Advances in Cell and Gene Therapy

Development of Novel Nonviral Gene Therapy Technologies

Lead Guest Editor: Carol Miao
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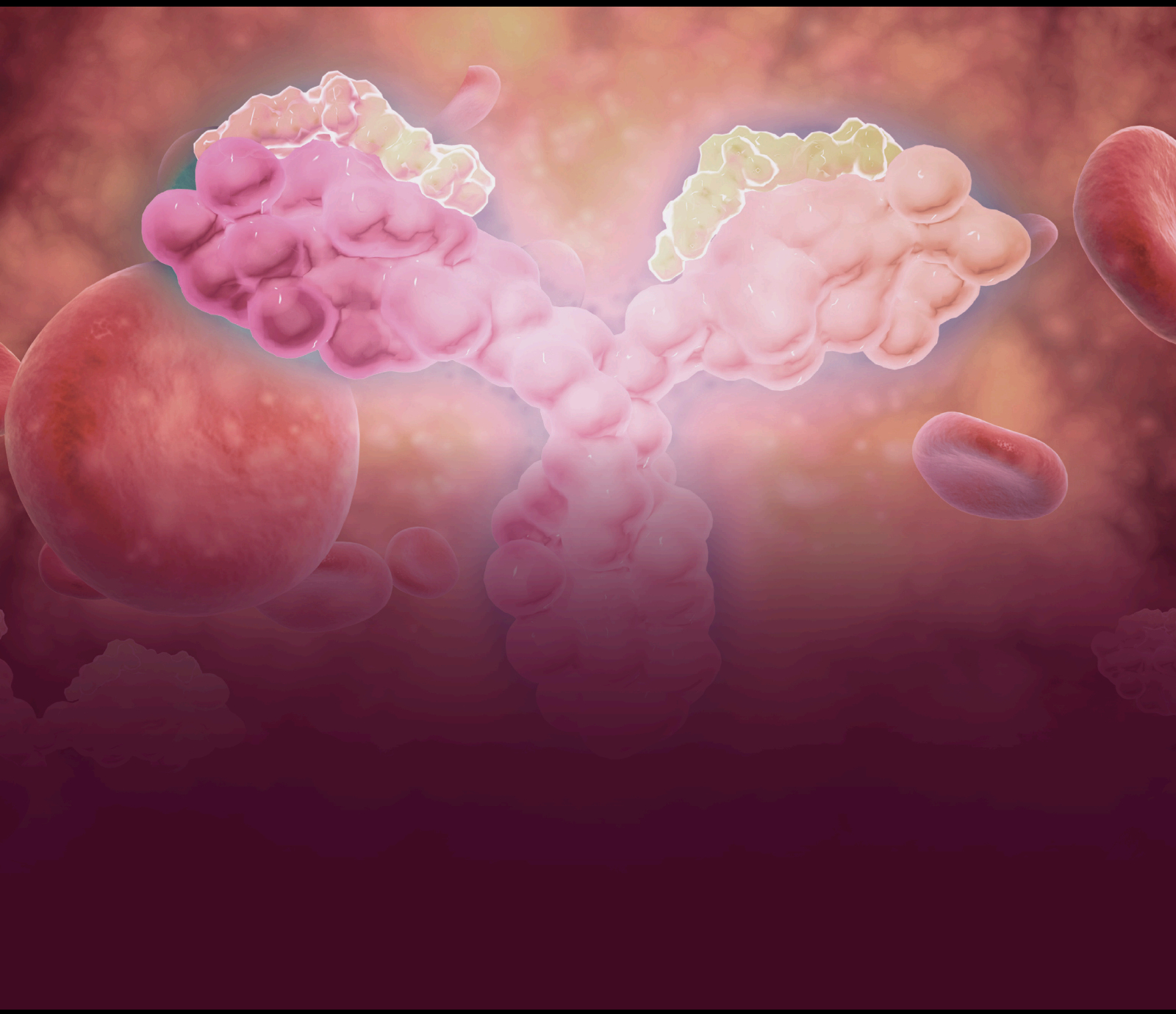

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Contents

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Research Article

Therapeutic Effect of Hydrodynamics-Based Delivery of Matrix Metalloproteinase-13 Gene on Thioacetamide-Induced Liver Fibrosis in Rats

