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Kyoto University
Positive correlation between the generation of reactive oxygen species and activation/reactivation of transgene expression after hydrodynamic injections into mice

1Naomi Takiguchi, 1Yuki Takahashi, 1Makiya Nishikawa, 1Yuriko Matsui 1Yasushi Fukuhara, 2Daihi Oushiki, 2Kazuki Kiyose, 2Kenjiro Hanaoka, 2Tetsuo Nagano and 1Yoshinobu Takakura

1Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan
2Department of Bioorganic Chemistry, Graduate School of Pharmaceutical Sciences and Chemical Biology Research Initiative, The University of Tokyo, Tokyo, Japan

Corresponding author: Yoshinobu Takakura, Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.
Tel: +81-75-753-4615; Fax: +81-753-4614
E-mail: takakura@pharm.kyoto-u.ac.jp

Running head: Correlation of ROS and transgene expression after hydrodynamic injections
Abstract (<200 words)

Purpose: Hydrodynamic injection has been shown to reactivate silenced transgene expression in mouse liver. In this study, the roles of inflammatory cytokines and reactive oxygen species (ROS) in the reactivation were examined.

Methods: Production of inflammatory cytokines and ROS by hydrodynamic injection of saline was examined in mice that had received a hydrodynamic injection of a plasmid expressing Gaussia luciferase. The level of reporter gene expression was used as an indicator of the reactivation. The involvement of cytokines and ROS was examined by depleting Kupffer cells or by pre-administration of antioxidants, respectively.

Results: A hydrodynamic injection of saline induced a significant production of interleukin (IL)-6. Depleting Kupffer cells using clodronate liposomes markedly reduced the IL-6 production but had no significant effect on the transgene expression. On the other hand, an injection of catalase or N-acetylcysteine significantly inhibited the hydrodynamic injection-induced reactivation of silenced transgene expression. The silenced expression was also reactivated by carbon tetrachloride, an inducer of oxidative stress in the liver, in a dose-dependent manner, and this reactivation was significantly inhibited by catalase.

Conclusions: These findings show a positive correlation between the generation of ROS and the reactivation of silenced transgene expression after hydrodynamic injections.

Key Words: hydrodynamic injection; liver; plasmid DNA; reactive oxygen species; inflammation
Introduction

Hydrodynamic gene delivery, which involves a rapid, large-volume injection of naked plasmid DNA, produces an extremely high level of transgene expression in mouse liver (1, 2). Transgene expression in the liver obtained by hydrodynamic injection is several orders of magnitude greater than that obtained by other nonviral methods, including electroporation-mediated gene delivery, direct injection of naked plasmid DNA into the liver and intraportal injection of plasmid DNA/cationic liposome complex (lipoplex) (3). Because such high levels of transgene expression are difficult to explain simply by the difference in the amount of plasmids delivered to the liver (4), some biological changes induced by the injection are thought to play an important role in the high level of transgene expression obtained by hydrodynamic gene delivery.

Recent findings showing that silenced transgene expression in mouse liver is reactivated by rapid, large-volume injection of an isotonic solution containing no plasmid DNA (5, 6) have shed a light on the mechanism of the hydrodynamic injection. In our previous study, we found that hydrodynamic injection of isotonic solution containing no plasmid DNA activates the transcription factor activator protein (AP)-1 and nuclear factor (NF)-κB in the liver. Plasmids with no binding sequences for these transcription factors failed to be reactivated by hydrodynamic injections, strongly suggesting that the activation of these transcription factors is a key process in the reactivation of silenced transgene expression. These findings also suggest an important issue, namely, that the activation of transcription factors in the liver by hydrodynamic injection makes a major contribution to the extremely high transgene expression produced by injection, although the detailed mechanism of how the hydrodynamic injection
activates transcription factors is still not clear. The elucidation of the mechanism underlying the reactivation produced by hydrodynamic injection will provide useful information for developing more efficient gene delivery methods.

