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Brief Communication

Pretreatment of Hepatocyte Growth Factor Gene Transfer Mediated by Octaarginine Peptide-Modified Nanoparticles Ameliorates LPS/D-Galactosamine-Induced Hepatitis

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We previously reported that an octaarginine- and pH-sensitive fusogenic peptide-modified multifunctional envelope-type nano device (R8-GALA-MEND) produces a high level of gene expression in the liver. In this study, we report on an examination of whether this gene delivery system exerts potent hepatoprotective effects against lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced acute liver injury. *In vivo*-jetPEI™-Gal, a commercially available *in vivo* transfection reagent, was used as a reference. The systemic administration of the R8-GALA-MEND or *in vivo*-jetPEI™-Gal showed that the latter was more toxic than the R8-GALA-MEND, indicating that R8-GALA-MEND is a safer system than *in vivo*-jetPEI™-Gal. Pretreatment with R8-GALA-MEND or *in vivo*-jetPEI™-Gal loaded with hepatocyte growth factor (HGF) pDNA inhibited serum GPT and GOT levels from becoming elevated. However, the survival rate of the mice was significantly enhanced in the case of R8-GALA-MEND, but not for the *in vivo*-jetPEI™-Gal treatment. These results demonstrate that R8-GALA-MEND has the potential for use in the pretreatment of an acute liver injury.

Introduction

Gene therapy, especially non-viral gene therapy, holds great promise because it has less toxicity and immunogenicity compared with viral vectors. In preclinical trials using non-viral vectors (Ditto et al., 2009); however, it is evident that their efficiencies remain low compared with viral vectors. Consequently, a need for more effective and efficient non-viral vectors that can lead to production of effective therapeutic effects continues to exist.

The objective of our ongoing research is to develop a multifunctional envelope-type nano device (MEND), consisting of a lipid bilayer encapsulating plasmid DNA (pDNA) or small interfering RNA (siRNA) particles that are condensed with a polycation (Kogure et al., 2004). Previous studies have shown that a MEND modified using octaarginine (R8) and a pH-sensitive fusogenic peptide (GALA) to produce an R8-GALA-MEND resulted in a high rate of gene expression (Khalil et al., 2011).

The liver is involved with a wide spectrum of diseases, such as metabolic disorders, hepatitis, cirrhosis, cancer, and infectious disorders; however, only a few treatment options are available for many of these diseases. In particular, it is well known that fulminant hepatic failure has a poor prognosis with high mortality rates (Hoofnagle et al., 1995), so a safe and effective preventive and therapeutic method is greatly needed.

Here we demonstrate the positive protective effects of a R8-GALA-MEND loaded with hepatocyte growth factor (HGF) pDNA, compared with that of *in vivo* jetpolyethylenimine-galactose (jetPEI™-Gal), which is a well-known commercially available gene transfer reagent to the liver, especially cells expressing galactose-specific membrane receptors such as hepatocytes. The preparation has potent hepatoprotective therapeutic effects against lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced acute liver injury.

Materials and Methods

Male, 5-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals. Mice were administered LPS (300 μg) (Sigma, St. Louis, MO) and D-galactosamine hydrochloride (D-GalN) (10 mg) (Toronto Research Chemicals Inc., Northe York, Canada) intraperitoneally in 500 μL of injection volume. Blood samples were collected from the tail vein and serum obtained by centrifugation (10,000 rpm, 4°C, 10

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minutes). Glutamate pyruvate transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) levels in serum were measured using a transaminase CII test kit (Wako Pure Chemicals, Osaka, Japan), according to the manufacturer's instructions.

HGF encoding pDNA (cat. EX-A0820-M02) was purchased from GeneCopoeia. A pcDNA3.1(+)-luc (7037 bp) encoding the firefly luciferase gene was used. MENDs encapsulating luc pDNA or HGF pDNA were prepared by the hydration method, as described previously (Khalil et al., 2011), except that we used shGALA (Sakurai et al., 2011), not GALA peptide. The average diameters and zeta-potentials of the nanoparticles were determined using a Zetasizer Nano ZS instrument (Worchestershire, UK). As a control, *in vivo*-jetPEI™-Gal reagents (Polyplus-transfection, New York) were used to form PEI/pDNA complexes (N:P ratio 10), according to the manufacturer's instructions. The free pDNAs that were dissociated from the MEND or *in vivo*-jetPEI were detected by the PicoGreen DNA quantification reagent. A 200-times-diluted PicoGreen reagent solution was added to both nanoparticle solution (833 ng/mL) at a ratio of 1:1 (v/v). Dextran sulfate (40 μg/mL) was added to measure the pDNAs that were present outside of the nanoparticles and the fluorescent intensity of the free pDNA was then detected by spectrofluorometry (excitation 495 nm, emission 525 nm). The pDNA entrapment ratio was calculated by subtracting the ratio of dissociated free pDNA under dextran solution from 100%.