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Liver cirrhosis is the final stage of chronic liver disease and can be life-threatening. Despite extensive studies on its treatment, a standard therapy is yet to be developed. Considering the complex mechanism of fibrogenic and fibrolytic processes in liver cirrhosis, combined therapy may have clinically significant effects on cirrhotic livers. In this study, we used thioacetamide (TAA) administration and *matrix metalloproteinase-13* (*MMP13*) gene delivery to induce extracellular matrix generation and degradation in rats. The aim of this study was to determine whether hydrodynamics-based gene delivery of *MMP13* to cirrhotic liver has regressive and suppressive effects on fibrogenesis. *MMP13*-encoding plasmids were hydrodynamically delivered to TAA-induced cirrhotic livers, and intravascular pressure was monitored. Therapeutic effect with and without continuous TAA exposure was assessed 8 weeks after the gene delivery. Test results indicated successful gene delivery and gene expression in the cirrhotic livers. Furthermore, microscopic imaging showed that *MMP13* delivery resulted in significant degradation of fibrotic areas. Quantitative analysis of hydroxyproline content supported the microscopic findings. These results suggest that transgene delivery of *MMP13* can be a promising candidate to treat liver fibrosis and that hydrodynamics-based gene delivery can be a good option for delivery of *MMP13* to cirrhotic livers.

1. Introduction

Liver cirrhosis is the final stage of liver fibrosis, which is caused by chronic liver injury [1, 2]. Although several studies on the treatment of liver fibrosis have been conducted, antifibrotic treatment remains an unmet medical need for liver cirrhosis, because the mechanisms of fibrosis are complex [2]. Treatment approaches for liver fibrosis include control of underlying liver disease, suppression of hepatic stellate cell activity, and degradation of the pathological extracellular matrix (ECM).

In our previous study, we demonstrated that the hydrodynamic gene delivery of *matrix metalloproteinase-13* (*MMP13*) gene [3], which is a collagenase secreted by various cells and capable of degrading the ECM, into the hepatocytes prevented the progression of fibrosis in the rat liver fibrosis model. Based on the above-mentioned preliminary results, the aim of this study was to investigate whether

hydrodynamics-based *MMP13* gene therapy in a thioacetamide- (TAA-) induced rat liver fibrosis model can be a candidate for fibrolytic therapy and show regression and suppression in advanced stage liver fibrosis.

2. Materials and Methods

2.1. Plasmid and Animal. *MMP13*-encoding plasmids (pBGI-MMP13) were prepared as described in our previous study [3]. All animal experiments were conducted in full compliance with the relevant regulations and were approved by the Institutional Animal Care and Use Committee of Niigata University (SA00263). Wistar rats (female rats weighing 200–230 g) were purchased from Charles River Laboratories Japan (Yokohama, Kanagawa, Japan). Figure 1(a) summarizes the animal model used in this study. The cirrhotic liver was developed by feeding TAA solution to rats for the first 12 weeks of the study, which was based on body weight changes (Figure 1(b)). The concentration of the TAA solution was adjusted once a week using the method described in a previous study [4] (Figure 1(c)). In brief, TAA was dissolved in drinking water, its concentration was uniformly set at 0.03% from day 1 to day 7, and appropriate adjustments were made every week based on the weight changes of individual rats. After the initial TAA exposure, rats that were fed TAA solution for 12 weeks (T12w rats) were euthanized and their livers were used to obtain baseline data. The other rats were divided into four groups, namely, TMO, TOO, TMT, and TOT. Rats in the TMO and TMT groups underwent hydrodynamics-based gene delivery of pBGI-MMP13. Rats in the TOO and TOT groups underwent a sham procedure, which has been summarized in Figure 1(a). Following a 1-week period of recuperation, rats in the TMT and TOT groups were again fed TAA solution of appropriate concentration. In contrast, rats in the TMO and TOO groups were fed normal water. The liver-targeted hydrodynamics-based gene delivery of pBGI-MMP13 (10 μg/ml in saline) to rats was performed as described in our previous study [3].

2.2. Detection of Plasmids and mRNA in Cirrhotic Liver. Whole DNA from the liver was eluted using the DNeasy Blood & Tissue Kit (Qiagen, Hilde, Germany), and PCR was performed using primers for *AmpR* sequence (F primer: tccttgagagttttcgcccc; R primer: cagtgctgcaatgataccgc) and PrimeSTAR Max DNA Polymerase (Takara, Shiga, Japan) to detect pBGI-MMP13. Amplification was performed as follows: 98°C for 2 min, 35 cycles of 98°C for 10 sec, 61°C for 5 sec, 72°C for 10 sec, and 72°C for 30 sec. For mRNA detection, whole RNA was collected using RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Hilde, Germany). QuantiTect SYBR Green RT-PCR Kit and QuantiTect Primer Assays for *MMP13* and *Gapdh* (Qiagen, Hilde, Germany) were used for mRNA detection. One-step RT-PCR was performed as follows: 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. The band shades of the *AmpR* and *MMP13* PCR products were represented as the peak, quantified, and compared using the MultiNA Viewer software.