Hydrodynamic injection induces a variety of changes in mouse liver. Suda et al. reported that the size of mouse liver increases soon after hydrodynamic injection up to about 240% compared with the original size (7). They also reported that the blood pressure in both the inferior vena cava and the portal vein of the mice that received a hydrodynamic injection was 3-fold higher than that of the control group. These changes in the physiological state would cause tissue damage, leading to a cascade of inflammatory responses. In addition, the oxygen supply to the liver would be reduced because saline or other isotonic solution, which is much less oxygenated than blood, remains in the liver for some time. This could lead to transient hepatic ischemia, which then results in hypoxia. Hypoxia increases the generation of reactive oxygen species (ROS) at mitochondrial complex III (8, 9).

These pieces of information suggest that reactivation of silenced transgene expression is mediated by the increased levels of proinflammatory cytokines and/or ROS, because both of these factors are known activators of AP-1 and NF-κB (10, 11). Therefore, in the present study, we investigated the roles of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and ROS in the reactivation of silenced transgene expression produced by a hydrodynamic injection of saline.
Materials and Methods

Chemicals

Dichloromethylenediphosphonic acid disodium salt (clodronate), lipopolysaccharide (LPS) and bovine liver catalase (40000-60000 units/mg protein) were purchased from Sigma Chemical (St Louis, MO, USA). N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade and used without further purification.

Plasmid DNA

pCMV-gLuc encoding Gaussia luciferase under CMV promoter was constructed by subcloning the EcoRV/XbaI Gaussia luciferase cDNA fragment from pGLuc-Basic vector (New England Biolad, Madison, WI, USA) into the multi-cloning site of pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). pCMV-Luc encoding firefly luciferase under the control was obtained as described previously (6). pTA-Luc, pAP-1-Luc and pNF-κB-Luc, plasmid DNA encoding firefly luciferase under the control of TATA-box only, TATA-box and AP-1 binding sites or TATA-box and NF-κB binding sites, respectively were purchased from Clontech (Mountain View, CA). Plasmid DNA was amplified in the E. coli strain DH5α, isolated, and purified using JETSTAR 2.0 Plasmid GIGA Plasmid Purification Kits (GENOMED GmbH, Löhne, Germany).

Animals
Male ICR (four-week-old, about 20 g) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and were maintained under conventional housing conditions. The protocols for animal experiments were approved by the Animal Experimentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University.

**Hydrodynamic injection**

Saline solution with a volume of 8% body weight containing no or only 10 μg naked pCMV-gLuc was injected into the tail vein of mice over 5 sec. To reactivate silenced transgene expression of pCMV-gLuc, mice that had received the hydrodynamic injection of pCMV-gLuc were injected with saline in a manner similar to the hydrodynamic injection.

**Preparation of clodronate liposomes**

Phosphatidylcholine (63.5 μmol) and cholesterol (10.3 μmol) were dissolved in chloroform and a thin lipid film was formed by low-vacuum rotary evaporation. This film was dispersed in 5ml of phosphate buffered saline (PBS) in which clodronate was dissolved at a concentration of 0.7 M. The suspension was maintained at room temperature for 2 h followed by ultrasonication under nitrogen gas for 3 min. After incubation for another 2 h at room temperature, the suspension was centrifuged at 22000 × g for 1 h at 10 °C to remove free clodronate, and then washed four times using centrifugation at 22000 × g for 25 min at 10 °C. The liposomes were then resuspended in PBS and stored at 4 °C until use.

**Transient depletion of Kupffer cells**
To deplete Kupffer cells, 100 μl of clodronate liposomes were injected into the peritoneal cavity of mice 48 h prior to the hydrodynamic injection of saline (12). In control mice, PBS was injected into the peritoneal cavity. The existence of Kupffer cells in the liver was evaluated by immunofluorescent staining of liver sections with F4/80, a macrophage specific antigen. In brief, frozen liver sections (8-μm thick) were obtained by a cryostat (Jung CM 3000, Leica Microsystems AG, Wetzlar, Germany), stained with FITC-conjugated rat anti-mouse F4/80 antibody (1:50 dilution; AbD Serotec, Oxford, UK) and observed using a fluorescence microscope (Biozero BZ-8000, KEYENCE).

