Analysis of transgene expression profile-dependent induction of transgene-specific immune response

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Contents

Preface

Gene therapy is expected to become a new treatment for refractory diseases such as cancers and virus infections [1-6]. The therapeutic effects of gene therapy depend on the expression profile and biodistribution of the transgene product and types of cells expressing transgene product [7-10]. Therefore, much effort has been made to develop methods that can regulate these factors. In previous studies performed in my labolatory, it was demonstrated that construction of plasmid vectors with reduced the number of CpG motifs and optimized promoter allows sustained transgene expression [11-13]. The transgene product is sometimes recognized by immune system, which causes immune response [14-16]. Induction of transgene-specific immune response could affect transgene expressing cells and cause adverse effects [17-18]. Induction of the immune response to transgene products or, in other words, the encoded therapeutic protein, is a serious concern in gene therapy [19-20].

It is generally known that induction of the immune response depends on several factors, including the antigenicity of the protein, types of transgene-expressing cells, transgene expression profile, and subcellular localization or secretion of the product [7, 9]. However, the exact nature of the relationship between the transgene expression profile and immune induction following gene transfer is unclear despite the fact that many efforts by our labolatory and others have been made to develop methods that regulates transgene expression profile [11-13].

In this study, I investigated the effects of transgene expression profile, type of transgene expressing cells and transgene expression level on the induction of transgene-specific immune response. In addition, I tried to identify the cells that work as antigen presenting cells (APCs) in the induction of transgene-specific immune response after gene delivery by the hydrodynamics-based procedure.

In Chapter I, the effect of transgene expression profile on the induction of immune response against the transgene product was examined. Firefly luciferase (fLuc) was selected as a model anitigen and *Gaussia* luciferase (gLuc) was chosen as a reporter protein. Two types of fLuc-expressing plasmid DNA, a long-term expression plasmid pCpG-fLuc or a short-term expression plasmid pCMV-fLuc, were injected into mice by hydrodynamic injections along with a gLuc-expressing long-term plasmid (pROSA-gLuc) whose transgene expression was used to evaluate the transgene expression profile of fLuc-expressing plasmids in the liver affected by immune response because simultaneous injection of the fLuc- and gLuc-expressing plasmid DNAs resulted in the expression of fLuc and gLuc in the same cells. In addition, the effect of transgene expression in splenic cells and macrophages on the induction of immune response was evaluated by spleen removal and macrophage depletion.

In Chapter I, it was found that high and sustained transgene expression of fLuc induced the immune responses to the antigenic protein. However, it was not investigated whether total amount of transgene product or the amount of transgene product per cell is important for the induction of

transgene-specific immune response. In Chapter II, the relationship between the amount of transgene product in each cell and the induction of immune response against the transgene product was examined in more detail. *Cypridina* luciferase (cLuc), a secretory reporter protein that has antigenisity, was used as a model antigen. gLuc-expressing plasmid was delivered with cLuc-expressing plasmid as above by one high-dose hydrodynamic injection or by three low-dose injections. Then, I examined whether the total level of transgene expression or the level of transgene expression per cell is important for the induction of transgene-specific immune responses. Finally, I investigated whether hepatocytes work as APCs after hydrodynamic injection of plasmid vectors using ovoalbumin (OVA) as a model antigen.

In this thesis, the results are presented in the following two chapters.

Chapter I
Elucidation of the effect of the duration of transgene expression on the induction of transgene-specific immune response

I-1 Introduction

The therapeutic effects of gene therapy depend on the types of transgene expressing cells, as well as the expression profile and biodistribution of the transgene, and much effort has been made to develop methods that control these factors. In previous studies prformed in my labolatory, it was reported that the plasmids with reduced numbers of CpG motifs or with optimized promoter and enhancer can be used for achieving sustained transgene expression [21]. It was also demonstrated that these novel plasmid vectors can be used to increase the therapeutic effects of interferon gene transfer against tumor metastasis, atopic dermatitis or hepatitis. A drawback of sustained transgene expression, however, is that it might increase the risk of induction of immune response to the transgene product. Immune response to the transgene product or, in other words, the encoded therapeutic protein, is a major concern in gene therapy because it would cause serious adverse effects and affect the transgene expression profile [22].

Induction of the immune response depends on several factors, including the antigenicity of the protein, the type of transgene-expressing cells, transgene expression profile, and the subcellular localization or secretion of the product. A previous study suggested that transgene expression in antigen-presenting cells (APCs) is a risk factor for eliciting immune response to the transgene [23]. On the other hand, studies investigating the relationship between the transgene expression profile and the immune response to the product are limited. Bates *et al*. reported that hydrodynamic injection of plasmid DNA expressing firefly luciferase (fLuc) driven by a cytomegalovirus (CMV) promoter, which resulted in a transient luciferase expression, hardly induced anti-fLuc antibody production, whereas administration of an fLuc expression vector driven by a ubiquitin promoter, which generated sustained transgene expression, induced anti-fLuc antibody production [24]. The same group also investigated whether incorporation of target sites for APC-specific microRNA (miRNA) in the plasmid and the use of cell type specific promoters would be effective approaches for reducing the risk of immune responses to the transgene product [25].

In general, the reduction in transgene expression could be accounted for by two different phenomena: the removal of transgene-expressing cells as a consequence of the immune response or a reduced efficiency of transgene expression by processes such as promoter inactivation. It is necessary to distinguish between these two phenomena to clearly understand the association between the transgene expression profile and the immune response. This issue, however, has not been fully addressed in previous

studies.

In the present study, I investigated the effect of the transgene expression profile on the induction of an immune response to the transgene product. Accordingly, different types of plasmid vectors were administered by hydrodynamic injections to obtain transient and sustained transgene expression profiles. fLuc was selected as a model antigen because (i) the amount of fLuc can be quantitatively determined using the luciferase assay and (ii) fLuc is immunogenic and elicits an immune response [26,27].

The immune response to fLuc-expressing cells was evaluated using *Gaussia* luciferase (gLuc) as a reporter, which was accomplished by co-administering a gLucexpressing plasmid along with the fLuc-expressing vectors. This approach was employed for several reasons: (i) our preliminary study indicated that hydrodynamic administration of the gLuc-expressing vector, pROSA-gLuc, resulted in sustained, high gLuc expression for more than 1 year (Takahashi Y and Matsui Y, unpublished data); (ii) the distribution of two vectors co-administered by hydrodynamic injection almost completely overlapped [28]; (iii) the expression levels of gLuc can be quantitatively and reproducibly determined by measuring serum gLuc activity without sacrificing the mice; and (iv) the use of gLuc protein as a marker for transgene-expressing cells enables us to monitor the removal of these cells as a consequence of the immune response to fLuc because the reduction in serum gLuc activity is ascribable to the removal of the transgene-expressing cells and not to any phenomenon reducing the transgene expression levels in the cells. Plasmids that stably (pCpG-fLuc) or transiently (pCMV-fLuc) express fLuc were co-administered with the pROSA-gLuc vector to mice by hydrodynamic injections. The cytotoxic immune response to fLuc-expressing cells was continuously evaluated by measuring serum gLuc activity without sacrificing the mice. The schematic image of the experimental design is shown as Figure 1.

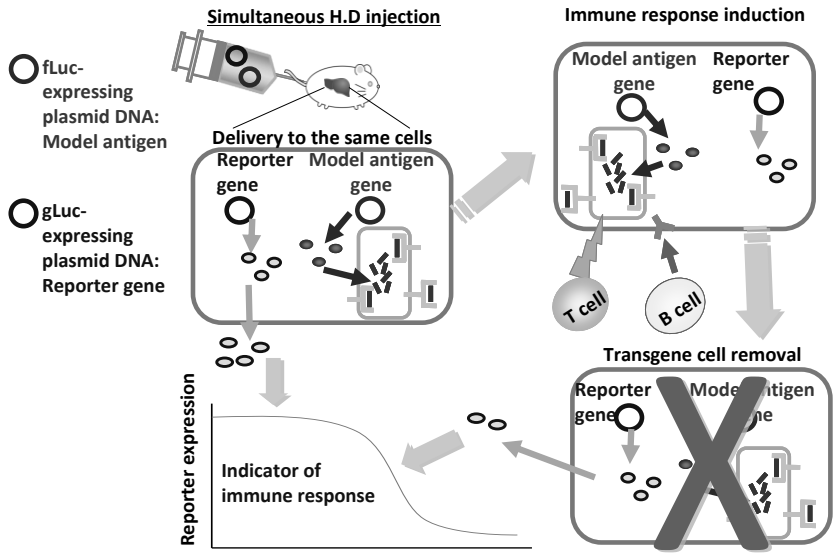

Figure 1. Experimental image for the evaluation of immune response for transgene product.

I-2 Material and Methods

Plasmid DNA

pCpG-mcs was purchased from Invivogene (San Diego,DA, USA). The fLuc-expressing plasmids, pCMV-fLuc and pCpG-fLuc, were constructed as described previously [21]. pROSA-gLuc and pROSA-fLuc, plasmid expressing gLuc and fLuc, respectively, were constructed using In-fusion Advantage polymerase chain reaction. pCMV-fLuc was amplified in the Escherichia coli strain DH5α, whereas pCpG-mcs, pCpG-fLuc and pROSA-gLuc were amplified in the E. coli strain GT115 Plasmid DNA was purified using JETSTAR 2.0 Plasmid MAXI Plasmid Purification Kits (GENOMED GmbH, Löhne, Germany). Characteristics of the plasmid DNAs used are summarized in Table 1.

Table 1. Properties of plasmid DNA used in Chapter I.

Plasmid	Size (kbp)	Number of CpG *	Enhancer	Promoter	cDNA
pCMV-fLuc	7.1	846	hCMV	hCMV	Firefly luciferase
pCpG-mcs	3.0	0	hCMV	hEF1	None
pCpG-fLuc	4.7	194	hCMV	hEF1	Firefly luciferase
pROSA-gLuc	5.8	598	None	hROSA26	*Gaussia* luciferase
pROSA-fLuc	6.7	622	None	hROSA26	Firefly luciferase

* The Numbers of CpG dinucleotides in plasmid DNA are indicated. CMV, human cytomegalovirus; EF1 human elongation factor 1.

Mice and plasmid DNA administration

Four-week-old female ICR mice and 6-week-old female C57/BL6 mice, weighing approximately 20 g each, were purchased from Japan SLC (Shizuoka, Japan). C57/BL6 mice were used only in the experiment in which interferon (IFN)-γ secretion from splenocytes was evaluated. All animal experiments were subject to deliberation and approval by the Ethics Committee for Animal Experiments at the

Graduate School of Pharmaceutical Sciences, Kyoto University. Plasmid DNA was administered to the mice using a hydrodynamics-based procedure, in which plasmid DNA dissolved in saline solution (whose volume equaled 8% of the total body weight) was injected into the tail vein of the mice within 5 s using a 26-gauge needle.

Cell culture

Murine melanoma B16BL6 cells or B16BL6 cells stably expressing fLuc (B16BL6/fLuc) was cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Co. Ltd., Tokyo, Japan) supplemented with 10 % heat-inactive fetal bovine serum (FBS). splenocytes was cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, gLucose, sodium pyruvate.