2.3. Measurement of Serum MMP13 Concentration. Blood samples were collected from the tail vein 2 weeks after gene delivery, and serum *MMP13* concentration was determined by performing enzyme-linked immunosorbent assay using the Human *MMP13* ELISA Kit (ELH-MMP13; RayBiotech Norcross, GA, USA).

2.4. Sirius Red Staining. Liver samples were collected at 12 or 20 weeks of the study period and fixed in 10% formalin and embedded in paraffin, and then, sections 10 μm in thickness were made. Sirius red staining was performed, and the fibrotic area was calculated using ImageJ (version 2.0.0; National Institutes of Health) as described in a previous study [5].

2.5. Quantification of Hydroxyproline Content. Hydroxyproline content in each liver lobe was determined using the QuickZyme Hydroxyproline Assay Kit (QZBHYPRO2; QuickZyme Biosciences B.V., Netherlands).

2.6. Statistical Analysis. Data from histological and biochemical analyses were statistically evaluated using Mann–Whitney U test.

3. Results

3.1. Successful Gene Delivery of pBGI-MMP13 into Cirrhotic Liver. Figure 1(a) depicts a summary of the development of animal models, and with details are provided in Materials and Methods. Briefly, TAA-induced rat liver fibrosis was developed by TAA feeding based on body weight changes (Figure 1(b)) and adjusting TAA concentration (Figure 1(c)). The five groups of rats used in the study were named as follows: T12w, fed TAA for 12 weeks; TOO, fed TAA for 12 weeks, sham procedure, following water feeding; TMO, fed TAA for 12 weeks, *MMP13* delivery, following water feeding; TOT, fed TAA for 12 weeks, sham procedure, following TAA feeding; and TMT, fed TAA for 12 weeks, *MMP13* delivery, following TAA feeding (Figure 1(a)). Liver-specific hydrodynamics-based gene delivery was performed in the animal models (Figure 1) via the inferior vena cava with intravascular pressure monitoring as described in an earlier study [6]. A representative time–pressure curve is shown in Figure 2(a). Portal vein (PV) pressure peaked at baseline plus 30 mmHg within 10 sec, and the injection volume was about 9 ml. To demonstrate successful delivery of pBGI-MMP13, whole DNA was extracted from pieces of the cirrhotic liver 8 weeks after the gene delivery. The results of polymerase chain reaction (PCR) (Figures 2(b) and 2(c)) show expected *AmpR* bands in the TMO (lane B) and TMT (lane D) groups, and the results of reverse transcription-polymerase chain reaction (RT-PCR) (Figures 2(d)–2(f)) show the *MMP13* band in the TMO (lane H) and TMT (lane L) groups. Figure 2(g) shows the level of serum MMP13, 2 weeks after the gene delivery. The mean *MMP13* concentrations in the TMO, TOO, TMT, and TOT groups were 65.1 ng/ml, 12.1 ng/ml, 42.2 ng/ml, and 9.6 ng/ml, respectively. These results indicate that hydrodynamics-based gene delivery of pBGI-MMP13 into the cirrhotic liver was successfully performed and that