**Preparation of cationic liposomes and plasmid DNA/cationic liposome complex (lipoplex)**

Cationic liposomes consisting of DOTMA and cholesterol were prepared as previously reported (13). In brief, the lipids mixed in chloroform at a molar ratio of 1:1 were dried as a thin film in a round-bottomed flask using a rotary evaporator, and then hydrated in 5% w/v dextrose by gentle vortexing. After hydration, the dispersions were sonicated for 2.5 min and passed through a Minisart® 0.45 μm filter unit (Sartorius K.K., Tokyo, Japan). The lipid concentrations of cationic liposomes were determined by the Cholesterol E-Test Wako kit (Wako Pure Chemical Industries). Cationic liposomes and pcDNA3.1 were mixed in 5% dextrose at a charge ratio of +2.24 and the mixture was left for at least 30 min at 37 °C to form a lipoplex.

**Injection of LPS or lipoplex into mice**

Five days after hydrodynamic injection of pCMV-gLuc, mice received an injection of LPS or lipoplex. LPS was injected into the peritoneal cavity of mice at the indicated doses. In
separate mice, lipoplex was intravenously injected at the indicated doses.

**Injection of catalase or N-acetylcysteine**

Saline (8% of body weight) containing 100-1000 U bovine liver catalase was hydrodynamically injected into mice that had received pCMV-gLuc transfer 5 days before the catalase administration. Catalase heat-inactivated at 60 °C for 30 min was used as a control. In separate mice, N-acetylcysteine (NAC) was injected into the peritoneal cavity of mice that had received pCMV-gLuc transfer 5 days before the NAC administration. Thirty minutes after NAC administration, mice received a hydrodynamic injection of saline.

**Carbon tetrachloride-induced reactivation of transgene expression**

Carbon tetrachloride (CCl₄) dissolved in olive oil was injected into the peritoneal cavity of mice at a dose of 1-3 ml CCl₄/10 ml olive oil/kg body weight. In another group of mice, catalase was injected at a dose of 1,500 U/mouse into the tail vein immediately after administration of 2 ml CCl₄/kg.

**Luciferase assay for gaussia luciferase**

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. Then, 10 μl of serum was mixed with the sea pansy luciferase assay buffer (PiccageneDual, Toyo Ink, Tokyo, Japan), and the chemiluminescence was measured with a luminometer (Lumat LB 9507; EG&G Berthold, Bad Wildbad, Germany).
Measurement of serum concentrations of inflammatory cytokines

The concentrations of IL-6 and TNF-α in serum were measured using enzyme-linked immunosorbent assay (ELISA) kits (BD OptEIA Mouse IL-6 ELISA set, BD Bioscience, San Jose, CA, USA; TNF-α Mouse ELISA Kit, R&D systems, Minneapolis, MN, USA).

Evaluation of transcription factor-specific gene expression

To examine whether AP-1 and NFκB are activated in the liver, mice received a hydrodynamic injection of 10μg of pTA-Luc, pAP1-Luc, pNF-κB-Luc or pCMV-Luc. Five days after gene transfer, mice received another hydrodynamic injection of saline or an intraperitoneal injection of CCL4. At 6 h after the treatment, mice underwent euthanasia and the liver was excised and homogenized in 5 ml/g lysis buffer (0.1 M Tris, 0.05% Triton-X-100, 2 mM EDTA, pH 7.8). The homogenate was centrifuged at 13000 g for 10 min at 4°C. Then the supernatant was mixed with the luciferase assay buffer, and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507).

