesia. One day 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 Suite (Leica Microsystems GmbH).

Macropinocytosis Enhancer Bovine fetuin (Calbiochem (Merck Ltd., Tokyo, Japan)) and mouse EGF (BD Biosciences, CA, U.S.A.) were used as macropinocytosis enhancers. Fetuin (2.5, 5 or 25 μg/5 μL in phosphate-buffered saline (PBS)) or EGF (0.3 or 3 ng/5 μL in 5% glucose solution) was instilled onto gastric serosal surface directly, then naked pDNA was instilled onto the same area. In addition, pDNA was instilled at intervals. In these experiments, the skin near the stomach was cut, and subsequently a 26 G×1/2″ needle (Nipro Co., Ltd., Osaka, Japan) was inserted *via* the abdominal wall. Then, fetuin (25 μg/5 μL in PBS) or EGF (3 ng/5 μL in 5% glucose solution) was injected onto gastric serosal surface. At an appropriate time interval (30 min, 12, 24 or 48 h), naked pDNA was administered in accordance with the above procedures.

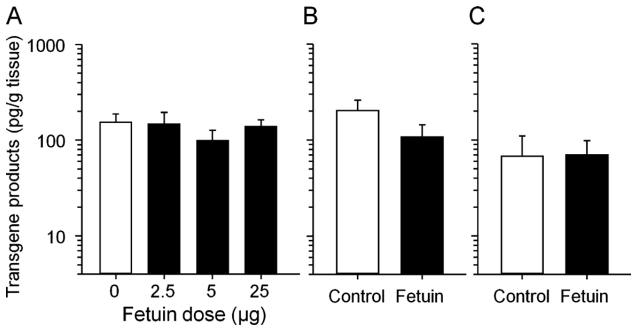

Fig. 1. Effect of Fetuin on Transgene Expression in the Stomach 6 h after Gastric Serosal Surface Instillation of Naked pDNA in Mice

(A) Effect of fetuin dose on transgene expression. (B) Effect of 30 min and (C) 24 h interval between fetuin treatment and pDNA instillation on transgene expression. Each bar represents the mean+S.E. of at least 6 experiments. There are no significant differences.

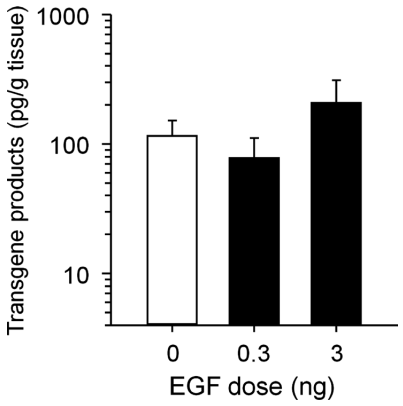

Fig. 2. Effect of EGF Dose on Transgene Expression in the Stomach 6 h after Gastric Serosal Surface Instillation of Naked pDNA in Mice

Each bar represents the mean+S.E. of at least 11 experiments. There are no significant differences.

Statistical Analysis Statistical comparisons were performed by the Wilcoxon rank sum test for two groups or by Steel's test for multiple comparisons with the control group.

RESULTS

Effect of Fetuin Treatment on Transgene Expression We previously demonstrated that the administration of naked pDNA onto the gastric serosal surface in mice resulted in stomach-selective transgene expression.[23,24] To enhance transgene expression, we utilized fetuin as a macropinocytosis enhancer. Figure 1A shows the effect of fetuin dose on transgene expression 6 h after instillation of naked pDNA. As a result, no improvement of transgene expression was observed. In this condition, naked pDNA was immediately instilled after fetuin treatment. We hypothesized that stimulation of macropinocytosis may require time intervals. Thus, we set time intervals

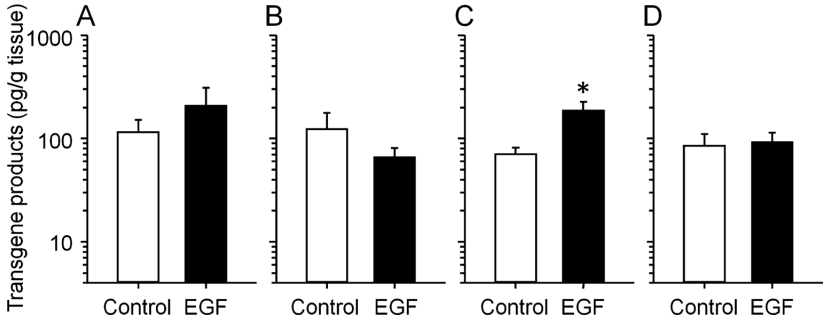

Fig. 3. Effect of Time Intervals between EGF Treatment and pDNA Instillation on Transgene Expression in the Stomach 6 h after Gastric Serosal Surface Instillation of Naked pDNA in Mice