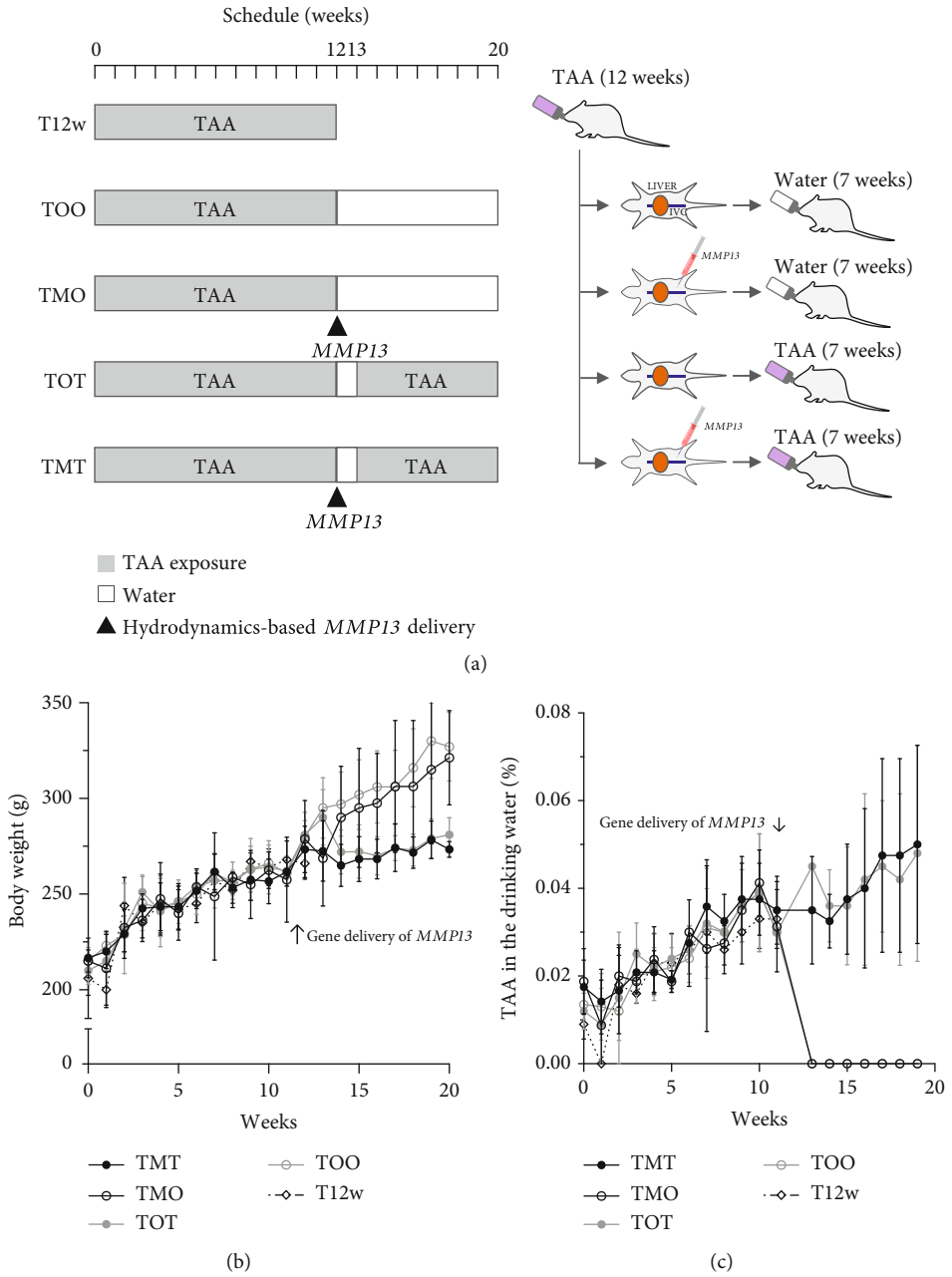

Figure 1: Scheme of thioacetamide (TAA) administration and *MMP13* delivery. (a) An appropriate concentration of TAA solution was prepared. For the first 12 weeks, rats in all groups were fed TAA solution. TMT and TMO rats were hydrodynamically injected with *MMP13* immediately after 12 weeks of TAA exposure. For the next week, all of the rats (those who received *MMP13* injections and those who did not) were fed water without TAA. The rats were fed TAA solution or water for the last 7 weeks depending on their group (rats in the TMT and TOT groups were fed TAA solution, and rats in the TMO and TOO groups were fed water). After euthanasia, liver samples were collected in week 20. T12w rats: rats fed TAA solution for 12 weeks; TMT group: rats fed TAA solution for 12 weeks, injected with *MMP13* immediately thereafter, fed water the following week, and again fed TAA solution for the last 7 weeks; TOT group: rats fed TAA solution for 12 weeks, fed water the following week, and again fed TAA solution for the last 7 weeks; TMO group: rats fed TAA solution for 12 weeks, injected with *MMP13* immediately thereafter, and fed water up to week 20; TOO group: rats fed TAA solution for 12 weeks and fed water (i.e., no *MMP13* injection or additional feeding with TAA solution) up to week 20. (b) Body weight distribution over time and (c) TAA concentration. A suitable concentration of TAA solution was prepared. TAA solution was fed to rats in all groups for the first 12 weeks. The TAA concentration and initial body weight range were 0.03% and 200–230 g, respectively. The TAA concentration was adjusted weekly based on weight change, with the goal of achieving a body weight of 250 ± 25 g. For the following 1 week, each rat (whether or not it had received gene therapy) was fed water. For the last 7 weeks, the rats were fed TAA solution or water according to their groups (the TMT and TOT groups were fed TAA solution, while the TMO and TOO groups were fed water). Values are expressed as mean ± standard deviation (SD).

