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Sustained Transgene Expression by Transfection of Renin Gene into Liver of Neonates

Naruya Tomita, Ryuichi Morishita, Jitsuo Higaki, Sawako Tomita, Motokuni Aoki, Yasufumi Kaneda,* and Toshio Ogihara[1]

*Department of Geriatric Medicine, Osaka University Medical School, Suita, Osaka 565, Japan; and *Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan*

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Although transfection of renin gene into adult liver resulted in increased blood pressure (BP) for 1 week, sustained transgene expression must be considered to produce a continuous hypertensive animal. We hypothesized that gene transfer into neonatal rats would result in long-term transgene expression, given with highly replicating hepatocytes in neonates. Initially, chloramphenicol acetyltransferase (CAT) vector was transfected into the liver of 1-day-old rats. Immunohistochemical staining showed positive staining of CAT throughout the liver. Therefore, we transfected renin vector to study biological effects. At 2, but not 4 and 8, weeks, a significant increase in plasma angiotensin II concentration was observed in rats transfected with renin vector. Expression of renin mRNA in the liver transfected with renin vector could be detected at least up to 6 weeks, while no significant changes in BP were observed. These results demonstrated that *in vivo* gene transfer into the neonatal liver resulted in sustained transgene expression, suggesting the potential use of *in vivo* gene transfer as a tool to produce a novel model.

Gene transfer has recently emerged as a potential and novel approach to study the pathophysiology of various human diseases. Therefore, many gene transfer methods such as viral vector and non-viral mediated methods have been developed (1-3). As previously reported, a gene transfer method mediated by Hemagglutinating Virus of Japan (HVJ; Sendai virus) and liposome is an efficient gene transfer method in various organs *in vivo* in adult animals (4-10). However, most of these methods, except retroviral and adeno-associated viral methods, cannot achieve integration of transgene into the host genome, resulting in transient transgene expression (1-3). Therefore, it would be worthy to prolong the period of transgene expression. If transgene expression can be sustained, the realm of gene therapy and/or utility of gene transfer as a tool in cardiovascular research would be strengthened. Therefore, we focused on the duration of transgene expression achieved by HVJ-liposome method. In this study, we attempted to transfect foreign genes into the liver of neonatal rats because of the following findings. It is reported that after 70% hepatectomy, the transfection efficiency of retroviral vector was enhanced (11,12), since after partial hepatectomy hepatocytes re-enter the cell cycle, leading to the integration of transgene into host genome. Of importance, the hepatocytes in neonatal rats are still replicating and growing in the absence of hepatectomy. Taken together, we hypothesized that replication in the liver of neonates may facilitate sustained transgene expression by HVJ-liposome method. In the present study, transfection of rat renin gene as a functional gene and chloramphenicol acetyltransferase (CAT) gene as a marker gene into neonatal liver was performed, to address the above issues.

[1] Correspondence. Department of Geriatric Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565, Japan. Fax: 81(Japan)-6-879-3859.

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METHODS

Plasmid Constructions

Rat renin plasmid. Rat renin cDNA (donated by Dr. Kazuo Murakami, University of Tsukuba) was cloned into pAct-c-myb (donated by Dr. Shunsuke Ishii, The Institute of Physical and Chemical Research) which contained the chicken β-actin promoter to generate pAct-rat renin (8).

CAT expression plasmid. We obtained this plasmid from a commercially available source (Promega Corporation, Madison, WI). CAT expression vector is driven by SV 40 promoter and enhancer.

Control plasmid. As the control, we constructed a control plasmid which had no rat renin cDNA in pAct-rat renin.

HVJ-Liposome Preparation

HVJ (Hemmagglutinating Virus of Japan) was propagated in chorioallantonic fluid of embryonated eggs (4-10). Briefly, HVJ was collected by centrifugation at 27,000 xg for 40 minutes and suspended in BSS (balanced saline solution) (−) (137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) overnight. This procedure was repeated at least twice. The resuspended HVJ was stored at −4 ℃ and used immediately. The hemagglutinating activity of HVJ was determined as described previously (4-10). HVJ suspension with A540 = I contained 1 mg/ml protein and was equivalent to 15,000 HAU/ml as an index of fusogenic property. Lipids (phosphatidylcholine, phosphatidylserine and cholesterol) were mixed at a ratio of 4.8:1:2 (w/w/w) as described previously (4-10). The lipid mixture (10 mg) in tetrahydrofuran was deposited in a rotary evaporator. Plasmids were incorporated into liposomes by shaking and sonication. The liposomes and HVJ, inactivated by ultraviolet irradiation (110 erg/mm^2/sec) for 3 minutes just before use, were incubated at 4℃ for 10 minutes and then at 37 ℃ for 30 minutes with gentle shaking. This solution was centrifuged on a sucrose gradient. The top layer was collected for use. One-day-old Wistar rats were injected with 0.1 ml HVJ-liposome complex containing expression vector into the liver from the outside of the body. All studies were performed with the permission of the Ethical Committee for Animal Research, Osaka University Medical School.