Time intervals: (A) 0 min, (B) 30 min, (C) 24 h, (D) 48 h. Each bar represents the mean+S.E. of at least 10 experiments. Significant difference from control (*$p<0.05$).

(30 min and 24 h) between fetuin treatment and naked pDNA instillation (Figs. 1B, C). Again, no improvement of transgene expression was observed.

Effect of EGF Treatment on Transgene Expression We chose EGF as another macropinocytosis enhancer. Even when we increased the EGF dose 10-fold, EGF did not change the transfection efficiency (Fig. 2). Next, we hypothesized that stimulation of macropinocytosis may require time intervals similar to fetuin experiments. Intervals of 24 h between EGF treatment and naked pDNA instillation significantly improved gastric transgene expression by 2.6-fold (Fig. 3C). The improving effect of EGF on transfection was stomach-selective (Table 1). The improvement of transgene expression lasted for 48 h (Fig. 4). Unfortunately, transgene expression level was decreased around the detection limit 96 h after naked pDNA administration.

Transgene-Positive Cells on the Stomach To evaluate whether EGF increased transgene-positive cells, we used pZsGreen1-N1, green fluorescent protein-expressing pDNA (Fig. 5). Not only the middle body of the stomach (instilled area), transgene expression was spreading to greater curvature. However, transgene expression was rare in the forestomach (data not shown). Then, we counted transgene-positive cells.

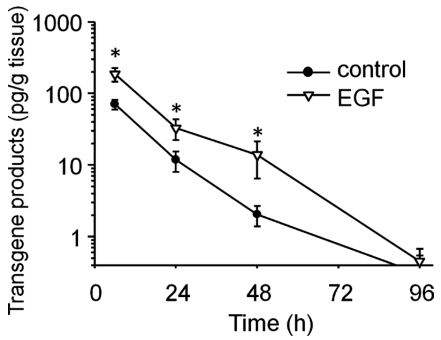

Fig. 4. Time Course of Transgene Expression in the Stomach after Gastric Serosal Surface Instillation of pDNA in Mice

Control group (closed circles), EGF pre-treatment group (24 h prior to pDNA administration, inverted triangles). Each symbol represents the mean±S.E. of at least 6 experiments. Significant difference from control (*$p<0.05$).

EGF treatment increased transgene-positive cells significantly (control; 96.3±43.1 cells, EGF; 507.0±85.6 cells, $p<0.01$).

DISCUSSION

Early stage gastric ulcer and gastric cancer occasionally

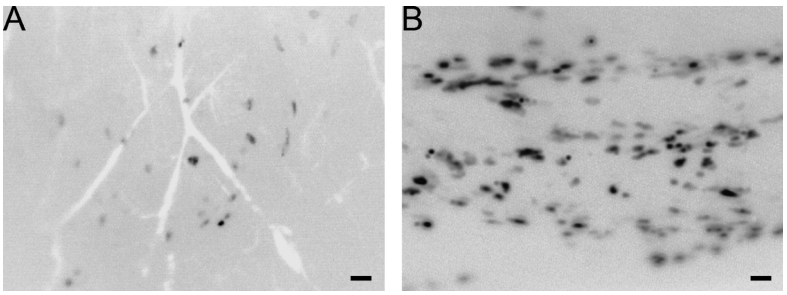

Fig. 5. Detection of Transgene-Positive Cells on the Stomach after Gastric Serosal Surface Instillation of pDNA in Mice

(A) Control group, (B) EGF pre-treatment group (24 h prior to pDNA administration). Twenty-four hours after pDNA administration, transgene expression of green fluorescent protein ZsGreen1 was observed. 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 into a black color). Scale bar, 100 μm.