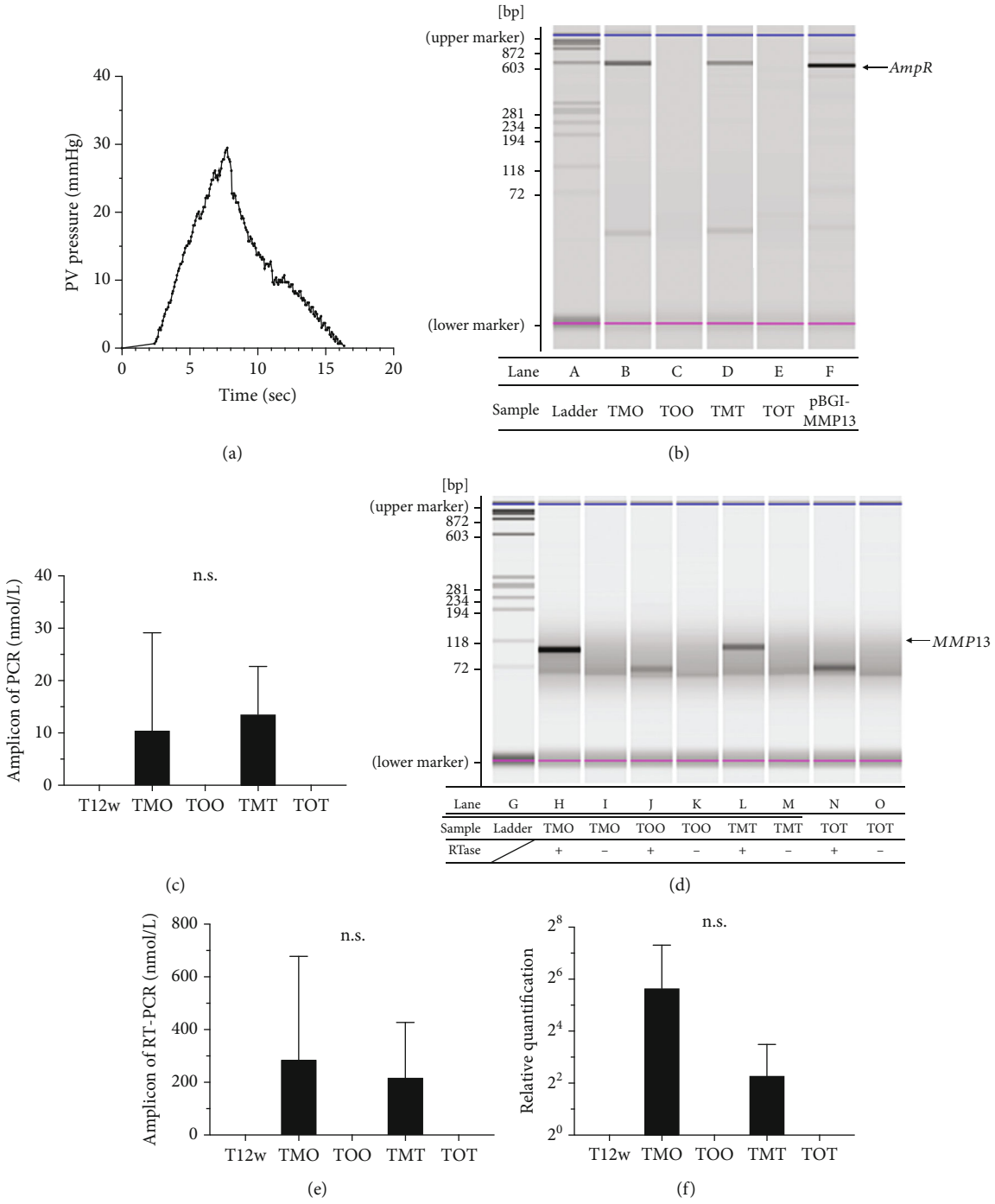

Figure 2: Continued.