Luciferase Assay

At indicated time points, blood was collected from the tail vein of mice. The blood samples were incubated at 4 °C for 2 h to allow clotting and then centrifuged at 8000 × g for 20 min to obtain serum samples. To measure luciferase activities in the liver, the liver was harvest and homogenized in 10 ml/g liver of lysis buffer (0.1 M Tris, 0.05 % TritonX-100, 2 mM EDTA, pH7.8), and the homogenates were centrifuged at 12000 × g for 10 min at 4 °C. Then, the supernatant was mixed with luciferase assay buffer (PicageneDual, Toyo Ink, Tokyo, Japan), and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany).

Quantitation of antibody titers

Serum samples were obtained as described above. The amount of fLuc-specific antibodies was measured by enzyme linked immunosorbent assay (ELISA) as described previously [29]. In brief, 96-well flat-bottom polystyrene plate was coated with 0.2 mg/ml of firefly luciferase (Promega, San Luis Obispo USA) by overnight incubation at 4 ºC. The wells were blocked with 5% bovine serum albumin (BSA)-containing phosphate buffered saline with Tween-20 (0.5 % Tween-20 in phosphate buffer saline (PBS)) for 1 h at 37 ºC. After washing, serially diluted serum samples were added to the wells. After 2 h incubation at 37 ºC and subsequent washing, horse raddish peroxidase (HRP)-labeled rabbit anti-mouse IgG (1:2000 dilution; Zymed Lab, San Francisco, CA) was added to each well. After 1 h incubation at 37 ºC and subsequent washing, freshly prepared o-phenylenediamine dihydrochloloride (Wako, Tokyo, Japan) solution containing H_2O_2 was added to each well. After 10 min incubation at room temperature, 10 % H_2SO_4 was added to each well to stop the reaction and measured absorbance at 490 nm.

IFN-γ secretion from splenocytes

C57BL6 mice received two injections of pROSA-gLuc with pCpG-mcs or pCpG-fLuc with a 3-week interval. One week after the second injection, splenocytes were isolated, purified and cultured in the presence of mitomycin C-treated murine melanoma B16BL6 cells or mitomycin C-treated B16BL6 cells stably expressing fLuc (B16BL6/fLuc) in 96-well culture plates for 2 days. The concentration of IFN-γ in supernatant was determined by an ELISA (Ready-SET-Go! Mouse IFN-γ ELISA; eBioscience, San Diego, CA, USA).

Measurement of serum alanine aminotransferase (ALT) activity

At the indicated time points after plasmid DNA administration, serum was collected as described above. Serum ALT level was measured using a quantification kit (Transaminase CII test Wako; Wako Pure Chemical, Osaka, Japan).

Hematoxylin and eosin staining

Mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then fixed in 4 % paraformaldehyde in PBS, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). The stained sections were examined using a microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan).

Histochemical analysis of the liver

Mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then embedded in Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and stored in 2-methyl butanol at -80 °C. Frozen liver sections (10 μm thick) were made using a cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany) by the routine procedure. The sections were fixed with 4% paraformaldehyde in PBS.

To detect $CD8^+$ cells and $CD4^+$ cells in the liver, the fixed sections were blocked with 20% FBS in PBS for 1 h at 37 °C and incubated with biotinylated Abs specific to mouse CD8 (Acris antibody, Herford, Germany) or CD4 (ebioscience, San Diego, CA, USA) for 1 h at 37 °C. Samples were examined under a fluorescence microscope (Biozero BZ-8000).

Spleen removal and macrophage depletion

For spleen removal, mice were anaesthetized and shaved. Then, a 2-cm incision was made in the skin at the left flank. The peritoneal membrane was opened, and the entire spleen was removed intact. The peritoneal membrane and the skin were closed separately with surgical silk-thread. This procedure ensures

that the spleen is removed in total and that no splenic fragments are left behind [30].

Macrophage depletion was performed by using clodronate-encapsulating liposome (clodronate liposome). Clodronate liposome was prepared by the method as described previously [31]. For macrophage depletion, mice received clodronate liposome administration 1 and 3 days before plasmid DNA administration, and then repeated every two days.

Statistical analysis

Differences were statistically evaluated by Student's t-test. The level of statistical significance was set at $P < 0.05$.

I-3 Results

I-3-a Hydrodynamic injection of pROSA-gLuc resulted in stable gLuc activity in the serum

To evaluate the stability of gLuc expression after hydrodynamic injection of pROSA-gLuc, serum gLuc activity was measured over time after hydrodynamic administration of 0.1, 1 or 10 μg of pROSA-gLuc, without sacrificing the mice. As shown in Figure 2A, stable gLuc activity was observed for more than 1 year, irrespective of the pROSA-gLuc dose administered. On the other hand, hydrodynamic injection of pCMV-gLuc (pCMV-based gLuc expressing plasmid) or pCpG-gLuc (pCpG-based gLuc expressing plasmid) also resulted in a long-term gLuc expression, although the time-dependent decline in gLuc activitywas greater than that after pROSA-gLuc administration (Figure 2B).

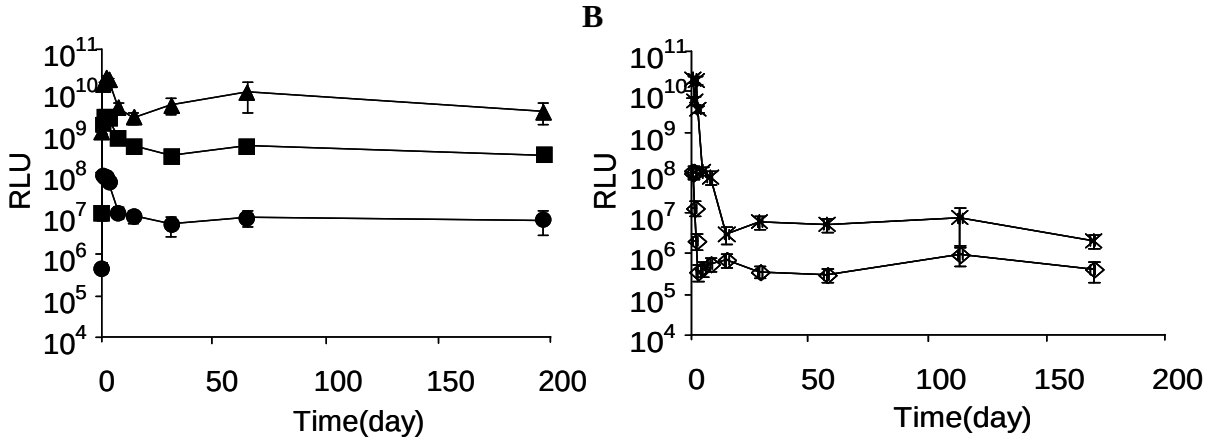

Figure 2. gLuc activity in the serum after hydrodynamic delivery.

(a) Time-course of gLuc activity in serum after hydrodynamic injection of 0.1 (circle), 1 (square) and 10 μg (triangle) of pROSA-gLuc. (b)Time-course of gLuc activity in serum after hydrodynamic injection of

10 (asterisk) of pCpG-gLuc and 10 μg ((open diamond) of pCMV-gLuc.The results are expressed as the mean ± SD of five mice.

I-3-b Hydrodynamic injection of pCpG-fLuc resulted in the stable fLuc activity in the liver at early time points and reduction of fLuc activity at later time points

After the administration of fLuc-expressing plasmid DNAs that show stable and transient expression profile (pCpG-fLuc and pCMV-fLuc, respectively) to mice by hydrodynamic injection, fLuc activity in the liver was high at immediately after administration of pCMV-fLuc, and it started to decline within the first day after injection. After the initial decline, fLuc activity in the liver was constant after pCMV-fLuc administration (Figure 3). On the other hand, fLuc activity in the liver was constant for the first 1 week after the administration of pCpG-fLuc. Drastic decline in the fLuc activity was observed in the mice that received 30, 10 and 3 μg of pCpG-fLuc at 10, 12, 14 days after the administration, respectively.

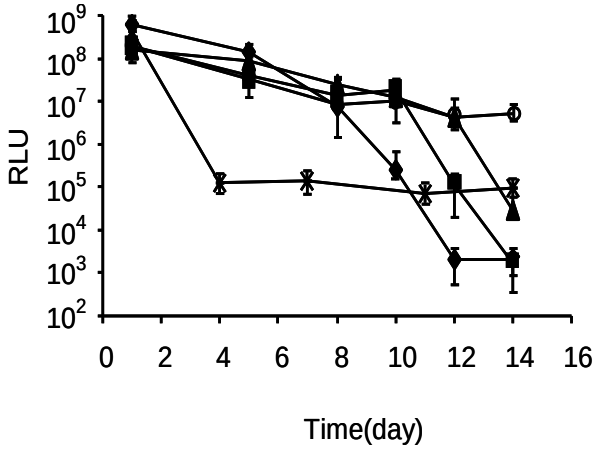

Figure 3. Time-course of fLuc activity in the liver after hydrodynamic injection of fLuc-expression plamids DNA at varying doses. Mice received administration of 10 μg of pCMV–fLuc (asterisk) or 1 (open circle), 3 (closed triangle), 10 (closed square) or 30 μg (closed circle) of pCpG-fLuc. The results are expressed as the mean ± SD of five mice. .

I-3-c Co-administration of fLuc-expressing plasmid affected gLuc activity from pROSA-gLuc

pROSA-gLuc was co-administered with 10 μg of pCMV-fLuc or 0.1, 0.3, 1, 3 or 10 μg of pCpG-fLuc, and serum gLuc activity was measured as an indicator of the number of cells expressing both gLuc and fLuc (Figure 4A). The time course of serum gLuc activity after co-administration of pROSA-gLuc with 10 μg of pCMV-fLuc or 0.1 μg of pCpG-fLuc was similar to that obtained after administration of pROSA-gLuc alone. On the other hand, serum gLuc activity showed a sharp decline approximately 1 week after co-administration of pROSA-gLuc with 1, 3 or 10 μg of pCpG-fLuc. This reduction in serum gLuc activity was initiated earlier as the dose of co-administered pCpG-fLuc was increased. A slight reduction in serum gLuc activity was also observed in mice that received pROSA-gLuc with 0.3 μg of pCpG-fLuc. These results suggest that sustained fLuc expression from the co-administered pCpG-fLuc induces the reduction in serum gLuc activity. Sustained expression of fLuc, an antigenic

protein, is capable of inducing a fLuc-specific immune response; I therefore hypothesized that the number of fLuc-expressing cells (which also simultaneously express gLuc) is reduced because of the removal of the transgene-expressing cells by the fLuc-specific immune response, which in turn is induced by sustained fLuc expression. The activities of both the luciferases (gLuc and fLuc) in the liver were measured 17 days after co-administration (Figure 4B). fLuc activity in the liver was higher in mice that received a low dose (0.1 or 0.3 μg) of pCpG-fLuc or 10 μg of pCMV-fLuc than in mice that received a higher dose (1, 3 or 10 μg) pCpG-fLuc, which suggests that the initial high and sustained expression obtained by a high dose pCpG-fLuc might induce a fLuc-specific immune response that removed the fLuc expressing cells. Similarly, gLuc activity in the liver of the mice that received pROSA-gLuc with 10 μg of pCMV-fLuc or 0.1 μg of pCpG-fLuc was much higher than the corresponding activity in mice that received higher doses of co-administered pCpG-fLuc, which strongly suggests the removal of transgeneexpressing cells by fLuc-specific immune response. Next, pROSA-gLuc was coadministered with pROSA-fLuc. Serum gLuc activity showed a sharp decline approximately 10 days after the co-administration of pROSA-gLuc with pROSA-fLuc (Figure 4C).