Detection of hypoxic conditions using a fluorescent probe

We used a fluorescent probe QCy5, which emit fluorescence under hypoxic conditions (14). Mice were anesthetized with pentobarbital in phosphate-buffered saline and mice received an intravenous injection of 10 nmol QCy5 dissolved in 100 μl saline. Soon after the injection of QCy5, a midline abdominal incision was made to expose the liver. Then, mice were left untreated or received a hydrodynamic injection of saline or a hepatic ischemia by clamping the
portal vein. Fluorescent images at excitation of 630 nm and detection at 655 nm were repeatedly obtained by using the NightOwl LB 981 Molecular Light Imager (EG&G Berthold) at the indicated time points after the treatment.

**Statistical Analysis**

Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Fisher’s PLSD test for multiple comparisons and by Student's t-test for two groups. A p-value of less than 0.05 was considered to be statistically significant.
Results

Production of proinflammatory cytokines after hydrodynamic injection

Fig. 1a shows the concentrations of IL-6 and TNF-α in mouse serum after hydrodynamic injection of saline into mice. A hydrodynamic injection caused a transient increase in serum IL-6 1-2 h after injection (closed symbol) with a peak level of 1500 pg/ml. In addition, a large volume injection of saline over 30 second or more (a slow injection) had no significant effects on the serum concentration of IL-6 (28.4 pg/ml at 1 h after injection). Then, the level quickly returned to normal while the injection produced hardly any increase in the serum TNF-α level (open symbol).

Effect of IL-6 on the level of transgene expression

IL-6 is mainly produced by Kupffer cells and involved in liver injury and regeneration (15). To examine whether IL-6 plays a key role in the increase in transgene expression by hydrodynamic injection, mice received a hydrodynamic injection of 10 µg pCMV-gLuc. Three days after hydrodynamic injection of pCMV-gLuc, mice received injections of clodronate liposomes into the peritoneal cavity to deplete macrophages including Kupffer cells and a hydrodynamic injection of saline was performed on day 5. F4/80 staining of liver sections clearly demonstrated that the number of F4/80-positive cells in the liver, most of which are Kupffer cells, were markedly reduced by the injection of clodronate liposomes (Fig.1b-c). Moreover, mice injected with clodronate liposomes showed decreased IL-6 production after the second hydrodynamic injection (Fig.1d), indicating that a large fraction of cells producing IL-6
was depleted by the treatment.

Then, the ratio of the expression levels (x-fold increase) before and 6 hr after hydrodynamic injection of saline was calculated and used to examine the effect of the clodronate liposomes on the reactivation of silenced transgene expression. The luciferase activity in serum was increased about 20-fold by hydrodynamic injection of saline in the PBS-treated mice (Fig.1e). A similar ratio was obtained in the clodronate liposome-treated mice, indicating that IL-6 production has no significant effect on the increase in transgene expression.

**Effects of LPS and lipoplex on the level of transgene expression**

To examine the effect of inflammatory cytokines, including IL-6, on the level of transgene expression, LPS or lipoplex, both of which are known to induce such cytokines, was administered to mice that had received a hydrodynamic injection of pCMV-gLuc 5 days before. Administration of LPS or pcDNA3.1 lipoplex increased the serum concentration of IL-6 to peak levels of 12000 and 15000 pg/ml, respectively, in dose-dependent manner (Fig.2a). Irrespective of the type of inducer, the luciferase activity in the serum was hardly affected by the treatment (Fig.2b-c). Taken together, these results indicate that IL-6 produced by hydrodynamic injections contributes very little to the increase in transgene expression.

**Effect of antioxidants on the increase in transgene expression**

To examine the role of ROS in the reactivation, catalase, an enzyme degrading hydrogen peroxide, was injected into mice together with saline by the hydrodynamic injection method 5 days after hydrodynamic administration of pCMV-gLuc. Fig.3a shows the luciferase activity
before the treatment and 6 h after hydrodynamic injection of saline containing catalase or inactivated catalase. About a 17-fold increase in the activity was observed in mice receiving a hydrodynamic injection of saline containing inactivated catalase. The x-fold increase was low when catalase-containing saline was injected; the injection-induced increase in luciferase activity in serum was partially inhibited by injection of saline containing a high dose of catalase at 1000 units/mouse (33 μg protein/mouse). Similar results were obtained when NAC was used instead of catalase (Fig.3b). These results suggest that ROS play a key role in the increase in transgene expression by hydrodynamic injection.