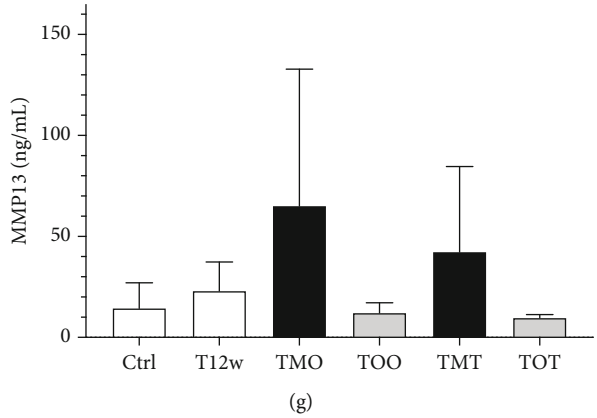

Figure 2: Delivery of *MMP13*-carrying plasmid into hepatocytes. (a) Time course of intravascular pressure during hydrodynamics-based gene delivery. (b) Electrophoresis of PCR products targeting *AmpR* on pBGI-MMP13. The plasmid of pBGI-MMP13 was analyzed as positive control. The expected band of *AmpR* is 581 bp. (c) Molar concentrations of *AmpR* on pBGI-MMP13. (d) Electrophoresis of RT-PCR amplicons targeting mRNA of *MMP13*. The expected band of *MMP13* is 97 bp. (e) Molar concentrations of PCR amplicons targeting *MMP13*. (f) Relative quantitation of real-time PCR for *MMP13* normalized to that of Gapdh for each sample. Values are expressed as mean ± SD ($n = 4$–5). Mann–Whitney *U* test. n.s.: not significant. (g) Serum MMP13 concentration 2 weeks after the gene delivery. The values are expressed as mean ± SD ($n = 4$–5). Ctrl: no TAA treatment; T12w: fed TAA for 12 weeks; TOO: fed TAA for 12 weeks, sham procedure, following water feeding; TMO: fed TAA for 12 weeks, *MMP13* delivery, following water feeding; TOT: fed TAA for 12 weeks, sham procedure, following TAA feeding; TMT: fed TAA for 12 weeks, *MMP13* delivery, following TAA feeding.

MMP13 expression in the liver resulted in the secretion of MMP13 in the serum.

3.2. Regressive Effect on Liver Fibrosis in the TMO Group. Sirius red staining of TMO and TOO livers revealed fibrotic areas of 2.4% and 7.0%. Compared to the TOO and T12w groups, the TMO group had significantly decreased fibrotic area ($p < 0.0001$, Figures 3(a) and 3(b)). Hydroxyproline also supported the evidence ($p < 0.0001$, Figure 3(c)). The above results suggest that the hydrodynamics-based *MMP13* gene delivery to cirrhotic liver improved fibrotic change without continuous TAA exposure.

3.3. Suppressive Effect on Liver Fibrosis in the TMT Group. After 12 plus 7 weeks of TAA exposure, rats of the TMT and TOT groups were euthanized. Fibrotic liver areas in the TMT and TOT groups increased to 8.7% and 14.8%. Although rats in the TMT and TOT groups developed more fibrotic tissue than rats in the T12w group (4.4%, $p < 0.0001$), fibrotic progression in the TMT group was significantly suppressed compared to that in the TOT group ($p < 0.0001$, Figures 3(d) and 3(e)). Analysis of hydroxyproline content in the fibrotic liver also supported the results (Figure 3(f)). These results suggest that the hydrodynamics-based *MMP13* gene delivery to the cirrhotic liver suppressed fibrotic progression under TAA exposure.

4. Discussion

The applicability of gene therapy has increased with the availability of genetic information regarding various diseases [7], including liver fibrosis [8]. In this study, we demonstrated the effect of *MMP13* gene therapy on liver fibrosis. *MMP13*, also known as collagenase 3, is a secreted protein [9] that is reported to be produced by hepatic stellate cells during the recovery phase of fibrosis [10] and by the Kupffer cells [11]. In addition to the activity of collagenase 3, the induction of MMP2, MMP9, and hepatocyte growth factor contributes to the improvement of liver fibrosis induced by CCl_4 and to liver regeneration [12]. According to a previous study on TAA-induced liver fibrosis, the negative regulation of *Mmp13* to *Tgfβ3* may be involved in the mechanisms underlying the antifibrotic effects of the treatment [13]. It is therefore logical to focus on *MMP13* gene delivery for potential gene therapy of liver fibrosis. Based on the above-mentioned reports, in our previous study, we showed the preventive effect of *MMP13* against rat bile duct ligation liver fibrosis model [3] using a hydrodynamics-based gene delivery [14–17]. The *MMP13* expression was sustained for 70 days after the gene delivery to a rat with a fibrotic liver [3]. In addition, the efficacy of the procedure was dependent on the amount of fibrotic tissue [18]. Based on these results, we evaluated whether hydrodynamics-based gene delivery of *MMP13* into advanced fibrotic livers with appropriate parameters could regress or suppress liver fibrosis. Indeed, *MMP13* was successfully delivered to hepatocytes in the fibrotic liver, and it reduced the ECM and suppressed further fibrogenesis. Although there is no statistical difference in plasmid DNA volume and *MMP13* expression between TMO and TMT (Figures 2(c), 2(e), and 2(f)), the tendency of lower *MMP13* expression in TMT could be due to the subsequent TAA administration, which caused damage to the transgene-delivered hepatocytes. Thus, this is the first study to show the efficacy of hydrodynamics-based gene delivery of *MMP13* to cirrhotic livers and its antifibrotic therapeutic effect on the livers. *MMP13* can be a promising candidate for clinical application. Simple and effective hydrodynamics-based gene delivery of *MMP13* has the potential to become a promising option of gene therapy for cirrhotic livers.