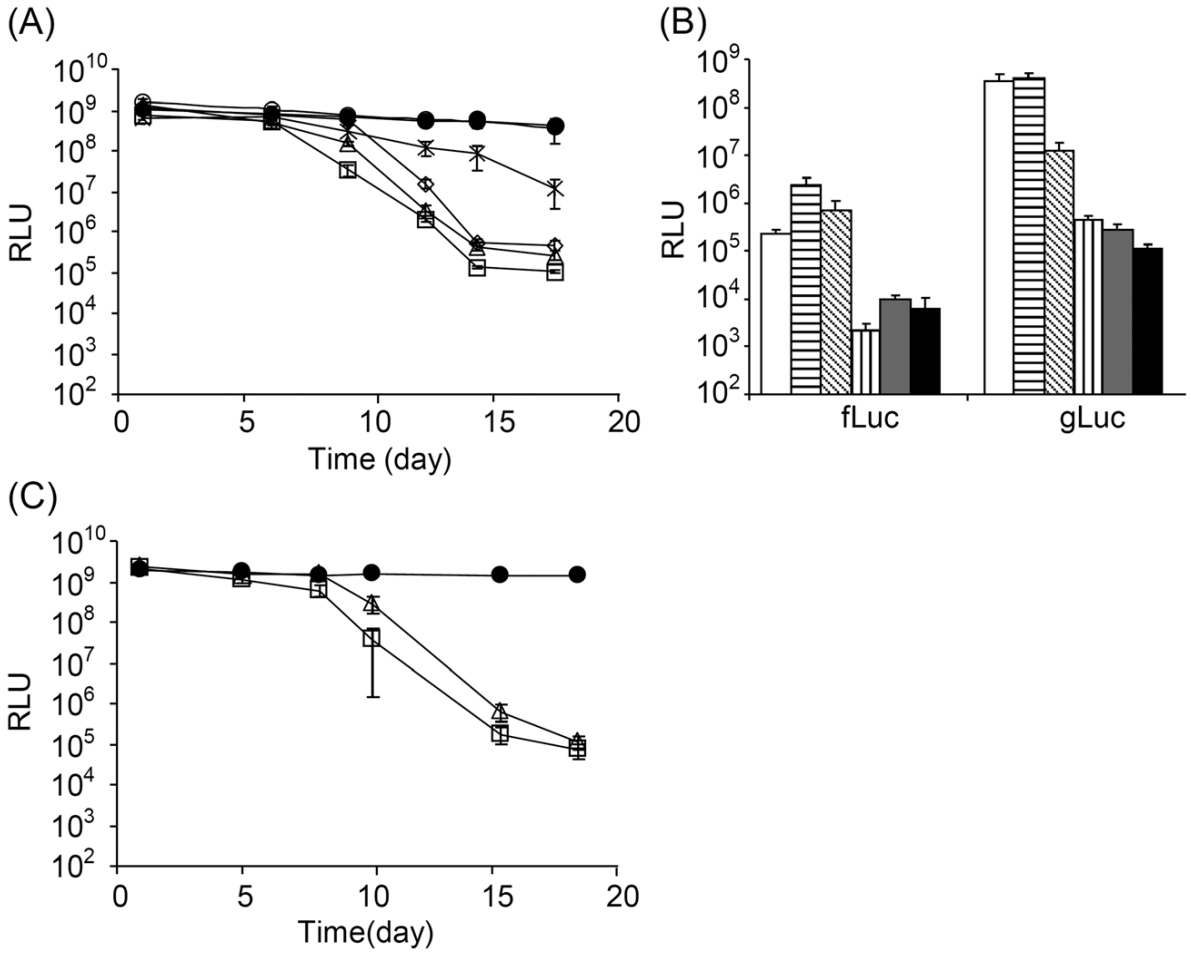

Figure 4. gLuc and fLuc activities in the serum and liver after co-administration of the respective

plasmids by hydrodynamic injection.

(A) Time course of serum gLuc activity after co-administration of 10 μg of pROSA-gLuc with 10 μg of pCMV-fLuc (closed circle) or 0.1 (open circle), 0.3 (asterisk), 1 (open diamond), 3 (open triangle) or 10 μg (open square) of pCpG-fLuc. (B) fLuc and gLuc activities in the liver 17 days after co-administration of 10 μg of pROSA-gLuc with 10 μg of pCMV-fLuc (open column) or 0.1 (horizontal lines), 0.3 (skewed lines), 1 (vertical lines), 3 (grey) or 10 μg (closed column) of pCpG-fLuc. (C) Time-course of gLuc activity in the serum after co-administration of 10 μg of pROSA-gLuc with 10 μg of pCpG-mcs (closed circle), pROSA-fLuc (open triangle) or pCpG-fLuc. The results are expressed as the mean ± SD of five mice

I-3-d fLuc-specific humoral and cellular immune response were induced in mice that received hydrodynamic injections of pCpG-fLuc

As a next step, I evaluated the fLuc-specific immune response in mice that received hydrodynamic injections of the fLuc-expressing plasmid DNA by measuring fLucspecific antibodies in the serum 14 days after pCpG-fLuc or pCMV-fLuc administration. As shown in Figure 5A, fLuc-specific antibodies were detected in the serum of mice that had been administered with 1, 3 or 10 μg of pCpG-fLuc. On the other hand, fLuc-specific antibodies were scarcely detected in the serum of mice receiving 10 μg of pCMV-fLuc.

To evaluate the cellular immune response specific for fLuc, I investigated IFN-γ production in spleen cells of immunized mice in response to the stimulation with fLuc. Spleen cells from mice immunized with pCpG-fLuc generated a large amount of IFN-γ. Splenocytes of mice receiving pCpG-fLuc produced significantly higher amountsof IFN-γ in response to fLuc than the splenocytes of mice (Figure 5B).

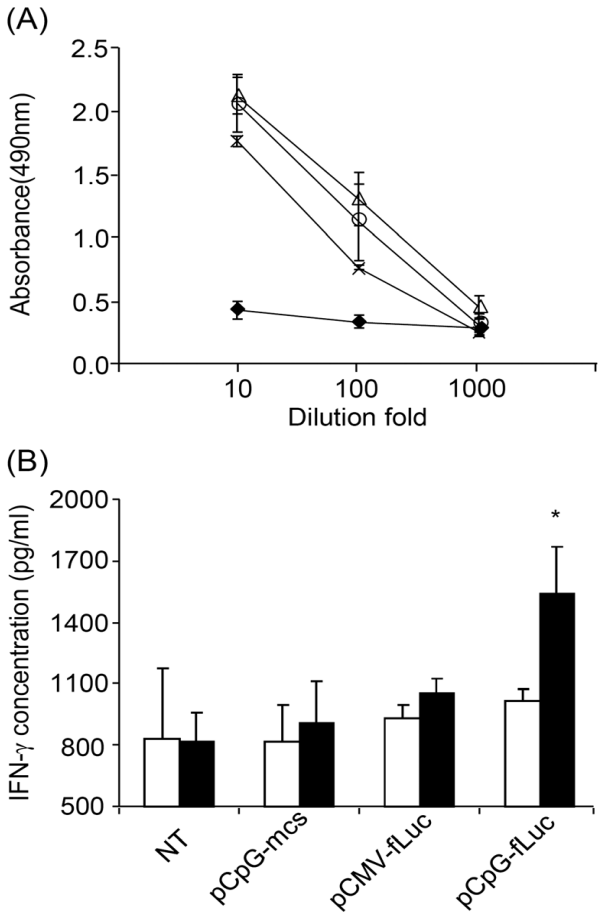

Figure 5. Induction of humral and cellular immune response specific for fLuc after hydrodynamic injection of fLuc-expression vector.

(A) Mice received hydrodynamic injections of 10 μg of pCMV-fLuc (closed diamond), 1 (asterisk), 3 (open triangle) or 10 μg (open circle) of pCpG-fLuc. Fourteen days after plasmid DNA administration, serum samples from the mice were collected, and antibody titers were measured by ELISA. The results are expressed as the mean ± SD of five mice. (B) Splenocytes collected from untreated mice or mice receiving pDNA administration were cocultured with B16BL6 (open columns) or B16BL6/fLuc cells (closed columns) for 2 days. IFN-γ concentration in the culture medium was measured by ELISA. The results are expressed as the mean ± SD of five mice. *$p<0.05$ compared to the B16BL6-stimulated group.

I-3-e Reduction in serum gLuc activity immediately after co-administration of pROSA-gLuc with pCpG-fLuc in mice pre-administered with pCpG-fLuc

I hypothesized that the fLuc-specific immune response induced by sustained fLuc expression eliminated fLuc expressing cells, thereby also resulting in decreased gLuc activity. This hypothesis was tested by pre-administering fLuc-expressing plasmid DNA, and evaluated whether the fLuc-specific immune response thereby induced affects the profile of gLuc expression from pROSA-gLuc that was subsequently co-administered with fLuc-expressing plasmid DNA. Specifically, saline, pCpG-fLuc or pCMV-fLuc was pre-administered through hydrodynamic injections, followed by co-administration of pROSA-gLuc with pCpG-fLuc or pCMV-fLuc 14 days later. As shown in Figure 6, a reduction in the gLuc activity was observed as early as 2 days after pCpG-fLuc co-administration in mice preadministered with pCpG-fLuc. In addition, serum gLuc activity began to decrease 7 and 9 days after co-administration of pROSA-gLuc with pCpG-fLuc in mice pre-administered with pCMV-fLuc or saline, respectively. On the other hand, co-administration of pROSA-gLuc with pCMV-fLuc in mice pre-administered with

pCpG-fLuc scarcely altered serum gLuc activity.

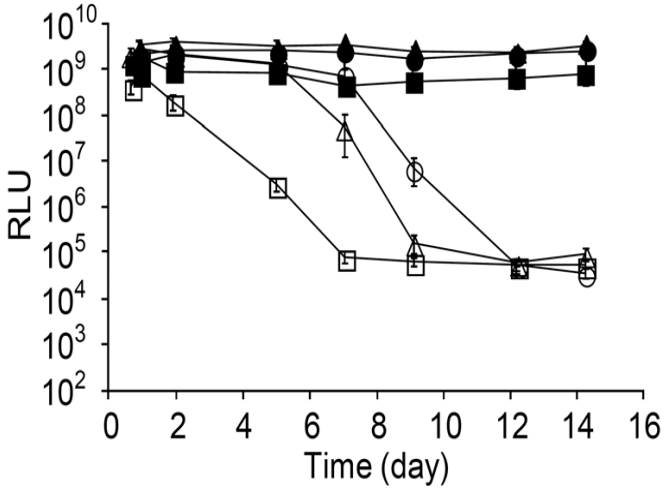

Figure 6. Time course of serum gLuc activity after repeated hydrodynamic injections.

The mice first received hydrodynamic injections of saline (circle), 10 μg of pCMV-fLuc (triangle) or 10 μg of pCpG-fLuc (square). Fourteen days after the first administration, the mice were co-administered 10 μg of pROSA-gLuc with 10 μg of pCMV-fLuc (closed symbols) or pCpG-fLuc (open symbols). The results are expressed as the mean ± SD of five mice.

