Original Contributions


Ryuichi Morishita, Gary H. Gibbons, Yasufumi Kaneda, Toshio Ogihara, and Victor J. Dzau

Although many in vitro gene transfer methods already exist, such as calcium phosphate precipitation, electroporation, or cationic liposomes, these methods cause significant cell injury and cell death. The study of the biology of endogenous autocrine-paracrine vasoactive systems such as the renin-angiotensin system in vascular cells is limited by the lack of a suitable gene transfer method with high efficiency of transfection and expression that will permit cell biology studies. Recently, the Sendai virus (hemagglutinating virus of Japan, HVJ)-liposome-mediated gene transfer method has been shown to be an efficient and nontoxic method of gene transfer. In this study, we characterized the efficiency and suitability of the HVJ method for vascular biology research. Using SV40 T-antigen complementary DNA (cDNA), we initially compared the efficiency of the HVJ method and lipofection for transfection of cultured vascular smooth muscle cells (VSMCs). We observed that after 35 minutes of incubation, the HVJ method exhibited a 10-fold higher efficiency of transfection than lipofection. We used this method to study vascular angiotensin converting enzyme (ACE) expression in cultured VSMCs and cultured rat carotid arteries in vitro. The HVJ method of transfection of human ACE cDNA into VSMCs and COS cells was significantly more efficient than lipofection. Using this method, we demonstrated that transfection of ACE cDNA resulted in increased DNA synthesis, which was inhibited by the specific angiotensin II receptor antagonist DuP 753 (10^{-9} M). Transfected human ACE cDNA into the rat carotid artery in organ culture yielded ACE expression in the tunica media as detected by immunohistochemistry at 3 days after transfection, whereas there was no positive staining in the control vector-transfected and untreated vessels. The present study demonstrates that the HVJ-mediated method is a very effective and efficient gene transfer method for VSMCs. Furthermore, the successful gene transfer into intact blood vessels in organ culture suggests that the HVJ-mediated gene transfer technique may provide a new and useful tool for in vivo study of endogenous autocrine-paracrine vascular modulators (such as the vascular renin-angiotensin system) and may be a useful delivery method for gene therapy for vascular diseases. (Hypertension 1993;21:894–899)

KEY WORDS • simian virus 40 T antigen • kininase II • muscle, smooth, vascular • organ culture • HVJ (hemagglutinating virus of Japan; Sendai virus)

The study of the effect of autocrine-paracrine vasoactive modulators (e.g., renin-angiotensin) on vascular smooth muscle cells (VSMCs) is very difficult in vivo, because in vivo studies are limited by 1) the multiplicity of coexisting variables, 2) the difficulties in manipulating individual components, and 3) the methodological limitations in studying the function of a locally produced modulator in the absence of any contribution by the circulatory system. Cell culture and gene transfer technologies have provided us with the opportunity to study cellular responses to the manipulation of the individual components (i.e., by overexpression or inhibition). Such an approach may increase our understanding of the biology and pathobiology of autocrine-paracrine systems. However, few in vitro gene transfer methods are suitable for these purposes. Current in vitro gene transfer methods such as calcium phosphate precipitation, diethylaminoethyl dextran, electroporation, and cationic liposomes can result in substantial cell injury and death and pose significant problems to the investigation of the role of potential autocrine mediators (e.g., angiotensin), especially in the regulation of cell growth. The utility of cationic liposomes for in vitro transfection and cell growth studies has been reported by many investigators including us, but this method requires a long incubation time (24 hours) and has a low efficiency of transfection. Infection of cells with retroviral or adenoviral vectors has established efficacy, but the problems of genomic integration and infectivity limit the applicability. Recently, the HVJ (hemagglutinating virus of Japan) method has been reported as an efficient and nontoxic method of gene transfer. In this study, we examined the usefulness of this method for vascular biology research. We compared the transfection efficiency of HVJ versus lipofection in transferring SV40 T-antigen...
or human angiotensin converting enzyme (ACE) complementary DNAs (cDNAs) into cultured rat aortic VSMCs. We studied the biochemical and physiological effects of overexpression of ACE on the biology of these cells. Finally, we applied HVJ gene transfer to the intact whole blood vessel in organ culture (i.e., rat carotid artery) and demonstrated its effectiveness. Taken together, our data demonstrate that the HVJ method is a very effective and suitable method for the study of autocrine-paracrine vascular modulators such as the vascular renin-angiotensin system.