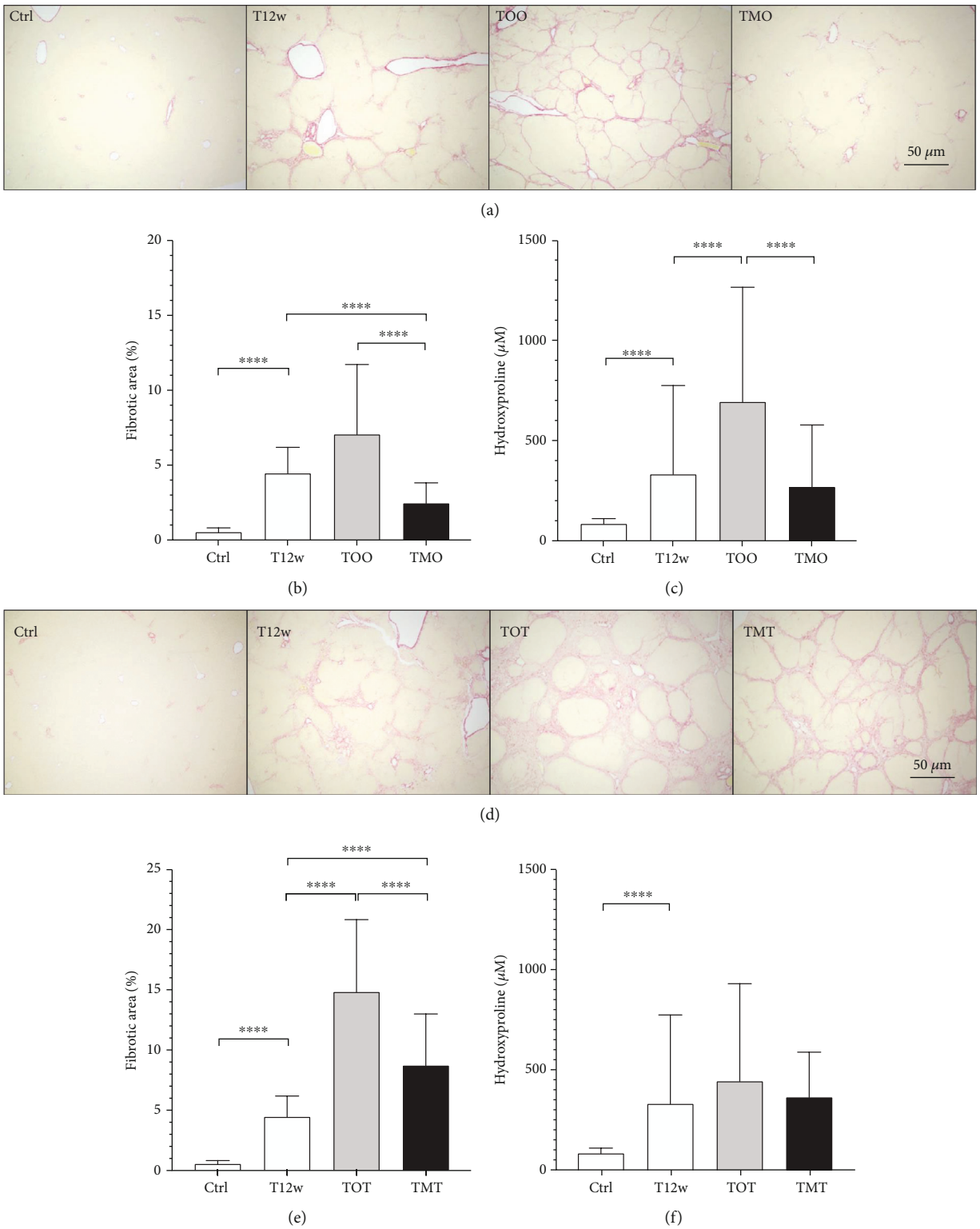

Figure 3: Regressive and suppressive effect of *MMP13* gene delivery on TAA-induced rat liver fibrosis. (a) Representative images of Sirius red staining of control, T12w, TOO, and TMO. The Ctrl group was not exposed to TAA. (b) Percentage of fibrotic area. (c) Quantification of hydroxyproline content. Values are expressed as mean ± SD ($n = 4$–5). Mann–Whitney U test. $^{****}p < 0.0001$. (d) Representative images of Sirius red staining of control, T12w, TOT, and TMT. The Ctrl group was not exposed to TAA. (e) Percentage of fibrotic area. (f) Quantification of hydroxyproline content. Values are expressed as mean ± SD ($n = 4$–5). Mann–Whitney U test. $^{****}p < 0.0001$. Ctrl: no TAA treatment; T12w: fed TAA for 12 weeks; TOO: fed TAA for 12 weeks, sham procedure, following water feeding; TMO: fed TAA for 12 weeks, *MMP13* delivery, following water feeding; TOT: fed TAA for 12 weeks, sham procedure, following TAA feeding; TMT: fed TAA for 12 weeks, *MMP13* delivery, following TAA feeding.

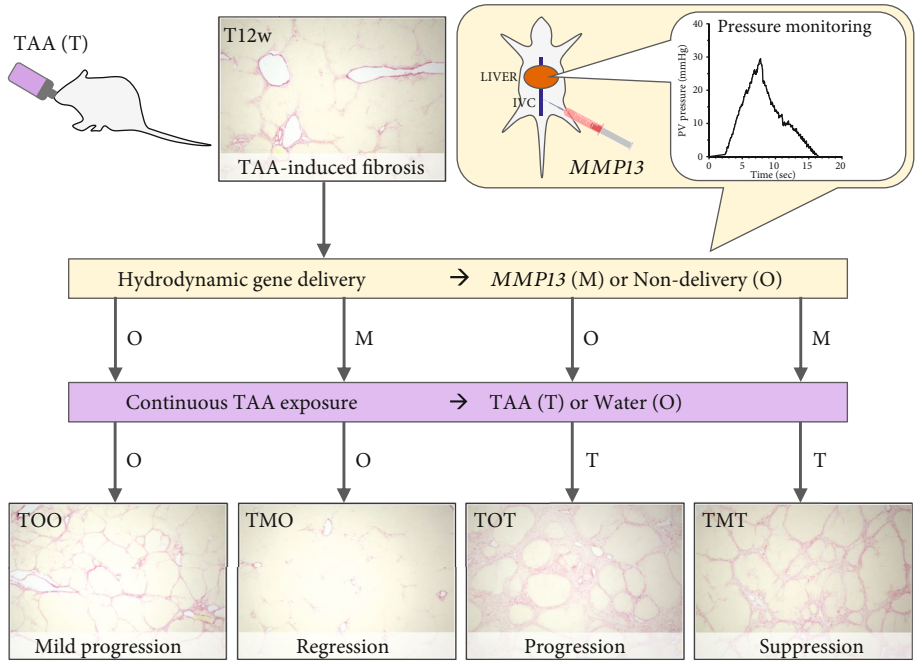

Figure 4: Graphical abstract of the study.

This study has limitations. Firstly, the results are entirely based on an *in vivo* study with one animal model and lack the molecular mechanism analyses. Secondly, the biological safety of *MMP13* overexpression was not fully investigated. Therefore, further studies using other fibrosis models, focusing on molecular mechanisms [19, 20], and *in vitro* studies are essential.

5. Conclusion

In conclusion, liver-specific hydrodynamics-based gene delivery of *MMP13* showed therapeutic effects on fibrotic livers. Thus, *MMP13* gene therapy could be a promising candidate for the treatment of liver fibrosis, and hydrodynamics-based gene delivery may be considered an option for the delivery of therapeutic genes to cirrhotic livers (Figure 4).

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors have no conflict of interest to declare.

Authors' Contributions

Takeshi Yokoo and Kenya Kamimura designed the study, interpreted the data, and wrote the manuscript. Takeshi Yokoo, Moeno Sugita, Osamu Shibata, and Ryosuke Nozawa prepared and fed TAA. Takeshi Yokoo collected and analyzed the data. Yuji Kobayashi, Hiroyuki Abe, and Shuji Terai supervised the scientific accuracy of the intellectual content. Hiromi Miura and Masato Ohtsuka generated pBGI-MMP13.

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