I-3-f Serum gLuc activity decreased only upon simultaneous co-administration of pROSA-gLuc and pCpG-fLuc

Next, it was investigated whether co-expression of fLuc and gLuc in the same cell was required for the reduction in gLuc expression observed after co-administration of pROSA-gLuc with pCpG-fLuc. To avoid co-expression of fLuc and gLuc in the same cells, I sequentially injected the two plasmids instead of a simultaneous injection because it was shown that the former protocol resulted in the expression of the transgenes in different cells with little overlap [28]. Mice were first co-administered pROSA-gLuc with pCpG-fLuc. Fourteen days after the first injection, pROSA-gLuc and pCpG-fLuc were co-administered or sequentially administered; the latter was administered with a 12-h interval (Figure 7A). Serum gLuc activity decreased in mice that received co-administration and not sequential administration. On the other hand, fLuc activity in the liver 14 days after the second injectionwas comparable between these two groups (Figure 7B), which suggests that the fLuc-specific immune response was induced in both groups.I further investigated whether reduction in gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc accompanies the reduction in gLuc expression from separately administered pROSA-gLuc. The mice first received hydrodynamic administration of 1 μg of pROSA-gLuc. One week after the first injection, the mice were administered saline (without any plasmid DNA) or coadministered 10 μg of pROSA-gLuc with pCpG-mcs or pCpG-fLuc (Figure 7C). Co-administration of pCpG-mcs hardly affected the gLuc expression from pROSA-gLuc. Serum gLuc activity was enhanced by the second administration of pROSA-gLuc, which reflects the fact that the dose of pROSA-gLuc was 10-fold higher

in the second administration than in the first administration. In the mice that received a second injection of pROSA-gLuc with pCpG-fLuc, serum gLuc activity began to decrease 7 days after the second injection and reached the levels found in the control mice that received saline during the second administration. This result implies that gLuc-expressing cells generated by the first pROSA-gLuc administration were not affected by the immune response induced by the second injection of ROSA-gLuc with pCpG-fLuc.

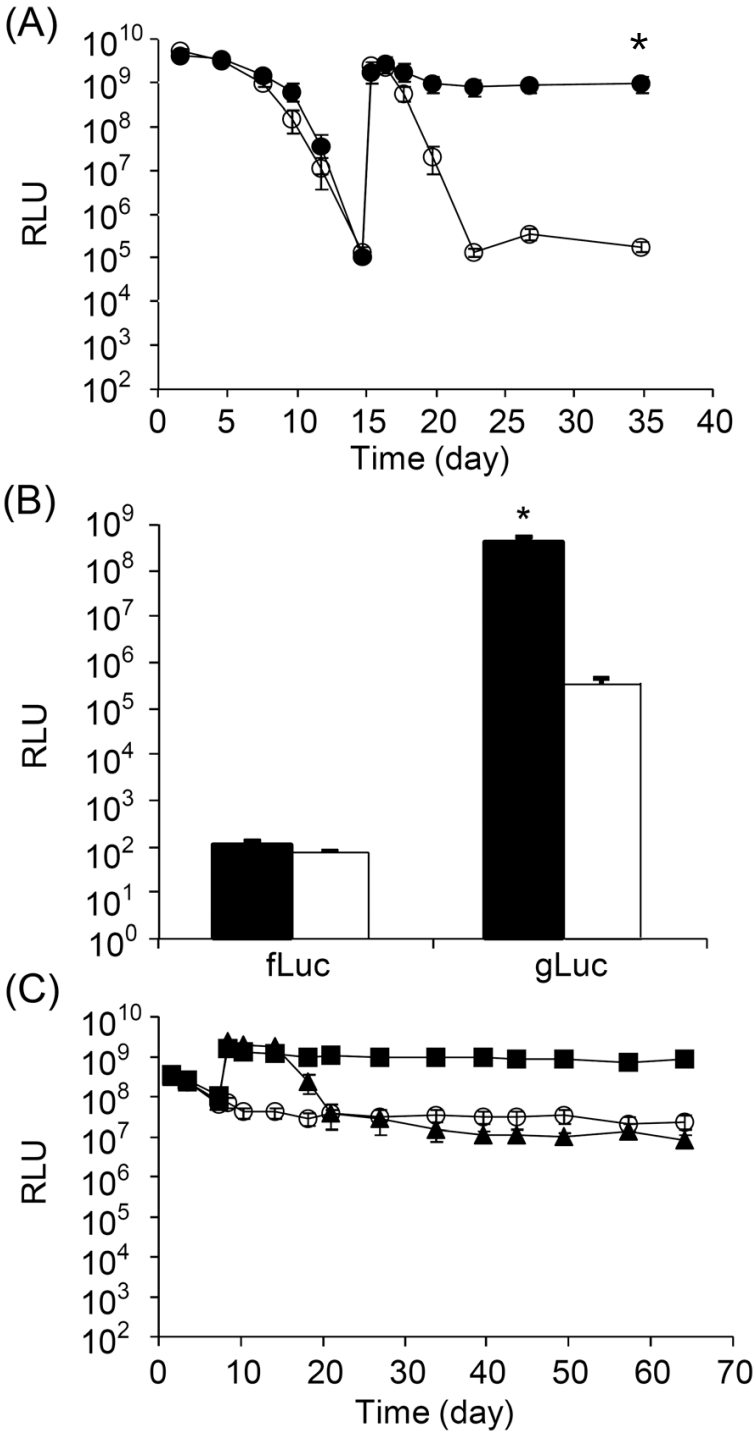

Figure 7. Time course of serum gLuc activity and activities of both gLuc and fLuc in the liver after simultaneous or sequential hydrodynamic delivery

(A) The mice initially received hydrodynamic injection of 10 μg of pROSA-gLuc with 10 μg of pCpG-fLuc. Fourteen days after the first injection, the mice received co-injection of 10 μg of pROSA-gLuc with 10 μg of pCpG-fLuc (open circles) or a sequential injection of 10 μg of pROSA-gLuc followed by 10 μg of pCpG-fLuc after a 12-h interval (closed circles). The results are expressed as the mean ± SD of five mice. *$p<0.05$ compared to the simultaneous injection group. (B) fLuc and gLuc activities in the liver 21 days after simultaneous (open column) or sequential (closed column) injections of pROSA-gLuc with pCpG-fLuc. The results are expressed as the mean ± SD of five mice. * $p<0.05$ compared to the simultaneous injection group. (C) Mice initially received hydrodynamic injection of 1 μg of pROSA-gLuc.

Seven days after the first injection, the mice received saline injection (open circle) or co-administration of 10 μg of pROSA-gLuc with 10 μg of pCpG-mcs (closed square) or pCpG-fLuc (closed triangle). The results are expressed as the mean ± SD of five mice.

I-3-g Inflammatory cells, including $CD8^+$ cells, were detected in the liver after pCpG-fLuc administration

Mice were co-administered pROSA-gLuc with pCpG-fLuc or pCpG-mcs twice with an interval of 2 weeks. The livers were collected from the mice 2, 4 or 6 days after the second injection, and liver sections were prepared to evaluate the effect of the fLuc-specific immune response. HE staining of the liver sections (Figure 8A) revealed a large number of infiltrating cells in the liver 2 days after the second administration of pCpG-fLuc. The number of infiltrating cells declined on day 4 and returned to a level comparable to that found in the pCpG-mcs group by day 6. The liver sections were stained with CD4- or CD8- specific antibodies to characterize the type of infiltrating cells (Figures 8B and 8C). A slight difference was observed in the number of $CD4^+$ cells between the pCpG-mcs and pCpG-fLuc groups. By contrast, the number of $CD8^+$ cells was higher in the pCpG-fLuc group than in the pCpG-mcs group 2 days after the second administration; the number decreased on day 4 and returned to a level comparable with that found in the pCpG-mcs group by day 6. Because it was hypothesized that the removal of transgene-expressing hepatocytes by the infiltrating cells was related to hepatic injury, the time-course of serum ALT level was measured.

As previously reported, an increase in serum ALT level was detected immediately after hydrodynamic injection, irrespective of the types of plasmid DNAs, indicating a transient increase in the permeability of cellular membrane by the injection. At 7 and 9 days after injection, the serum ALT level of mice receiving pCpG-fLuc was significantly higher than that of mice receiving pCpG-mcs (Figures 8D). This result also implies that hepatocytes expressing fLuc are damaged by the fLuc specific immune response.

I-4 Discussion

In the present study, single pROSA-gLuc administration was found to result in serum gLuc activity that was stable for more than 1 year, implying that sustained gLuc expression did not induce a gLuc-specific immune response such as anti-gLuc antibody production. On the other hand, sustained fLuc expression induced a fLuc-specific immune response, suggesting that the antigenicity of fLuc protein is higher than that of the gLuc protein.

Co-administration of high doses of pCpG-fLuc with pROSA-gLuc resulted in a reduction in serum gLuc activity, which is likely caused by the removal of transgeneexpressing cells as a consequence of the fLuc-specific immune response induced by sustained fLuc expression. On the other hand, co-administration of high doses of pCMV-fLuc, which results in a high but transient transgene expression, induced neither a detectable fLuc-specific immune response, nor a reduction in the serum gLuc activity. This suggests that the duration of fLuc expression would be an important factor for eliciting the fLuc-specific immune response, which in turn eliminates the fLuc-expressing cells. The results obtained in the present study indicate that long-term expression of a transgene has a higher risk of eliciting an immune response than short-term expression.

Aubert *et al.* [33] demonstrated that a cytotoxic immune response was the chief mechanism responsible for the removal of transgene-expressing cells after retroviral-mediated β-galactosidase gene transfer in the liver. In addition, an ex vivo study suggested that a cytotoxic immune response resulted in the removal of transgene (green fluorescent protein)-expressing cells in concert with the helper T cells after an epidermal gene transfer [34]. In the present study, hydrodynamic injection of pCpG-fLuc induced a cellular immune response specific to fLuc. In addition, $CD8^+$ cell infiltration wasobserved in the liver of themice that received pCpG-fLuc administration. These results suggest that fLuc-specific cytotoxic T lymphocytes play an important role in eliminating the fLuc-expressing cells, which is in agreement with previous studies [33]. In addition, hydrodynamic injection of pCpG-fLuc and pROSA-gLuc resulted in the increase in serum ALT levels at approximately 1 week after the administration, when the decline in gLuc activity occurred, also suggesting that hepatocytes expressing both fLuc and gLuc were damaged. Furthermore, fLuc-specific antibodies may be involved in the removal of the fLuc-expressing cells.

A reduction in serum gLuc activity was observed only when pROSA-gLuc was co-administered with pCpG-fLuc. On the other hand, gLuc expression from preadministered pROSA-gLuc was scarcely affected by the second injection of pROSA-gLuc with pCpG-fLuc. From these results, it is likely that the decrease in serum gLuc activity after the simultaneous injection of pROSA-gLuc and pCpG-fLuc is caused by the elimination of cells expressing both gLuc and fLuc, as a consequence of the fLuc-specific immune response. This fact suggests that co-administration of pROSA-gLuc with a vector expressing a

gene of interest could be a useful method for monitoring the effect of an immune response to the product of interest in transgene-expressing cells. However, there is a possibility that transgene expression level from a plasmid vector may be affected by the simultaneously delivered plasmid vector via the interference in transgene expression process [35]. Detailed molecular studies would be required to exclude this possibility.