To estimate toxicities of the nanoparticles in mice, luc pDNA loaded MEND or *in vivo*-jetPEI™-Gal nanoparticles were administered through the tail vein at a dose of 50 μg pDNA. In experiments related to acute liver injury, the mice were treated with HGF pDNA loaded MENDs in a volume of 400 μL via the tail vein. The mice were then treated with the solution of LPS and D-GalN intraperitoneally 1.5 hours after the initial treatment with nanoparticles. Blood samples were collected 9 hours after the LPS and D-GalN treatment and GPT and GOT levels in serum were measured.

Results and Discussion

The physical characteristics of R8-GALA-MEND and *in vivo*-jetPEI™-Gal nanoparticles are shown in Table 1. The *in vivo*-jetPEI™-Gal nanoparticles were smaller in size, and their pDNA entrapment ratio was significantly lower compared with R8-GALA-MEND. The marked differences in the pDNA entrapment ratio could be explained by the different methods used in producing the nanoparticles; the pDNA in the MEND system is highly concentrated in the lipid envelope

Table 1. Physical Properties of the Two Different Hepatocyte Growth Factor pDNA Loaded Nanoparticles

	Size	*Zeta-potential*	*PdI*	*pDNA entrapment ratio (%)*
MEND	139 ± 4	36 ± 1	0.18 ± 0.02	91 ± 1
in vivo-jetPEI-Gal	82 ± 11	28 ± 2	0.19 ± 0.02	34 ± 3

Data are presented as the mean ± standard deviation ($n = 3$–5).
jetPEI-Gal, jetpolyethylenimine-galactose; MEND, multifunctional envelope-type nano device; PdI, polydispersity index; pDNA, plasmid DNA.

Table 2. Mice Survival Rate Six Hours After the Treatment

MEND	100% (8/8)
in vivo-jetPEI-Gal	18.2% (2/11)

(Kogure et al., 2004), while the pDNA in *in vivo*-jetPEI ™-Gal nanoparticles are associated with polycations, which we call a polyplex system. When they were intravenously injected into normal mice at a dose of 50 μg HGF pDNA, 9 out of 11 mice were dead after 6 hours. In contrast, all of the mice survived in the case of the R8-GALA-MEND treatment (Table 2). This overall toxicity can be explained by the fact that *in vivo*-jetPEI™-Gal nanoparticles induced liver damage (Akita et al., 2011). It can therefore be concluded that the R8-GALA-MEND is safer as a non-viral gene delivery system. Further experiments were performed in a dose of 25 μg HGF pDNA in order to compare the usefulness of the R8-GALA-MEND and *in vivo*-jetPEI™-Gal nanoparticles in treating an acute liver injury.

Model mice with fulminant hepatic failure were produced by the intraperitoneal injection of both LPS and D-GalN, which causes the over production of tumor necrosis factor-alpha (TNF-alpha) from activated macrophages and blocking of the nuclear factor-kB-induced expression of the cell survival gene, leading to acute liver damage. These animals selectively develop a more severe form of hepatic failure than is commonly observed in humans (Kosai et al., 1999). Figure 1 shows that GPT levels were slightly increased 5.5 hours after the injection and sharply increased between 5.5 to 8.5 hours. Although HGF is a promising antiapoptoic agent for preventing fulminant hepatic failure, the rapid clearance of HGF from the blood circulation may be a major problem in terms of its clinical use (Liu et al., 1992). Thus, HGF gene transfer to the

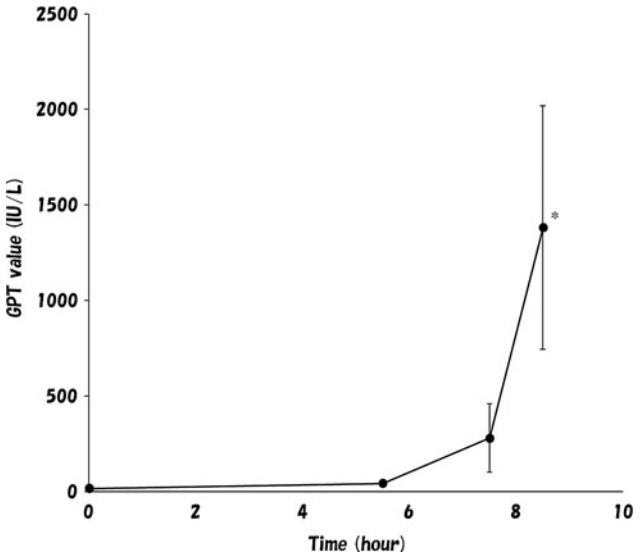


FIG. 1. Changes in plasma GPT levels after the injection of lipopolysaccharide/D-galactosamine (LPS/D-GalN). Data are expressed as mean ± standard error of the mean (SEM) ($n = 6$). The statistical significance of mean comparisons was performed by one-way analysis of variance (ANOVA) ($P < 0.05$), followed by Dunnett test. *$P < 0.05$. GPT, glutamate pyruvate transaminase.