Methods

Construction of Plasmids

The pUC-CAGGS expression vector plasmid (kindly provided by J. Miyazaki, Tokyo University) was cut with the restriction enzyme EcoRI. The EcoRI fragment containing human truncated ACE cDNA of RB 35-15 including two putative active sites (kindly provided by P. Corvol, INSERM, France) was inserted into the EcoRI site in this vector by filling EcoRI ends with T4 polymerase. pAct-SV was subcloned as previously reported.4 CAGGS as control vector contains the entire envelope region open reading frame consisting of three translation initiation codons, which represent the N-terminals of the large, middle, and major surface (S) polypeptides downstream of the cytomegalovirus enhancer and the chicken \( \beta \)-actin promoter.

Cell Culture

Wistar-Kyoto rat (12 weeks old) VSMCs (passages 4–10) were isolated and cultured according to the method of Owens and Thompson.5 COS cells were obtained from the American Type Culture Collection, Rockville, Md. VSMCs and COS cells were maintained in Waymouth's medium (GIBCO, Grand Island, N.Y.) with 5% calf serum or Dulbecco's modified Eagle's medium with 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (1,000 \( \mu \)g/mL), respectively. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% \( \text{CO}_2 \) with media changes every 2 days.

Preparation of Hemagglutinating Virus of Japan Liposomes

Liposomes containing plasmid DNA and high mobility group 1 (HMG-1) were constituted as previously reported.2,4 Briefly, dried lipid (phosphatidylserine, phosphatidylcholine, and cholesterol combined in a weight ratio of 1:4.8:2) was mixed with plasmid DNA (200 \( \mu \)g) (previously incubated at 20°C for 1 hour with HMG-1–2,4) shaken vigorously, and sonicated to form liposomes. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm\(^2\) per second) for 3 minutes just before use. The liposome suspension mixed with HVJ was incubated at 4°C for 10 minutes and at 37°C for 30 minutes. HVJ-liposome complex was collected for 5 minutes and then 37°C for 30 minutes. Alternatively, the DNA (10 \( \mu \)g) and HMG-1 complex was mixed with Lipofectin reagent DOTMA (n1-[2,3 dioleoyloxy]propyl)-n,N,n-trimethylammonium chloride) (BRL Life Technologies) dissolved in the same volume of water in a ratio of 1:3 (wt/wt) and incubated for 24 hours at 37°C. After incubation, the medium was changed to fresh medium, and immunohistochemistry was performed after 2 days (day 3 after transfection) by using anti-SV40 large T-antibody. Cells were fixed with 3% paraformaldehyde and stained with an enzyme immunohistochemical kit (Histostain-SP kit, Zymed Laboratories Inc., South San Francisco) with mouse monoclonal antibody against SV40 large T antigen (Oncogene Science, Inc., Manhasset, N.Y.). Stained cells were counted under a microscope and calibrated by total cells in randomly selected areas. COS 7 cells were used as a positive control for constitutive T-antigen expression. Each value represented a mean of 10 selective areas.

Measurement of Angiotensin Converting Enzyme Activity

For measurement of ACE activity, VSMCs or COS cells were maintained in medium with serum, which had previously been inactivated at 60°C for 1 hour and 58°C for 1 hour. This method effectively eliminates ACE activity in serum. Cells (1 \( \times \)10\(^5\)) were seeded onto 60-mm Petri dishes and grown to confluence. After cells were washed with balanced saline solution containing 2 mM CaCl\(_2\), the HVJ-liposome complex including plasmid DNA (approximately 20 \( \mu \)g DNA was enwrapped in liposomes) was incubated at 4°C for 5 minutes and then 37°C for 30 minutes (total, 35 minutes). The DNA (30 \( \mu \)g) and HMG-1 complex with lipofectin dissolved in the same volume of water in a ratio of 1:3 (wt/wt; 30 \( \mu \)g DNA and 90 \( \mu \)g per 4 mL media lipofectin) was incubated for 24 hours at 37°C. After incubation, the cells then were maintained in medium with 5% calf serum, andACE enzymatic activity was measured after 2 days (day 3 after transfection). ACE activity (per milligram of protein), expressed as hippuryl-L-histidyl-L-leucine hydrolyzing activity in cell homogenates, was determined by the modified method of Cushman and Cheung.6 With this protocol, measured ACE activity is completely abolished by either quinaprilat (a specific ACE inhibitor) or neutralizing antibodies to ACE (data not shown).