Immunohistochemistry

A small portion of the liver transfected with CAT plasmid was fixed with 4% paraformaldehyde, embedded in paraffin and cut into 5 mm-thick sections. After deparaffinization, tissue sections were incubated with monoclonal CAT antibody (5-prime 3-prime, Inc., CO) at 4℃ overnight. After washing with phosphate buffered saline (PBS), they were incubated with biotinated anti-rabbit IgG antiserum, then with avidin-biotinate horseradish peroxidase complex using an enzyme immunohistochemical kit (Histostain-SP kit, Zymed Lab. Inc., CA), and counterstained with hematoxylin.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was extracted from rat renin plasmid- or control plasmid-treated liver by RNAzol (Tel-Test, TX) 2, 4 and 6 weeks after transfection of HVJ-liposome. Levels of renin and β-actin mRNA were measured by RT-PCR. The renin 5′ primer was 5′-ACA-GCA-GGG-AGT-CCC-ACC-TGC-T-3′; 3′ primer was 5′-TCA-TCG-TTC-CTG-AAG-GGA-TT-3′ (13). The 5′ primer complementary to the rat β-actin gene was 5′-TTG-TAA-CCA-ACT-GGG-ACG-ATA-TGG-3′: the 3′ primer was 5′-GAT-CTT-GAT-CTT-CAT-GGT-GCT-AGG-3′ (14). Extreme care was taken to avoid contamination of tissue samples with trace amounts of experimental RNA. Aliquots of RNA derived from cultured cells were amplified simultaneously by PCR and compared with a negative control (primers without RNA) (14). Aliquots of RNA were amplified simultaneously by PCR (30 cycles) performed with step-cycle program set to denature at 94℃ for 1 minute, anneal at 50℃ for 1 minute and extend at 72℃ for 2 minutes, and they were electrophoresed through 2% agarose gels and stained with ethidium bromide.

Measurement of Plasma Renin Activity and Angiotensin II Concentration

Blood samples were collected at 2, 4 and 6 weeks after injection of HVJ-liposome complex. Blood samples were pre-chilled in cooled tubes containing disodium-ethylenediamine tetraacetic acid (1 mg/ml) and centrifuged at 4 ℃ immediately. Rat plasma renin activity (PRA) was measured as described previously (15). The extraction of angiotensin (Ang) I and II was performed by high-performance liquid chromatography (HPLC), and Ang II was measured as described previously (8,16).

Blood Pressure and Heart Rate Measurement

Blood pressure was measured by the tail cuff method as described previously (8). Unanesthetized rats were warmed for several minutes in a box thermomatically controlled at 37-38 ℃, and then introduced into a small holder for the measurement of blood pressure and heart rate.

RESULTS

To confirm the feasibility of gene transfer into the liver of neonatal rats, we initially transfected CAT plasmid as a marker. Immunohistochemical staining was performed to detect CAT expression in the liver at 4 days after transfection. As shown in Figure 1A, CAT protein was readily detected in the liver of rats transfected with CAT vector, indicating successful gene transfer by HVJ-liposome method into the liver, without opening the abdomen. In contrast, no staining for CAT was detected in the liver of rats transfected with control vector (Fig. 1B) and untransfected rats (data not shown). No staining was observed in control sections treated with nonimmune serum as negative control. This result indicated the possibility to achieve neonatal gene transfer, since the liver of 1-day-old rats was clearly seen from outside of the body.