When the transgene product is a secretory protein, the transgene-expressing cells secrete the products so that they may be taken up by APCs to elicit an immune response [36–39]. fLuc is a nonsecretory protein and is scarcely released from the fLuc-expressing cells. Therefore, a fLuc-specific immune response induced after pCpG-fLuc administration is likely a result of the direct gene delivery of plasmid DNA into APCs. By using a target sequence for miRNA-142-3p, a miRNA highly expressed in APCs, Brown *et al*. [40] demonstrated that transgene expression in APCs, resulting in sustained transgene expression, is the chief reason for the induction of an immune response to the transgene products. Hydrodynamic injection also delivers plasmid DNA into Kupffer cells in the liver and spleen cells, both of which function as APCs; I therefore evaluated the role of Kupffer and splenic cells in the induction of the immune response. I found that, even in mice that had been subjected to both spleen removal and macrophage depletion, serum gLuc activity declined after hydrodynamic administration of pROSA-gLuc with pCpG-fLuc, which implied that the fLuc-specific immune response could eliminate fLuc-expressing cells in the absence of macrophages in the liver (Kupffer cells) or spleen (splenic macrophages), or in fact any splenic cells. It is known that the liver has some dendritic cells; gene transfer to the dendritic cells might occur after hydrodynamic injection of plasmid DNA [41] and these dendritic cells might function as APCs. In addition, hepatocytes might also function as APCs after hydrodynamic injection because hepatocytes have been previously reported to exhibit antigen presenting ability [42–44]. Further studies are required for determining which types of cells functioned as APCs in the induction of the fLuc-specific immune response after hydrodynamic injection of pCpG-fLuc.

Although induction of an immune response to a transgene product is a serious problem in gene therapy, eliciting the immune response is, in turn, desirable for DNA vaccination. My results demonstrate that single pCpG-fLuc administration could induce a strong cytotoxic immune response against fLuc; therefore, hydrodynamic administration of the recombinant pCpG vector encoding an antigenic protein might prove to be potent as a DNA vaccine because it also shows sustained transgene expression. On the other hand, in the development of DNA vaccine by hydrodynamic gene delivery, the administration of adjuvant is desirable to induce stronger immune response because the degree of immune activation is limited even after hydrodynamic delivery of CpG-rich plasmid DNA [45].

In conclusion, the present study demonstrates that high levels of sustained expression of a transgenic antigen induce an immune response and that the cells expressing the transgene product are eliminated,

probably as a result of the transgene product-specific immune response.

Chapter II
Importance of transgene expression level in each cell on the induction of transgene expression-specific immune response

II-1 Introduction

The immune response to the encoded therapeutic protein is a serious concern in gene therapy because it can cause serious adverse effects and affect the transgene expression profile that reduces therapeutic effect [46, 47]. Induction of the immune response depends on several factors, including the antigenicity of the protein, types of transgene-expressing cells, transgene expression profile, and subcellular localization or secretion of the product [48]. In Chapter I, I demonstrated that high levels of sustained expression of a transgene product with antigenesity induced an immune response specific to the transgene product and that the cells expressing the transgene product are eliminated by the immune response [49]. In that study, I found that not only sustained transgene expression but also high level of transgene expression was important for induction of transgene-specific immune response. In addition to my results, the relationshop between the level of transgene expression and the induction of the immune response has been reported. In the development of DNA vaccine, it was shown that high levels of antigen expression induced cellular immune responses that in turn rapidly down-regulate antigen expression [50]. Zhang *et al*. also reported that transgene expression levels and kinetics are the primarily risks for the induction of humoral immune response in factor IX gene therapy [51]. However, in these previous studies, the detailed relationship between transgene expression level and the induction of immune response was not investigated. In these previous studies, transgene expression level was evaluated by measuring the amount of transgene product in organ or in the blood, which is the product of multiplication of transgen expression level per cell times the number of transgen-expressing cells. It is necessary to investigate whether total amount of transgene product or the level of transgene expression per cell is important for the induction of immune response in order to clearly understand the relationship between the transgene expression level and the immune response.

In Chapter I, I found that spleen removal or macrophage depletion hardly affected immune response induced by hydrodynamic gene transfer [49]. Wolff *et al*. also reported that transgene-specific immune response caused after hydrodynamic gene transfer could not be prevented by reducing transgene expression in denderitic cells (DCs) by utilization of DC-specific micro RNA [25]. Based on these results, I hypothesized that hepatocytes, which are the major transgene-expressing cells after hydrodynamic gene transfer, work as APCs after hydrodynamic gene transfer. If this is the case, transgene expression level in each hepatocyte is important factor for the induction of transgene-specific immune response as enough

Mice and plasmid DNA administration

Four-week-old female ICR mice (approximately 20 g body weight), six-week-old female BALB/c mice (approximately 20 g body weight) were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were brought under deliberation and approved for the Ethics Committee for Animal Experiments at the Graduate School of Pharmaceutical Sciences, Kyoto University. Administration of plasmid DNA to mice was performed by the hydrodynamics-based procedure in which plasmid DNA dissolved in 8 % vol/ body weight of saline were injected into the tail vein of mice over less than 5 s.

Cell culture

CD8 OVA1.3 cells, T hybridoma cells against SIINFEKL-Kb [60], were a generous gift from Dr. C. V. Harding (Case Western Reserve University, Cleveland, OH).

Hepatocytes were isolated from mice according to a standard two-step perfusion protocol [62,63]. Briefly, mice were anesthetized with i.p. administration of pentobarbital (50 mg/kg), the thoracic inferior vena cava was cannulated, and the portal vein was opened for drainage. The liver was sequentialy perfused in situ with two solutions. The pre-perfusion solution was compose of solution A (136 mM NaCl, 5.3 mM KCl, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 9.1 mM HEPES, and 4.1 mM $NaHCO_3$ (pH 7.4)) supplemented with 0.5 mM EGTA and 5 mM D-gLucose and was administered for 6 min at a flow rate of 5 ml/min. The perfusion solution (solution A supplemented with 0.05% collagenase A (Sigma-Aldrich, St.Louis, MO, USA), 0.004% DNase I (Sigma-Aldrich), 5 mM $CaCl_2$, 0.005% trypsin inhibitor (Sigma-Aldrich), and 1% bovine serum albumin (BSA; Sigma-Aldrich)) was administered for 6 min at a flow rate of 5 ml/min. Perfused liver tissue was gently dispersed in a Hanks buffer and filtered through a nylon mesh. Viable hepatocytes were purified by three consecutive, low-speed centrifugations (50 × g for 2 min at 4 ℃).

CD8OVA1.3 T cell were cultured in Dulbecco's modified Eagle medium (Nissui Co.Ltd, Tokyo, Japan) supplemented with 10 % heat-inactive fetal bovine serum (Equitedh-Bio, Kerrville, TX), 0.5 mM monothioglycerol, 2 mM L-glutamine, antibiotics and nonessential amino acid (all from Life Technologies, Carlsbad, CA). CD8OVA1.3 T cell (5×10^6 cells/well) and primary hepatocytes (5×10^5 cells/well) were co-cultured in RPMI 1640 medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented as described for Dulbecco's modified Eagle medium.

Luciferase Assay

At indicated time points, blood was collected from the tail vein of mice. The blood samples were incubated at 4 ℃ for 2 h to allow clotting and then centrifuged at 8000 × g for 20 min to obtain serum samples. To measure luciferase activities in the liver, the liver was harvest and homogenized in 10 ml/g

liver of lysis buffer (0.1 M Tris (pH 7.8), 0.05 % TritonX-100, 2 mM EDTA), and the homogenates were centrifuged at 12000 × g for 10 min at 4 ℃. Then, the supernatant was mixed with Cypridina luciferase assay buffer (Thermo Fisher Scientific, Rockford, USA) or luciferase assay buffer (PicageneDual, Toyo Ink, Tokyo, Japan) to measure cLuc activity and gLuc activity, respectively. The chemiluminescence produced was measured in a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany).

Detection of antibody

Serum samples were obtained as described above. The amount of cLuc-specific and OVA-specific antibodies was measured by enzyme linked immunosorbent assay (ELISA) as described previously. In brief, 96-well flat-bottom polystyrene plate was coated with 0.2 mg/ml of cypridina luciferase (purified from pET-22b-cLuc in BL21(DE3) plysS) or 1 mg/ml of OVA (purchased from Sigma-Aldrich) by overnight incubation at 4 ºC. The wells were blocked with 5% BSA-containing phosphate buffered saline (PBS) with Tween-20 (0.5 % Tween-20 in PBS) for 1 h at 37 ºC. After washing, serially diluted serum samples were added to the wells. After 2 h incubation at 37 ºC and subsequent washing, horse raddish peroxidase (HRP)-labeled rabbit anti-mouse IgG (1:2000 dilution; Zymed Lab, San Francisco, CA) was added to each well. After 1 h incubation at 37 ºC and subsequent washing, freshly prepared o-phenylenediamine dihydrochloloride (Wako, Tokyo, Japan) solution containing H_2O_2 was added to each well. After 10 min incubation at room temperature, 10 % H_2SO_4 was added to each well to stop the reaction and measured absorbance at 490 nm.

Interferon (IFN)-γ production

Indicated days after gene transfer, spleens were isolated from the immunized mice and single cell suspensions were prepared. Cells were placed in 24-well plates and adjusted to a concentration of 1×10^6 cells/well in a RPMI 1640 medium supplemented with 10 % FBS, antibiotics and 0.5 mM monothioglycerol. They were stimulated by addition of 0.1 mg/ml cLuc or 0.5 mg/ml OVA for 4 days. After the cells were centrifuged at 420 × g for 10 min, the supernatants were harvested and freeze-thawed. Then, IFN-γ levels in the supernatant were measured by ELISA using antibodies for capture and detection in accordance with the Manufacturer's protocol (Ready-SET-Go! Mouse IFN-γ ELISA, eBioscience, San Diego, CA, USA).

PKH26 cell labeling

PKH26 was purchased from Sigma-Aldrich, and used to label cells as described in manufacturer's protocol. In brief, CD8 OVA 1.3 T cell (1×10^7 Cell per ml) were incubated with PKH26 in dilute C for 5 min, and washed three times. Cells were seupended in Hank's balanced salt solution and 2×10^6 cells were

intravenously administrated into mice.

Histochemical analysis of the liver

Mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then embedded in Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and stored in 2-methyl butanol at -80 °C. Frozen liver sections (10 μm thick) were made using a cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany) by the routine procedure. The sections were fixed with 4% paraformaldehyde in PBS.

To detect DCs in the liver, the fixed sections were blocked with 20% FBS in PBS for 1 h at 37°C and incubated with biotinylated Abs specific to anti-mouse CD11c (ebioscience) for 1 h at 37°C. Samples were examined under a fluorescence microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan).

Antigen presentation assay

Hepatocytes (5×10^5 cells/well) were purified as described above and co-incubated with CD8 OVA1.3 T cells (5×10^6 cells/well) in RPMI 1640 medium described above in 24-well plate at 37 °C and 5 % CO_2. After 24 h incubation, culture supernatants were collected. The response of CD8OVA1.3 T cells was determined by measuring Interleukin-2 (IL-2) levels in the supernatants with ELISA (BD OptiEIA Mouse IL-2, BD Biosciences, San Diego, CA) [62-64].