Increase in transgene expression by CCl4 and its suppression by catalase

CCl4 is metabolized to the trichloromethyl radical (·CCl3) in the liver and induces oxidative stress (16). To examine the effects of oxidative stress on the transgene expression, the change in the level of transgene expression after CCl4 injection was first examined. Mice that had received a hydrodynamic injection of 10 μg pCMV-gLuc received an intraperitoneal injection of 1-2 ml/kg CCl4 on day 5. CCl4 increased the level of luciferase activity in the serum in a dose-dependent manner (Fig.4a). However, this CCl4-induced increase in activity was significantly suppressed by an intravenous injection of catalase at the dose of 1500 units/mouse (50 μg protein/mouse) (Fig.4b), indicating that ROS, especially hydrogen peroxide, induced by CCl4 reactivates silenced transgene expression. To determine the time course of the increase in transgene expression after hydrodynamic injection of saline or CCl4 administration, the luciferase activity in the serum was determined at the indicated time points (Fig.4c). As some mice did not survive the CCl4 treatment, the results of survived mice were shown. The luciferase
activity had a peak value at 6 h to 1 day after the treatment, and decreased thereafter. The profiles were almost identical between the two treatment groups.

To evaluate whether ROS production in the liver activate transcription factors including NF-κB and AP-1, plasmid DNA with binding sites for these transcription factors was injected. In addition, pCMV-Luc and pTA-Luc were used as a positive and negative control, respectively. A hydrodynamic injection of saline or intraperitoneal injection of CCl_4 increased the luciferase activity in mice that had treated with pCMV-Luc, pAP-1-Luc or pNF-κB-Luc, suggesting that the transcriptional activity of AP-1 and NF-κB is increased by the treatment. On the other hand, neither treatment increased the luciferase activity in mice treated with pTA-Luc, a plasmid DNA lacking the binding sites for transcription factors.

Detection of hypoxia in mouse liver

To investigate whether hydrodynamic injection induces hypoxia in mouse liver, a fluorescent probe QCy5, which emits fluorescence only under hypoxic conditions, was intravenously injected to mice. Only a weak fluorescence, which might be derived from food, was observed in the gastrointestinal tract of untreated mice (Fig.5b). A time-dependent increase in fluorescent intensity was observed in the liver of mice with the portal vein being clamped. Moreover, hydrodynamic injection also increased the fluorescence intensity of the liver, suggesting that the liver is under hypoxic conditions.
Discussion

Hydrodynamic injections produce a variety of changes in the body, especially within the liver, which include, but are not limited to, a transient increase in the size of the liver, increased blood pressure across the liver, reorganization of the cytoskeleton (unpublished observation), and the activation of transcription factors, such as AP-1 and NF-κB (6, 7). In addition to these events, our present study showed that the levels of IL-6 and ROS are increased by hydrodynamic injection.