Next, we transfected the rat renin plasmid into neonatal liver to analyze the biological effects of the transgene. Expression of rat renin mRNA was examined in the liver at different time points, 2, 4 and 6 weeks after transfection, as detected by RT-PCR. As shown in Figure 2, at 2 and 4 weeks after transfection, renin mRNA was readily detected in liver transfected with rat renin gene, whereas no signal was observed in liver transfected with control vector and untransfected liver (data not shown). Moreover, even 6 weeks after transfection, mRNA expression of rat renin was still detectable in the liver transfected with rat renin gene. On the other hand, in liver transfected with control vector and untransfected liver, rat renin mRNA expression was not observed throughout the experiment. In control amplification using rat β-actin primers, there was no significant difference between each sample at any time points. Therefore, we examined the changes in plasma Ang II concentration 2, 4 and 6 weeks after transfection, because Ang II is the final product of the renin angiotensin system and is a very strong vasoactive substance. As shown in Figure 3, at 2 weeks after transfection, plasma Ang II concentration in rats transfected with rat renin plasmid was significantly increased as compared to rats transfected with control vector. However, there was no significant difference in plasma Ang II concentration between rats transfected with renin and control vectors both 4 and 6 weeks after transfection. Similarly, at 4 and 6 weeks after transfection no significant differences were observed in plasma renin activity between rats transfected with renin gene and control vector (Table 1). Finally, the effect of renin gene transfection on blood pressure was examined by the tail cuff method. The measurement of blood pressure was not performed until 4 weeks after transfection, because rats younger than 4-weeks-old were too small to measure blood pressure by the tail cuff method. As shown in Table 1, there were no significant differences in blood pressure and heart rate between rats transfected with rat renin and control vectors at 4 and 6 weeks after transfection.

DISCUSSION

The renin angiotensin system plays an important role in the regulation of blood pressure and electrolyte balance (17,18). Transgenic technology has been widely used to study the role of the renin angiotensin system (19). Transgenic technology has many advantages such as 1) the study of specific gene function as systemic and developmental effects, and 2) testing specific gene function chronically. Nevertheless, transgenic technology has several disadvantages: 1) it is time consuming and costly, 2) the effect of the over-expressed transgene is exerted throughout development, and 3) it is very difficult to target transgene expression in a tissue-specific manner. Alternatively, we therefore employed *in vivo* gene transfer technique for study of the renin angiotensin system. As previously reported, transfection of the renin gene into adult rat liver resulted in a transient increase in blood pressure for around 7 days, followed by a return to normal level (8). Therefore, sustained transgene expression would be ideal. In this study, we hypothesized that transfection of foreign genes into neonatal rats resulted in the