Statistical analysis

Differences were statistically evaluated by Student's t-test. The level of statistical significance was set at $P < 0.05$.

II-3 Results

II-3-a Hydrodynamic injection of high doses of long-term cLuc-expressing plasmid vectors resulted in the sudden decrease in the cLuc activity in the serum

To evaluate expression profile of cLuc after hydrodynamic injection of cLuc-expressing plasmid vectors with different doses and promoters, cLuc-expressing plasmid DNAs that show stable (pCpG-cLuc, pROSA-cLuc) and transient expression profile (pCMV-cLuc) were constructed and administrated into mice by hydrodynamics-based procedrue. cLuc activity in the serum was constant for the first 1 week after the administration of pCpG-cLuc (Figure 11A). cLuc activity drasticaly decreased from 9 days after the administration of 10 μg of pCpG-cLuc while cLuc activity in the serum did not decrease with time after

the administration of 0.1 μg pCpG-cLuc. In addition, cLuc activity started to decrease on the day 9 after the administration of 20 μg and 30 μg of pROSA-cLuc (Figure 11B). On the other hand, serum cLuc activity was maintained after the administration of low dose (1 or 10 μg) of pROSA-cLuc. After administration of pCMV-cLuc, serum cLuc activity was high at early time points after the administration and it started to decline immediately. cLuc activity in the serum was constant after the initial decline (Figure 11C).

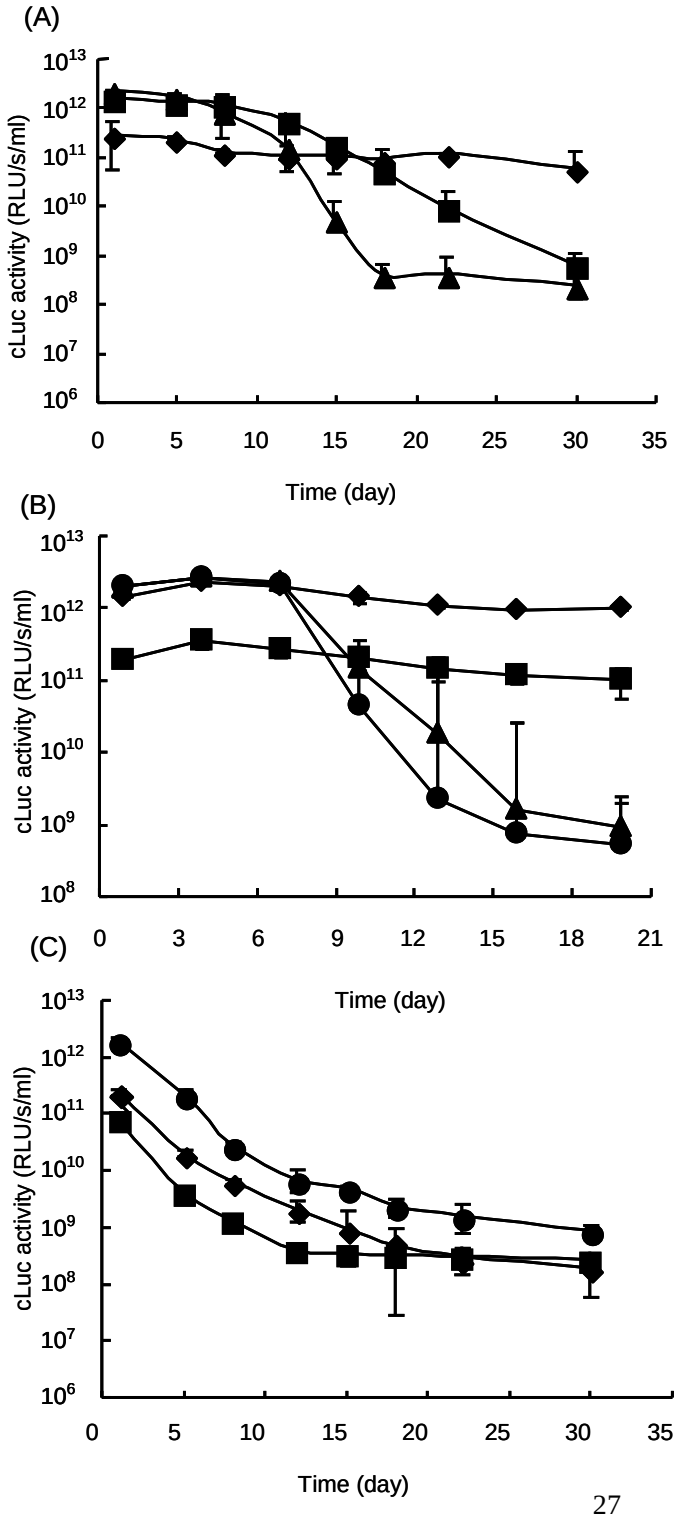

Figure 11. cLuc activity in the serum after hydrodynamic gene transfer.

(A) Time-course of cLuc activity in serum after hydrodynamic injection of 0.1 (diamond), 1 (square) and 10 μg (triangle) of pCpG-cLuc. (B) Time-course of cLuc activity in serum after hydrodynamic injection of 1 (diamond), 10 (square), 20 μg (triangle) and 30 μg (circle) of pROSA-cLuc. (C) Time-course of gLuc activity in serum after hydrodynamic injection of 0.1 (square), 1 (diamond) and 10 μg (circle) of pCMV-cLuc. The results are expressed as the mean ± SD of five mice.

II-3-b gLuc activity from pROSA-gLuc was affected by co-administration of high dose of pROSA-cLuc

In order to investigate if the reduction in cLuc activity after cLuc plasmid transfer was caused by the removal of transgene-expressing cells by cLuc-specific immune response, pROSA-gLuc was co-administrated with different doses of pROSA-cLuc and serum activities of gLuc and cLuc were measured (Figure 12). Time course of gLuc activity in the serum was stable after co-administration of pROSA-gLuc with 1 and 10μg pROSA-cLuc (Figure 12A). On the other hand, serum gLuc activity greatly declined approximately 9 days after the co-administration of pROSA-gLuc with 20 and 30 μg of pROSA-cLuc. In aggreement with the reduction in gLuc activity, serum cLuc activity declined at approximately 9 days after the co-administration of pROSA-gLuc with 20 and 30 μg of pROSA-cLuc while serum cLuc activity was constant after co-administration of pROSA-gLuc with 1 and 10 μg pROSA-cLuc (Figure 12B), which implies that the reduction in gLuc activity was caused by removal of cells expressing both cLuc and gLuc by cLuc-specific immune.

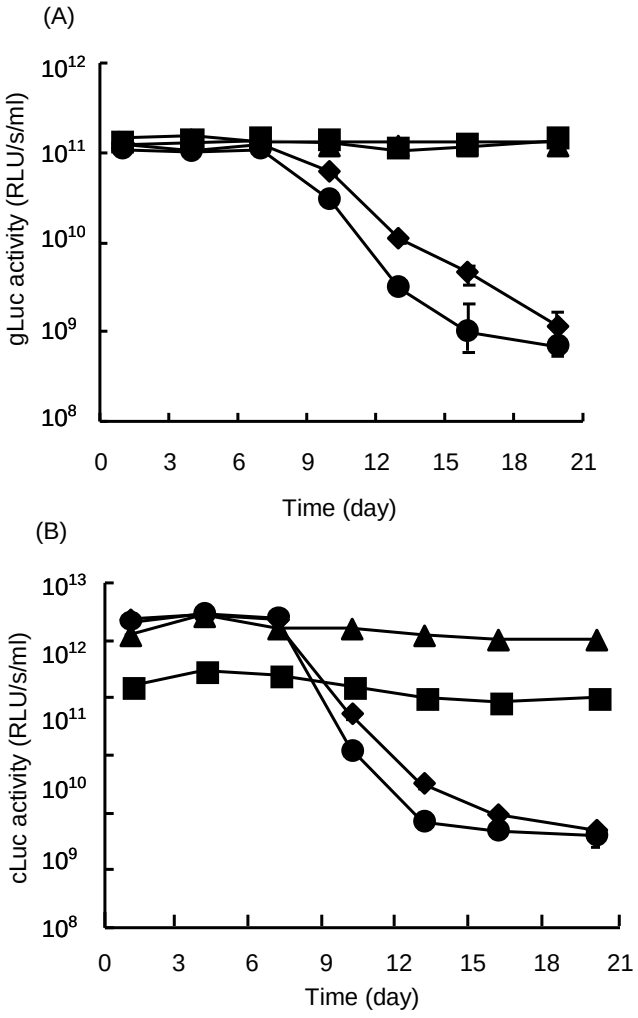

Figure 12. gLuc and cLuc activities in the serum after co-administration of the pROSA-cLuc with different doses of pROSA-gLuc by hydrodynamic injection.

(A) Time course of serum gLuc activity after co-administration of 10 μg of pROSA-gLuc with 1 (square), 10 (triangle), 20 (diamond) or 30μg (circle) of pROSA-cLuc. (B) Time course of serum cLuc activity after co-administration of 10 μg of pROSA-gLuc with 1 (square), 10 (triangle), 20 (diamond) or 30 μg (circle) of pROSA-cLuc.The results are expressed as the mean ± SD of five mice.

II-3-c Single administration of high dose of pROSA-cLuc result in the reduction of cLuc while serum cLuc activity did not decreased after repeated injection of low dose of pROSA-cLuc

To investigate whether transgene expression level per cell or total amount of transgene expression level is important for the induction of immune response against the transgene product, the time-courses of serum cLuc activity after single injection of high dose pROSA-cLuc (30 μg) or 3 times injection of low dose pROSA-cLuc (10 μg) were investigated (Figure 13). After single administration of 30 μg of pROSA-cLuc, serum cLuc strated decline approximately 10 days after the administration. On the other hand, after 3 times administration of 10 μg pROSA-cLuc, serum cLuc activity was stable for 3 weeks although the cLuc level in the serum ater 3 times injection of 10 μg pROSA-cLuc was equal or higher than that after single injection of 30 μg pROSA-cLuc. These results clearly suggest that the high transgene level per cell, not the total amount of transgene product, is important in the induction of transgene-specific immune response after hydrodynamic gene transfer.

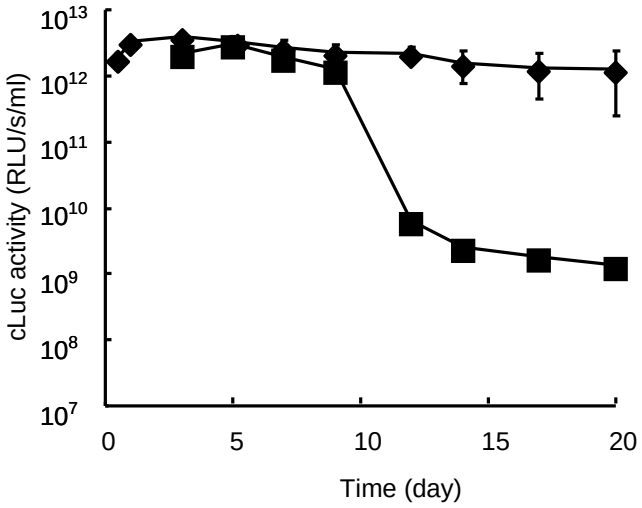

Figure 13. cLuc activity in the serum after single administration of high dose of pROSA-cLuc and repeated injection of low dose of pROSA-cLuc.