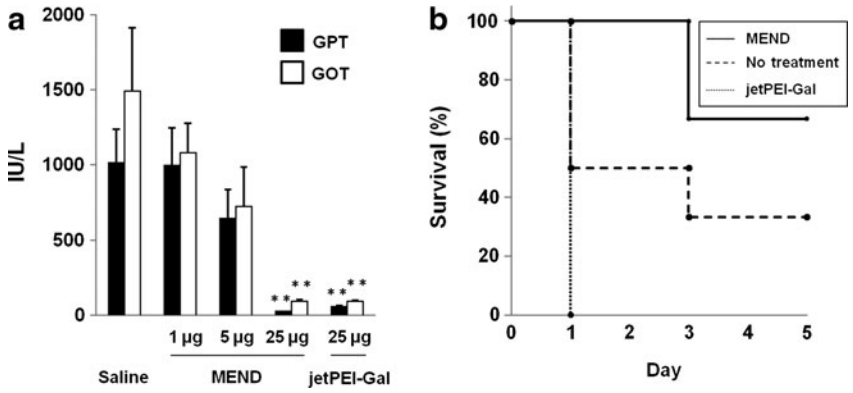


FIG. 2. Pharmacological effects of HGF pDNA on acute liver inflammation. **(a)** Dose-dependent pharmacological effect for treatment with the octaarginine- and pH-sensitive fusogenic peptide-modified multifunctional envelope-type nano device (R8-GALA-MEND) and *in vivo* jetPEI-Gal. Black and white bars represent GPT and GOT values respectively. Serum samples were collected 9 hours after LPS and D-GalN injection. Data are expressed as the mean ± SEM ($n = 6$). The statistical significance of mean comparisons was determined by one-way ANOVA ($P < 0.01$), followed by Dunnett test. *$P < 0.01$. **(b)** Survival rate of mice with no treatment and with R8-GALA-MEND or *in vivo* jetPEI-Gal treatment (25 μg). The statistical significance of the 2 groups was determined by the Kaplan Meier analysis (Gehan-Breslow test) ($P < 0.005$), followed by multiple comparisons. In each group $n = 6$.

liver would be expected to constitute an alternative preventive and therapeutic method because the concentration of HGF is locally increased, leading to a more prolonged pharmacological effect.

As shown in Fig. 2A, a dose of 25 μg HGF pDNA significantly decreased the serum levels of GOT and GPT, while 1 μg and 5 μg were not sufficient to significantly inhibit these values in the case of the R8-GALA-MEND treatment. On the other hand, a similar pharmacological effect was observed in the case of *in vivo* jetPEI-Gal at a dose of 25 μg HGF pDNA. However, the survival rate for the R8-GALA-MEND treatment group was significantly higher than that for the *in vivo* jetPEI-Gal treatment group ($P = 0.0027$) (Fig.2b), demonstrating that the R8-GALA-MEND system is more promising for preventing acute liver injury. The poor survival rate of the *in vivo* jetPEI-Gal treatment can be attributed to its toxicity (Akita et al., 2011) as described above. The survival rate of the R8-GALA-MEND treatment group was higher than that of the no-treatment group over time (100% vs. 50% at day 1, 67.5% vs. 33.3% at day 3 and day 5, respectively), though differences were not significant ($P = 0.113$). Two factors are considered to be involved in mice survival rate at day 3 and day 5; the degree of acute liver inflammation induced by LPS/D-GalN, and the resistance of the mice to this acute phenomenon. The former shows a broad range in each mouse as shown in Fig.1. The latter can be improved in Fig 2a; however, the improvement effect, that is, HGF pDNA delivery efficiency, is not sufficient for mice to acquire full resistance against acute liver inflammation. We note that fulminant hepatic failure could not be treated by HGF pDNA-loaded R8-GALA-MENDs after the administration of both LPS and D-GalN (data not shown). Further development of the R8-GALA-MEND will clearly be required to produce therapeutic effects.

We considered 2 mechanisms for improving acute liver injury through HGF pDNA-loaded R8-GALA-MEND. The first is that HGF pDNAs delivered to the liver produce an antiapoptotic effect through the dual induction of BVcl-xL and Cox-2, which suppresses caspase-3 activity (Nomi et al., 2000). The second is that HGF exerts a strong mitogenic action for hepatocytes, which enhances liver regeneration (Ishiki et al., 1992, Roos et al., 1992, Fujiwara et al., 1993) and stimulates the synthesis of hepatic protein responsible for liver-specific functions, including fibrinogen and albumin (Takehara et al., 1992, Matsuda et al., 1997).

In conclusion, a R8-GALA-MEND loaded with HGF pDNA has measurable preventive effects against acute liver injury with less toxicity compared with *in vivo* jetPEI-Gal. To our knowledge, this is the first report to demonstrate that a single systemic administration of a non-viral vector loaded with HGF pDNA ameliorates LPS/D-galactosamine-induced hepatitis. Thus, R8-GALA-MEND system represents a promising non-viral preventive device.

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Author Disclosure Statement

No competing financial interests exist.

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