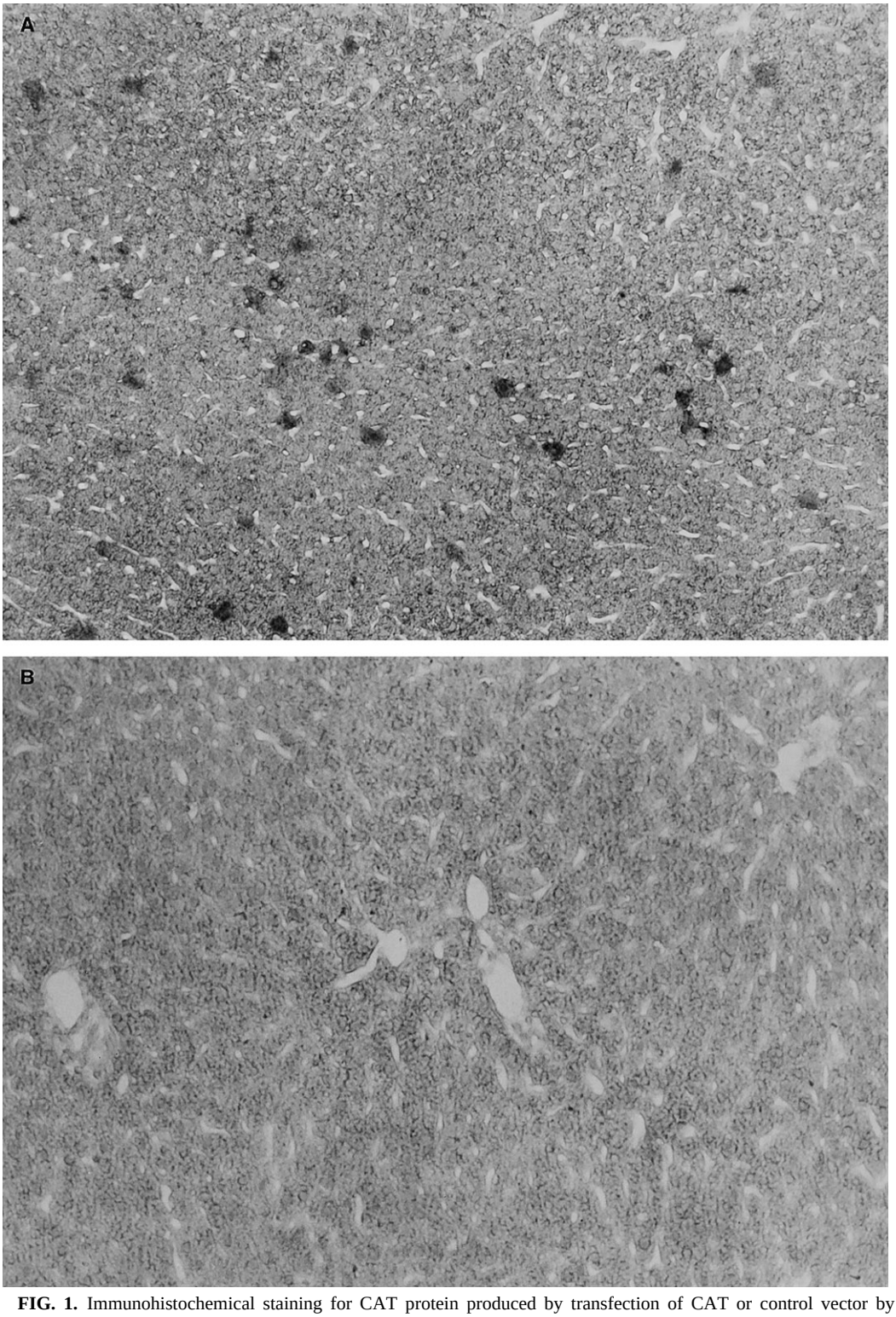

FIG. 1. Immunohistochemical staining for CAT protein produced by transfection of CAT or control vector by HVJ-liposome method in the liver at 4 days after transfection. (A) Liver of rat transfected with CAT vector. Dark dots represent CAT protein expression. (B) Liver of rat transfected with control vector.

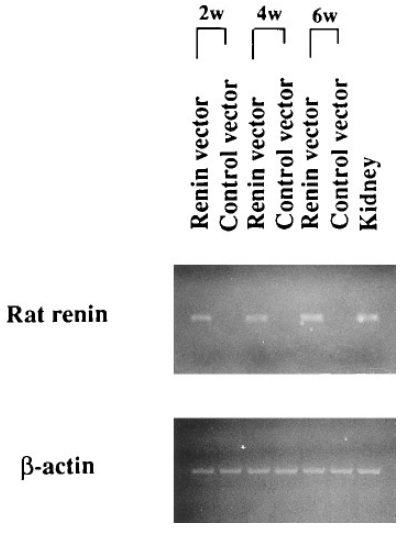

FIG. 2. Expression of rat renin mRNA in liver after transfection of rat renin gene or control vector assessed by RT-PCR. Kidney mRNA was employed as positive control (kidney lane). mRNA was extracted from the kidney of a normal Wistar rat, and then RT-PCR was performed with the same protocol. For the electrophoresis, the final PCR product from kidney mRNA was added to the gel after 1:5 dilution.

prolonged transgene expression. As shown in Figure 1, transfection of foreign genes into neonatal liver resulted in widespread transgene expression in the liver. Previously, we have reported that infusion of HVJ-liposome complex containing β-galactosidase, SV 40 T antigen and insulin genes into the portal vein resulted in widespread transgene expression in the whole liver in adult rats (4-6,8,9). However, there is no report about *in vivo* gene transfer into the liver of neonatal animals by HVJ-liposome method. In this study, we confirmed the feasibility