Time-course of cLuc activity in the serum of mice serum after single administration of 30 μg of pROSA-cLuc (square) or 3 times administration of 10 μg of pROSA-cLuc (diamond). The results are expressed as the mean ± SD of five mice.

II-3-d cLuc-specific humoral and cellular immune response were more strongly induced in the mice that received single administration of 30 μg pROSA-cLuc than that in mice receiving thrice injection of 10 μg pROSA-cLuc

To evaluate cLuc-specific humoral immune response in mice that received hydrodynamic injection of pROSA-cLuc, the level of cLuc-specific antibody in the serum was measured 14 days after single administration of 30 μg pROSA-cLuc or 3 times injection of 10 μg pROSA-cLuc. cLuc-specific antibody was detected in the serum of mice that had received pROSA-cLuc injection (Figure 14A). The level of cLuc-specific antibody in the serum was higher in the mice that received single administration of 30 μg

pROSA-cLuc than that in mice that received 3 times injection of 10 μg pROSA-cLuc.

To evaluate cLuc-specific cellular immune response after gene transfer of cLuc, the amount of IFN-γ production from splenocytes collected from the gene-delivered mice was measured after cLuc stimulation (Figure 14B). Splenocytes of mice receiving single administration of 30 μg pROSA-cLuc produced significantly higher amounts of IFN-γ in response to cLuc than the splenocytes of mice receiving 3 times injection of 10 μg pROSA-cLuc. In addition, splenocyte collected from mice receiving 3 times injection of 10 μg pROSA-cLuc hardly produced IFN-γ in response to cLuc, which suggests that cellular immune response was hardly induced after 3 times injection of 10 μg pROSA-cLuc.

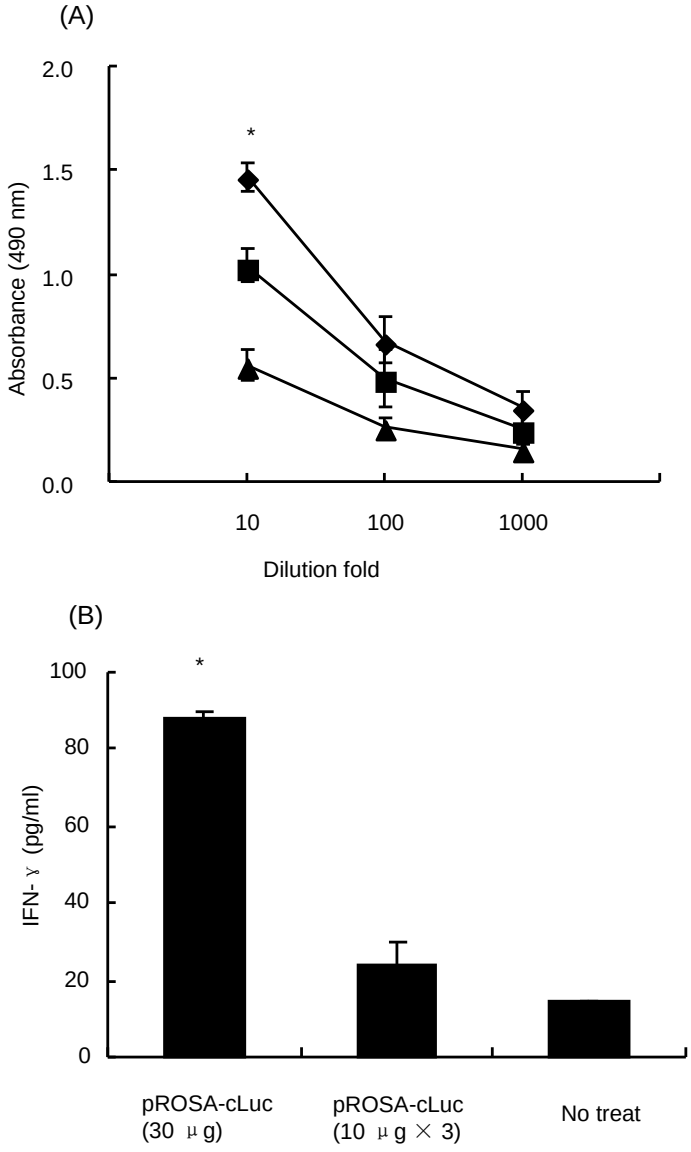

Figure 14. Induction of humral and cellular immune response specific for cLuc after hydrodynamic injection.

(A) Mice received hydrodynamic injections of 30 μg (diamond) of pROSA-cLuc, 3 times 10 μg (square) of pROSA-cLuc or No treat (triangle). Fourteen days after plasmid DNA administration, serum samples from the mice were collected, and antibody titers were measured by ELISA. The results are expressed as the mean ± SD of five mice. (B) Splenocytes collected from No treat mice or mice receiving plasmid DNA administration were cocultured with cLuc protein for 4 days. IFN-γ concentration in the culture medium was measured by ELISA. The results are expressed as the mean ± SD of five mice. *p<0.05 compared to the 3 times 10 μg plasmid DNA injection group.

II-3-e Serum cLuc activity declined after co-admnistration of 1 μg pROSA-cLuc with pROSA-gLuc following pre-administration of pROSA-cLuc while cLuc and gLuc activities in the serum and liver

declined after co-administration of 10 μg pROSA-cLuc with pROSA-gLuc following pre-administration of pROSA-cLuc

To further investigate the effect of immune response on the transgene expression profile, mice received pre-administration of 30 μg pROSA-cLuc and time-courses of cLuc and gLuc activities in the serum and the liver after co-administration of 10 μg pROSA-gLuc with 1 μg or 10 μg pROSA-cLuc were investigated (Figure 15A and 15B). Both in the liver or serum, the cLuc activity and gLuc activity declined after co-administration of 10 μg pROSA-cLuc with 10 μg pROSA-gLuc. After co-administration of 1 μg pROSA-cLuc with 10 μg pROSA-gLuc, only the cLuc activity in the serum decreased, and cLuc activity in the liver and gLuc activities in the serum and liver did not decrease.

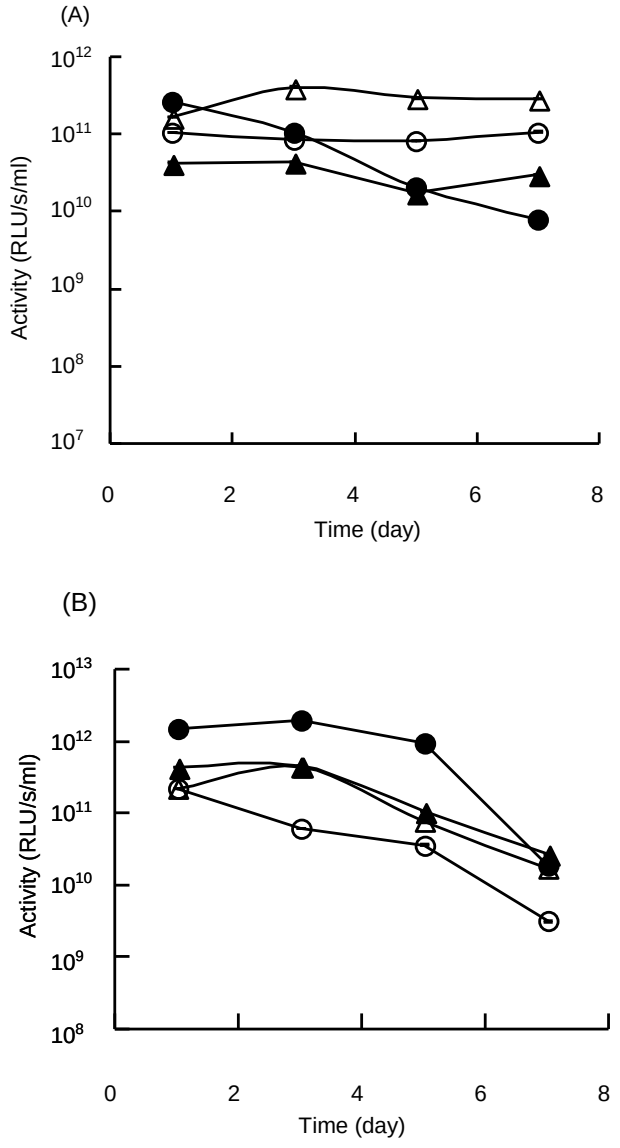

Figure 15. Time course of gLuc and cLuc activities in serum and liver after co-administration of pROSA-cLuc with different doses of pROSA-gLuc after pre-admininstration of pROSA-cLuc. The mice initially received hydrodynamic injection of 30 μg of pROSA-cLuc. Fourteen days after the first injection, the mice received co-injection of 10 μg of pROSA-gLuc with (A) 1 μg of pROSA-cLuc or (B) 10 μg of pROSA-cLuc. The time course of cLuc (closed symbol) and gLuc (open symbol) in the serum (circles) and in the liver (triangle) were examined. The results are expressed as the mean ± SD of five mice.

II-3-f OVA-specific cellular and humoral immune response was induced by the administration of pCpG-OVA

With the result that high level of transgene expression per cell is important for the induction of

transgene-sepcific immune response, I hypothesized that hepatocytes, which is the major transgene-expressing cells after hydrodynamic gene transfer, worked as APCs after hydrodynamic gene transfer. To investigate the hypothesis, OVA was selected as model antigen and OVA-TCR-transgenic CD8-T cell which expressing a TCR specific for the OVA epitope (CD8 OVA 1.3 T cell) was used as model T cell to examine the antigen presentation by hapotocytes.

To evaluate whether OVA-sepcific immune response was induced by hydrodynamic injection of OVA-expressing plasmid vectors, time-course of serum gLuc activity and OVA-sepcific immune response were evaluated after co-admininstration of pROSA-gLuc with pCpG-OVA or pCMV-OVA. Serum gLuc activity decreased after co-adminsitration with pCpG-OVA while serum gLuc activity did not decrease after co-admininstration with pCMV-OVA (Figure 16A). In addition, high level of OVA-specific antibody in the serum and IFN-γ production from splenocytes in response to OVA was observed in the mice receiving pCpG-OVA (Figure 16B and 16C). These results clearly indicate that OVA-sepcific immune response was induced by hydrodynamic administration of pCpG-OVA.