Partial hepatectomy leads to regeneration of the organ to its original size within a week or so (17). Liver regeneration is linked with the proliferation of liver cells where the expression of a variety of genes is markedly upregulated. In one of our previous studies, we found that hydrodynamic injection of saline increases the mRNA expression level of c-fos, c-jun and c-myc in the liver whereas it decreases the level of cyp2e1 (6), which is a similar gene regulation to those observed in the process of liver regeneration. Of the genes upregulated during liver regeneration, IL-6, the expression of which is markedly increased when the liver is damaged (18), plays important roles in the regeneration (15). For example, it was reported that hepatocytes are primed by inflammatory cytokines, such as IL-6, to competent for the replication (19). In the present study, we found that hydrodynamic injections increase the level of serum IL-6 (Fig.1a), which was comparable with that observed after partial hepatectomy (20). These results imply that hydrodynamic injection induces some liver damages that could trigger responses similar to those occurring during liver regeneration. Therefore, IL-6 induced by hydrodynamic injection may be involved in activating AP-1 and NF-κB, then in increasing the expression from plasmids. However, the reduced IL-6 level in mice whose Kupffer cells were
depleted hardly affected the level of reactivation (Fig. 1b-e). Moreover, increasing the IL-6 production by stimuli other than hydrodynamic injection, such as LPS and lipoplex, was not effective in reactivating the silenced transgene expression (Fig. 2). These results suggest that hydrodynamic injection induces the production of IL-6 from Kupffer cells, but the induced IL-6 has no significant effect on the reactivation of silenced transgene expression by hydrodynamic injection. On the other hand, it is known that LPS or lipoplex treatment activates transcription factors, including NF-κB. These treatments increased the serum concentration of IL-6, indicating that these treatments induced inflammatory responses (Fig. 2a). Considering the fact that hepatocytes are hardly involved in the initiation of inflammatory response, the activation of transcription factors by the treatment of LPS or lipoplex could take place mainly in inflammation-related cells, such as Kupffer cells, but not in hepatocytes.

Oxidative stress is associated with a large number of events in the liver, including hepatitis, viral infection, drug-induced liver injury and ischemia/reperfusion injury (21, 22). In the present study, we found that administration of catalase or NAC significantly suppressed the reactivation of transgene expression by hydrodynamic injections (Fig. 3), which strongly suggests that ROS are produced by hydrodynamic injections and that the ROS produced activate the transgene expression. In addition, the experimental results of CCl₄, an inducer of oxidative stress, also supported the hypothesis that ROS production in the liver triggers the reactivation of the transgene expression (Fig. 4). The following is one possible explanation for the mechanism of ROS production by hydrodynamic injections. After hydrodynamic injection, the oxygen supply to the liver would be reduced because isotonic saline solution, which is much less oxygenated than blood, remains in the liver for some time (7, 23). This could lead to transient
hepatic ischemia, which then results in hypoxia and increases the generation of ROS at mitochondrial complex III (8, 9). In accordance with this hypothesis, we detected that a hydrodynamic injection of saline induced hypoxic conditions in mouse liver (Fig.5b). As ROS are well-known intracellular second messengers that activate a variety of transcription factors, including AP-1 and NF-κB, it is reasonable that hydrodynamic injection-induced ROS activate these transcription factors, then these factors bind to their sites in the promoter/enhancer regions of plasmids, leading to a high level of transgene expression. In the present study, we also confirmed that CCl₄ administration activated the transcription activity of AP-1 and NF-κB (Fig.5a).

In our preliminary experiments, we found that hepatic ischemia/reperfusion could also reactivate silenced transgene expression (unpublished observations). In the ischemic liver, xanthine oxidase and hypoxanthine accumulate, and both of them produce a large amount of superoxide anions when the liver is reperfused (24). Therefore, this finding also supports the hypothesis that hepatic ROS production increases the transgene expression in the liver. Therefore, any type of stimuli that produces ROS can be used to increase the transgene expression.

Reactivation of silenced transgene expression is an interesting event. It suggests that plasmid DNA delivered to the liver remains for a long time in a form that can be activated transcriptionally by some stimuli. A previous study in our laboratory showed that the reactivation of transgene expression occurs even 3 months after hydrodynamic gene delivery (6). Thus, the reactivation of transgene expression could be an effective approach to achieving desirable transgene expression without repeated gene transfer. The requirement of ROS for
reactivation may raise some concern about tissue damage, but the damage induced when the reactivation stress is applied should be less than that when plasmid DNA is delivered to cells by other means. Developing methods that increase oxidative stress topically at the site where plasmid DNA is delivered will increase the availability of the reactivation technique for prolonged transgene expression without repeated administration of plasmid DNA.