Table 1. Effect of Pretreatment with EGF on Selectivity of Transgene Expression in the Stomach against That of Other Organs after Instillation of pDNA onto Gastric Serosal Surface in Mice

Ratio	St/Li	St/Sp	St/LK	St/RK	St/D
Control	1.6	6.2	4	22	2.1
EGF	18	22	75	80	13

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

respond to conventional pharmacological treatment. In addition, 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.[28] 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 a strategy using an adenoviral vector with a β-catenin/T-cell factor-responsive promoter, which is an activated pathway in gastric cancer cells.[29] However, the immunogenicity of adenoviruses restricts their safety and efficacy for repeated administration. Furthermore, 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 demonstrated that *in vivo* transfection efficiency to the stomach was successfully improved by treatment with EGF prior to naked pDNA instillation onto gastric serosal surface in mice, while fetuin did not affect the transfection efficiency significantly. Not only transgene expression but also the number of transgene-positive cells was increased by EGF treatment (Figs. 3–5). It was reported that fetuin, also known as alpha2-HS glycoprotein, enhanced phagocytosis of apoptotic cells and macropinocytosis by human macrophages.[30] Furthermore, Nagayama *et al.* demonstrated that fetuin mediated hepatic uptake of negatively charged nanoparticles.[31] In this study, however, fetuin was ineffective to improve transfection efficiency. On the other hand, EGF has been used as a macropinocytosis enhancer.[32] We found that time interval between EGF treatment and pDNA administration was an important factor to determine the enhancing effect of EGF on transgene expression (Fig. 3). Enhancing effect of EGF on macropinocytosis appears several minutes after treatment.[32] On the other hand, macropinocytosis enhancer fetuin did not enhance transgene expression (Fig. 1). Thus, not only the enhancing effect on macropinocytosis but also other effects of EGF may have contributed to enhanced transgene expression in this study. Especially, stimulation of proliferation by EGF might affect transfection efficiency of naked pDNA since disappearance of nuclear envelop during proliferation could theoretically enhance nuclear import of pDNA.

We have demonstrated that the solution composition of pDNA is an important factor when a glass-made cylindrical diffusion cell is applied to limit application area.[33] Hypotonic solution enhanced the transfection efficiency of naked pDNA administered into the diffusion cell, while hypertonic solution inhibited the transfection efficiency. However, when we simply instilled several solution compositions of naked pDNA, transfection efficiency did not change. In view of clinical use, a diffusion cell was troublesome and impractical for repeated administration. Here we demonstrate that EGF pre-treatment improved naked pDNA transfer to the stomach without the diffusion cell; as a consequence, this method should be favorable for clinical use. Recently, medical instruments, such as endoscopes and laparoscopes, have been developed. Therefore, 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.[34]

Stomach-selective or -specific gene transfection methods are expected to be safe and effective treatments against refractory gastric ulcer and gastric cancer.[28] 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.[35] 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*.[2,36–38] Moreover, gene therapy has been tried for gastric cancer *in vitro* and *in vivo* with various strategies, such as transfer of suicide genes,[39] the p51A gene,[40] dominant negative insulin-like growth factor I receptor gene,[41] and RhoA and RhoC short interfering RNA.[42] 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.

Pharmacological effects of EGF may affect outcome from therapy against gastric ulcer and cancer. Especially, proliferative effect of EGF may cause positive reaction against ulcer healing and negative reaction against cancer. However, administered EGF on serosal side of the stomach can hardly migrate to mucosal side in which both ulcer healing and cancer generation occur. Thus, effect of EGF on outcome from therapy against gastric ulcer and cancer may be limited.

Recently, we discovered a simple method to improve not only the transfection efficiency but also the duration of transgene expression, which rubbing the gastric serosal surface using a medical spoon immediately after instillation of naked pDNA onto the gastric serosal surface in rats and mice.[43] 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 pDNA did not improve transfection. pDNA should enter effectively into cells during rubbing. In this study, mechanism of the effect of EGF on transfection was unclear. But, if EGF improves nuclear import as mentioned above, combination of rubbing and pretreatment with EGF may be useful to enhance transfection.

In summary, we demonstrated that macropinocytosis enhancer EGF pre-treatment significantly enhanced *in vivo* naked pDNA transfer to the stomach in mice, while macropinocytosis enhancer fetuin failed to improve naked pDNA transfer. Timing of EGF-treatment was an important factor. Information in the present study is valuable for future clinical application of gene therapy against severe gastric ulcer and cancer.

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