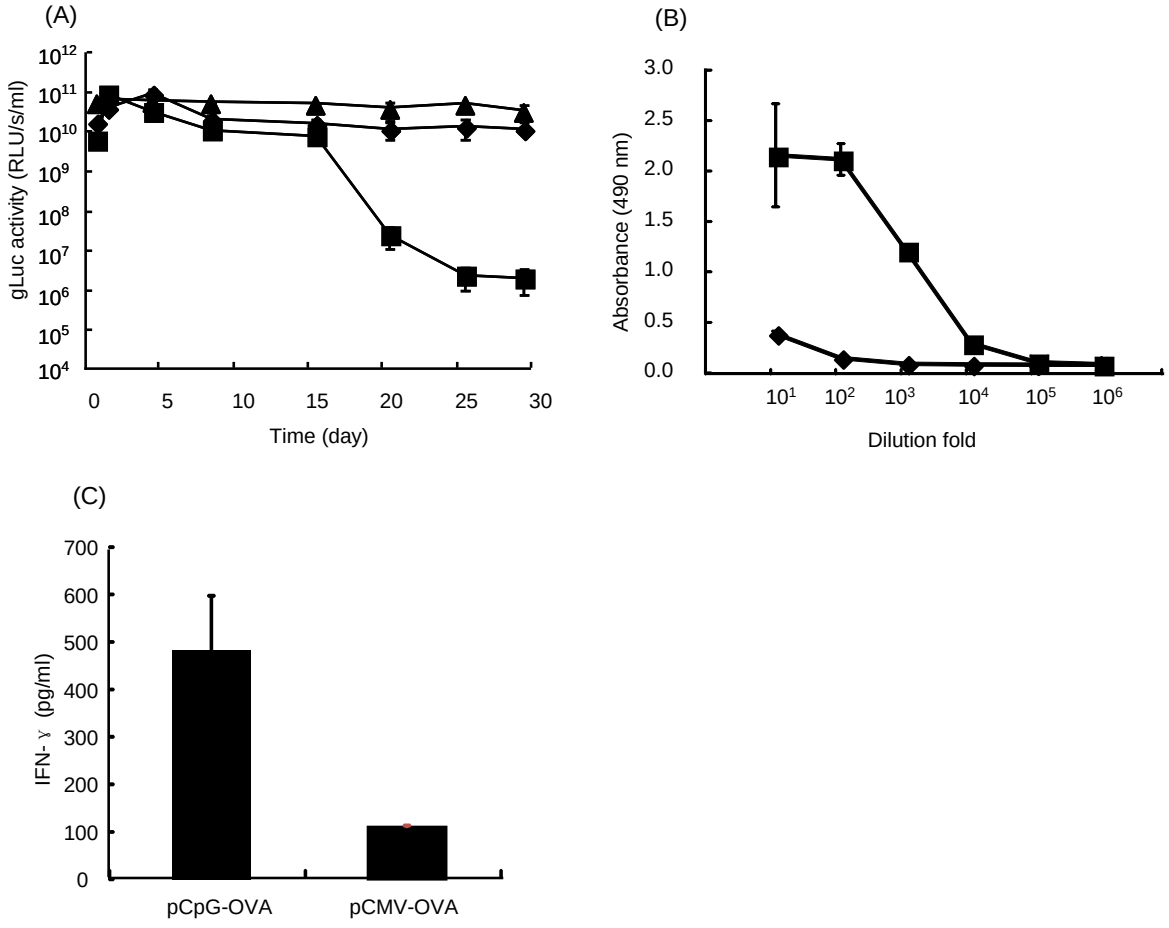

Figure 16. gLuc activities in the serum and OVA-specific immune responses after co-administration of the pROSA-gLuc with OVA-expressing vectors by hydrodynamic injection.

(A) Time course of serum gLuc activity after co-administration of 10 μg of pROSA-gLuc with 10 μg of pCMV-OVA (circle), pCpG-mcs (triangle) or pCpG-OVA (square). (B) Mice received hydrodynamic injections of 10 μg of pCMV-OVA (diamond) or 10 μg of pCpG-OVA (square). Twenty five days after plasmid DNA administration, serum samples from the mice were collected, and antibody titers were measured by ELISA. (C) Splenocytes collected from untreated mice or mice receiving plasmid DNA administration were cocultured with OVA for 4 days. IFN-γ concentration in the culture medium was measured by ELISA.

II-3-g CD8 OVA1.3 T cells were attracted to the liver after hydrodynamic gene transfer of pCpG-OVA

Antigen presentation is mediated through direct contact of APCs with T cells. To investigate whether T cells were captured in the liver after hydrodynamic gene transfer, CD8 OVA1.3 T cells labeled by PHK26 were intravenously administered into mice that had received plasmid DNA admininstration. The liver was harvest 3 days later and the liver frozen section was prepared. As a result, number of CD8 OVA1.3 T cells in the liver was lareger in the mice receiving pCpG-OVA than that in the mice receiving pCpG-mcs or pCpG-cLuc (Figure 17A).

The antigen presentation after hydrodynamic gene transfer might be caused by DCs in the liver. To evaluate the possibility, the liver section collected from the mice receiving CD8 OVA1.3 T cell injection after pCpG-OVA administration was fluorescently stained with DC-sepcific antibody. The number of DC in the liver was very small, and contact of CD8 OVA1.3 T cells with DC was not observed in the sections (Figure 17B).

cLuc-specific immune response.

Serum cLuc activity started to decline about 1 week after single injection of high dose (30 μg) of pROSA-cLuc while serum cLuc activity was stable after 3 times injection of a low dose (10 μg) of pROSA-cLuc, which indicates that cLuc-specific immune response was induced in the former situation. Evaluation of cLuc-specific humoral and cellular immune response also indicates that sigle administration of high dose of pROSA-cLuc induced stronger cLuc-specific immune response than thrice injection of low dose of pROSA-cLuc. Thrice injection of low doses of pROSA-cLuc induced humoral immune response and hardly induced cellular immune response while cLuc activity was stable after the thrice injection. This fact implies that reduction in cLuc activity, which is considered to reflect the removal of cLuc-expressing cells, was mainly caused by cellular immune response, not by humoral immune response. This result is in good agreement with the results reported by Ian J. *et al* that cellular immune response is more important than humoral immune response in the removal of transgene-expressing cells [65]. In addition, these results also suggest that the induction of cellular immune response requires higher transgene level per cell than the induction of humoral immune response does.

After the pre-admministration of 30μg pROSA-cLuc, co-administration of pROSA-gLuc with 1 μg pROSA-cLuc resulted in the decrease in the serum cLuc activity alone while coadministration of pROSA-gLuc with 10 μg pROSA-cLuc resulted in the decrease in the both cLuc and gLuc activities in the liver and in the serum. Considering the fact that the both humoral and cellular immune response specific for cLuc was induced by the preadministration, reduction in the both cLuc and gLuc activities in the liver and in the serum after coinjection of pROSA-gLuc with 10 μg pROSA-cLuc is highly likely to reflect the removal of transgene-expressing cells by cellular immune response. On the other hand, transgene-expressing cells was hardly affected after the coadministration of pROSA-gLuc with 1 μg pROSA-cLuc, which implies that cLuc-antigen presentation level in transgene-expressing cells was too low to be recognized by cellular immune response. In addition, reduction in the serum gLuc activity may indicate the acceralation of the clearance of cLuc from blood circulation by cLuc-specific antibody [66,67].

From the result that hig level of transgene expression level per cell is necessary to induce immune response specific for the transgene product, it was hypothesized that hepatocytes works as APC after hydrodynamic gene transfer. After pCpG-OVA administration, which induces OVA specific immune response, a lot of intravenously-injected CD8 OVA1.3 T cells were found in the liver during antigen-recogniton phase after the gene transfer, which implies that antigene presentation after hydrodynamic gene transfer might take place in the liver. In addition, detection of DC by immunostaining suggests that CD8 OVA1.3 T cells that accumulated in the liver did not attach to DCs in the liver.

It was demonstrated that hepatocytes collected from mice receiving pCpG-OVA administration by

hydrodynamic injection activated CD8 OVA1.3 T cells to secrete IL-2, which indicates that hepatocytes work as APC after hydrodynamic gene transfer. This result is in agreement with the previously reported results that the hepatocytes induce specific CD8 T cell proliferation [42]. However, as detailed molecular mechanism such as co-stimulatory molecules of hepatocytes were not clear, further studies are required to understand the APC function of hepatocytes.

In conclusion, the present study has demonstrated that the high level of transgene expression per cell is important in causing the immune response after hydrodynamic gene transfer. Moreover, hepatocytes with high level of transgene expression may work as APC to prime transgen-specific immune response.

Summary

In this thesis, the effects of transgene expression profile and types of transgene expressing cells on the induction of transgene-specific immune response were investigated. The main findings obtained in each chapter are as follows.

I. Elucidation of the effect of the duration of transgene expression on the induction of transgene-specific immune response

A high level of sustained fLuc expression in the liver triggered antigen-specific immune responses while short-term expression of the fLuc elicits little, if any, immune response. Moreover, fLuc-specific immune response induced by the sustained transgen fLuc expression removed the cells expressing fLuc in turn. When gLuc-expressing vector was coadminstered with sustained fLuc-expressing vector, sudden decrease in gLuc expression at approximately 1 week after the coadministration occurred, which indicates that cells expressing both fLuc and gLuc were recognized and attacked by fLuc-specific immune response. By histological analysis of the liver sections of mice, $CD8^+$ cell infiltration was observed, implying that the transgene-expressing hepatocytes were removed by the infiltrating cells. In addition, spleen removal and macrophage depletion did not change the profile of serum gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc, suggesting that fLuc-specific immune response induced by sustained fLuc expression is not dependent on the transgene expression in these types of cells.

II. The importance of transgene expression level in each cell on the induction of transgene expression-specific immune response

In Chapter II, the importance of transgene expression level in each cell on the induction of transgene expression-specific immune response was investigated. By using pROSA-cLuc, it was found that the transgene expression level per cells, not the total amount of transgene, is important in the induction of the transgene-specific immune response after hydrodynamic gene transfer. In addition, it was also suggested that higher level of transgene-expresion level per cells was required for the induction of transgene-specific cellular immune response than that was required for the induction of transgene-specific humoral immune response. In addition, it was found that high level of transgne expression is required to be recognized by transgene-specific cellular immune response.

After pCpG-OVA hydrodynamic gene transfer, CD8 OVA1.3 T cells accumulated in the liver while nearly no CD8 OVA1.3 T cells were found after pCpG-cLuc and pCpG-mcs injection, which suggests that CD8 OVA1.3 T cells received antigen presentation in the liver via direct contact. IL-2 secretion from CD8 OVA1.3 T cells co-cultured with hepatocytes collected from mice receiving pCpG-OVA implies that

hepatocytes expressing high level of an antigenic transgene product work as APCs to prime transgene-specific immune response after hydrodynamic gene transfer.

In conclusion, I demonstrated that a high level of sustained expression of antigenic transgene induces an immune response that removes the cells expressing the transgene product and that the transgene expression level per cells, not the total amount of transgene product, is important in the inducition of transgene-specific immune response after hydrodynamic gene transfer. In addition, transgene expression in the spleen and macrophages is not important in the induction of transgene-specific immune response after hydrodynamic gene transfer, while high level of transgene expression per cell is important in the immune induction. Moreover, it was demonstrated that hepatocytes with high level of transgene expression may prime transgene-specific immune response after hydrodynamic gene transfer. These results provide useful information to treat with the immune response in gene therapy.

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List of Publications

Removal of transgene-expressing cells by transgene-specific immune response induced by sustained transgene expression

Yalei Yin, Yuki Takahashi, Norifumi Ebisuura, Makiya Nishikawa, Yoshinobu Takakura

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Induction of transgene-specific immune response dependent on the transgene expression level per cell after hydrodynamic gene transfer

Yalei Yin, Yuki Takahashi, Makiya Nishikawa, Yoshinobu Takakura

Manuscript in preparation

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