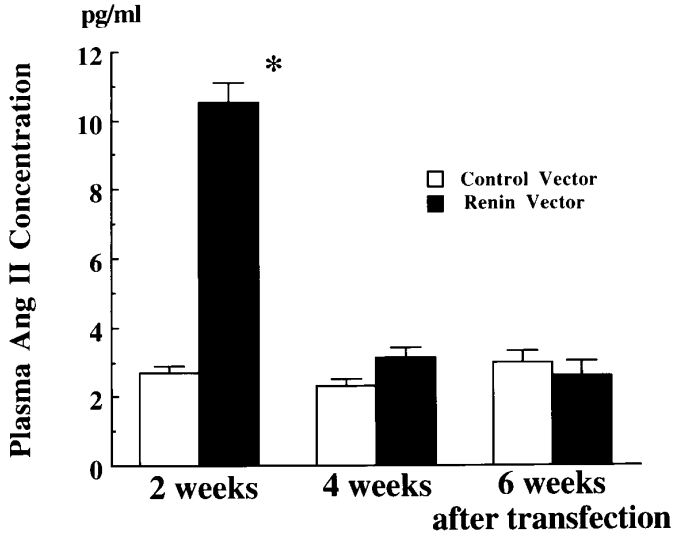

FIG. 3. Plasma Ang II concentration in rats transfected with renin and control vectors after transfection. Values represent mean ± SEM of 6 animals. *$p<0.01$ vs. control vector. Control Vector = rats transfected with control vector, Renin Vector = rats transfected with rat renin vector.

TABLE 1
Changes in Blood Pressure and Heart Rate 4 and 6 Weeks after Transfection of HVJ-Liposome

	PRA (ngAng I/ml/hr)	Blood pressure (mmHg)	Heart rate (beats/min)
		4 weeks	
Control plasmid	10.0 ± 0.5	121.8 ± 9.0	387.2 ± 29.9
Rat renin plasmid	9.1 ± 0.8	117.3 ± 6.8	406.4 ± 24.
		6 weeks	
Control plasmid	10.0 ± 0.6	119.6 ± 7.2	422.7 ± 13.2
Rat renin plasmid	9.5 ± 0.6	115.2 ± 3.7	408.0 ± 9.8

Note. Blood pressure and heart rate were measured by the tail cuff method. Unanesthetized rats were warmed for several minutes in a box thermostatically controlled at 37°C and then introduced into a small holder for measurement of blood pressure and heart rate. Values represent mean ± SEM of 6 rats in each group.

of *in vivo* gene transfer into neonatal liver by direct injection of HVJ-liposome complex without operation. Although we did not compare the efficiency of transgene expression between neonatal and adult rats, transgene expression is expected to be higher in neonatal liver as compared to adult liver, since gene expression is usually higher in proliferating cells as compared to non-dividing cells (20,21).

Therefore, we tested the biological actions of transgene using the renin gene. At 2 weeks after transfection, plasma Ang II concentration was increased in rats transfected with renin gene as compared to rats transfected with control vector. Probably, an excess amount of renin produced in the liver after transfection of rat renin vector cleaved angiotensinogen to generate Ang II locally or systemically. In contrast, in adult rats no significant difference in plasma Ang II level was observed between rats transfected with renin vector or control vector at 7 days after transfection, whereas plasma Ang II concentration was markedly increased in rats transfected with renin vector at 4 days after transfection. On the other hand, regarding the period of transgene expression, renin mRNA expression was still detected up to at least 6 weeks after single transfection. It is apparent that transfection of renin gene into neonatal liver resulted in prolongation of transgene expression as compared to transfection into adult liver. However, we failed to show a biological effect of transfected renin gene, since there was no significant difference in blood pressure and heart rate between rats transfected with renin or control vector. These unexpected data were also confirmed by the lack of differences in plasma renin activity and Ang II concentration at 4 and 6 weeks after transfection. The present study cannot address the reason why neonatal rats transfected with renin gene showed no changes in blood pressure, but the level of produced renin may have not been enough to increase blood pressure through Ang II production. Alternatively, the discrepancy between the responses of blood pressure and sustained renin gene expression in the liver was probably due to the action of compensatory systems such as the central nervous system and tissue RAS (22).

In this study, we failed to show the mechanisms of sustained long-term expression of transgene in the liver of neonatal rats transfected by HVJ-liposome method. One possible explanation is that the transgene may be integrated into the host genome, although our previous studies showed no integration of transgene into the host genome with HVJ-liposome method in adult animals (4-10). Another possible explanation for the long-term expression is lack of immunogenicity in neonatal animals, as DNA itself causes some immunogenic reactions (23,24). We have found that HVJ itself has little immunogenicity, as evidenced by successful

repeated administration of transgenes and negligible production of antibodies (25,26). Since the exact mechanism of the long duration of transgene expression has not been clarified in this study, further studies are necessary. Overall, this study demonstrated that sustained transgene expression was achieved by transfection into neonatal liver by HVJ-liposome method, but failed to show a sustained increase in blood pressure. To obtain hypertensive animals, further advances in *in vivo* gene transfer besides transgenic technology are needed.

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