**Conclusion**

The present study clearly demonstrates that hydrodynamic injection induces the production of both inflammatory cytokines and ROS, but only ROS is associated with reactivation of silenced transgene expression by hydrodynamic injection, whereas inflammatory cytokines are less likely to be associated with the reactivation.

**Acknowledgments**

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**References**


Legend to Figures

Fig.1 Inflammatory response following hydrodynamic injection and effect of Kupffer cell depletion on hydrodynamic injection-induced responses

(a) Time-courses of the concentrations of IL-6 and TNF-α in mouse serum after a rapid, large-volume injection of saline. Blood samples were sequentially obtained from the tail vein, and the concentrations of IL-6 (closed symbols) and TNF-α (open symbols) were measured. Results are expressed as mean ± SEM (n≥ 4). (b) and (c) Immunostaining of Kupffer cells in the liver after the administration of clodronate liposome. Liver sections were prepared 2 days after intraperitoneal administration of PBS (b) or clodronate liposomes (c) and stained with FITC-labeled F4/80 specific antibody. Scale bars = 100μm. (d) and (e) Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. On day 3, they were intraperitoneally injected with PBS or clodronate liposomes and, on day 5, they received a hydrodynamic injection of saline. (d) Time-course of the concentration of IL-6 in mouse serum after the hydrodynamic injection of saline on day 5. Closed symbols: PBS group, open symbols: clodronate liposome group. Results are expressed as mean ± SEM (n = 4). *P<0.05 compared with the PBS-injected group. (e) Gaussia luciferase activity in the serum was measured just before (open column) and 6 h after the hydrodynamic injection of saline (closed column), and the x-fold increase was calculated by dividing the value 6hr after injection by that before injection. Results are expressed as mean ± SEM (n = 4).

Fig.2 Effects of LPS and lipoplex on reactivation of silenced transgene expression. Mice received a hydrodynamic injection of 10 μg pCMV-gLuc. Five days after the administration of
plasmid DNA, mice received a rapid injection of a large volume of saline (saline HD), intraperitoneal injection of indicated doses of LPS or intravenous injection of lipoplex at a dose of 12.5-50 µg as the pcDNA3.1 amount. (a) Time-courses of the concentrations of IL-6 and TNF-α in mouse serum after saline HD, LPS administration or lipoplex injection. Results are expressed as mean ± SEM (n = 4). (b) and (c) Gaussia luciferase activity in the serum was measured just before (open column) and 6 h after saline HD, LPS administration or lipoplex injection (closed column). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean ± SEM (n ≥ 3). *P<0.05.

**Fig.3 Effect of antioxidant agents on reactivation of silenced transgene expression.** Mice received a hydrodynamic injection of 10 µg pCMV-gLuc. (a) On day 5, they received a hydrodynamic injection of saline containing 100 or 1000 U/shot bovine catalase or its inactivated derivative (inactivated catalase). Gaussia luciferase activity in the serum was measured just before (open column) and 6 h after the hydrodynamic injection of saline (closed column). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean ± SEM (n = 3). The shown data is representative result from the separate experiments that are performed twice. (b) NAC (400 or 600 mg/kg) was intraperitoneally injected into mice 30 min before a hydrodynamic injection of saline 5 days after pCMV-gLuc transfer. Gaussia luciferase activity in the serum was measured just before (open column) and 6 h after the hydrodynamic injection of saline (closed column). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean ± SEM (n ≥ 4). The shown data is representative result from the separate experiments that are performed twice.
**Fig. 4** Reactivation of transgene expression by CCl₄ and its suppression by catalase. Mice received a hydrodynamic injection of 10 µg pCMV-gLuc. (a) On day 5, they received an intraperitoneal injection of 1, 1.5 or 2 ml/kg CCl₄. Gaussia luciferase activity in the serum was measured just before (open column) and 6 h after the hydrodynamic injection of saline (closed column). The x-fold increase was calculated as described in the legend to Fig. 1e. (b) Catalase or inactivated catalase (1,500 U/shot) was intravenously injected into mice soon after an injection of 2 ml/kg CCl₄ into the peritoneal cavity. Gaussia luciferase activity in the serum was measured just before (open column) and 6 h after CCl₄ injection (closed column). The x-fold increase was calculated as described in the legend to Fig. 1e. Results are expressed as mean ± SEM (n ≥ 3). *P<0.05. (c) On day 5, mice received hydrodynamic injection of saline or intraperitoneal injection of 2 ml/kg CCl₄. At indicated time points after the treatment, Gaussia luciferase activity in the serum was measured. Results are expressed as mean ± SEM (n = 4).

**Fig. 5** Effect of hydrodynamic injection of saline on transcription factors and oxygen supply to the liver. (a) Mice received a hydrodynamic injection of 10 µg of pTA-Luc, pAP1-Luc, pNF-κB-Luc or pCMV-Luc. On day 5, they received an intraperitoneal injection of 3 ml/kg CCl₄ or hydrodynamic injection of saline. Six hours after the treatment, luciferase activity in the liver was determined. Results are expressed as mean ± SEM (b). *P<0.05 vs No Treat groups. (b) Mice received intravenous injection of 10 nmol QCy5 dissolved in 100 ul saline. Soon after QCy5 administration, mice were untreated or received hydrodynamic injection of saline or hepatic ischemia by clamping the portal vein. At the indicated time periods after the
initiation of the treatments, the fluorescent images at excitation of 630 nm and detection at 655 nm were obtained by using the NightOwl LB 981.
Figure 1

(a) Serum Concentration (pg/ml) vs. time (h)
- IL-6
- TNF-α

(b) Photograph of experimental results

(c) Photograph of experimental results

(d) Serum IL-6 (pg/ml) vs. time (h)
- PBS
- Clodronate Liposome

(e) RLU/s/ml
- 22-fold
- 20-fold

Legend:
- PBS
- Clodronate Liposome

* denotes statistical significance.
Figure 2

(a) Serum IL-6 (pg/ml) over time (h) for different groups:
- Saline HD
- pDNA 12.5 µg
- pDNA 25 µg
- pDNA 50 µg
- 1 µg/kg LPS
- 100 µg/kg LPS

(b) and (c) show RLU/s/ml for different treatments:
- Saline
- LPS 1 µg/kg
- LPS 100 µg/kg
- Saline HD
- Lipoplex pDNA 12.5 µg
- Lipoplex pDNA 25 µg
- Lipoplex pDNA 50 µg
- Saline HD

* indicates a significant difference.
Figure 3

(a) 15-fold 10-fold 6-fold

RLU/s/ml

Inactivated catalase 100U 1000U

Catalase

(b) 18-fold 16-fold 10-fold

RLU/s/ml

Saline 400mg/kg 600mg/kg

N-acetylcysteine
Figure 4

(a) Bar graph showing RLU/s/ml with different concentrations of CCl₄ (1ml/kg, 1.5ml/kg, 2ml/kg) compared to a vehicle control. The graph indicates a 2-fold increase at 1ml/kg, a 6-fold increase at 1.5ml/kg, and a 25-fold increase at 2ml/kg.

(b) Column chart showing RLU/s/ml for CCl₄ (2ml/kg) with and without catalase treatment. The chart indicates a 20-fold increase without catalase and a 5-fold increase with catalase.

(c) Graph showing RLU/s/ml over time (d) for Saline HD and CCl₄. The graph shows a decrease in RLU/s/ml over time for both groups, with a steeper decline for CCl₄.
Figure 5

(a)

(b)

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Digitalized image

- No Treat
- CCl4
- Saline HD

RLU/s/mg protein

1.E+00 1.E+01 1.E+02 1.E+03 1.E+04 1.E+05 1.E+06