Determination of DNA Synthesis

After confluence, cells were washed three times with buffered saline solution containing 2 mM CaCl\(_2\). Then, 500 \( \mu \)L HVJ-liposome complex (1.3 mg lipids and 5 \( \mu \)g encapsulated DNA) was added to the wells. The cells were incubated at 4°C for 5 minutes and then at 37°C for 30 minutes, and after a change to fresh medium with 5% calf serum, they were incubated overnight in a CO\(_2\) incubator. The studies on VSMC growth were conducted on confluent quiescent cells maintained in a defined serum-free medium containing insulin (5 \( \times \)10\(^{-7}\) M), transferrin (5 mg/mL), and ascorbate (0.2 mM) for 48 hours as previously reported.7 The relative rate of
DNA synthesis was assessed by determination of tritiated thymidine incorporation into trichloroacetic acid, precipitable material, as previously reported. Quiescent rat VSMC cells grown in 12-well Costar culture were pulsed for 24 hours (12 to 36 hours after the stimulation) with tritiated thymidine (2 µCi/mL).

Organ Culture
After balloon injury with a 2F embolectomy catheter, the carotid arteries of Sprague-Dawley rats (weighing 400 to 450 g) were dissected as previously reported and were immediately transferred to Waymouth’s medium without serum. A 20-mm segment of the carotid artery was isolated and occluded at both ends by ligation, and 500 µL HVJ-liposome complex or control vector plasmid DNA (approximately 2.5 µg encapsulated DNA) was introduced into the lumen and incubated for 5 minutes at 4°C and for 30 minutes at 37°C. After transfection, both ends were opened and the vessels were placed in Waymouth’s medium with 30% fetal calf serum, penicillin (100 units/mL), streptomycin (1,000 µg/mL). Previous studies in our laboratory and others have documented viability of the intact vessel maintained under these conditions. At 3 days after transfection, the vessels were fixed with 4% paraformaldehyde and embedded in paraffin. The sections were stained with an enzyme immunohistochemical kit (Histostain-SP kit) with polyclonal antibody against human ACE. Nonimmune serum was used as control.

Materials
Angiotensin II (Ang II) type 1 receptor antagonist (DuP 753) was a gift from Parke-Davis Pharmaceutical Co.

Statistical Analysis
All values are expressed as mean±SEM. All experiments were repeated at least three times. Analysis of variance with subsequent Duncan’s test was used to determine significant differences in multiple comparisons. A value of p<0.05 was considered statistically significant.

Results
Figure 1 shows the percentage of VSMCs that stained positively with antibody to SV40 T antigen (35-minute incubation period) as an index of gene transfection and expression. With the HVJ method (approximately 5 µg DNA), approximately 20% of cells stained positive for T antigen, whereas the lipofection method (10 µg DNA) resulted in only 2% of the cells with positive immunostaining. No staining was detected in cells exposed to DNA (10 µg) alone or vehicle (control). These results are readily reproducible because of the increased efficiency of transfection of the HVJ method.

Figure 2 shows ACE activity in the ACE cDNA-transfected and CAGGS (control vector)-transfected cultured COS cells (Figure 2A) and VSMCs (Figure 2B) after a 35-minute incubation. ACE-transfected COS cells using the HVJ method (approximately 20 µg encapsulated DNA) showed a 10-fold higher ACE activity than control vector-transfected cells, whereas there was no significant change in ACE- and control vector-transfected cells using the lipofection method (30 µg DNA). On the other hand, after 24 hours of incubation, the transfection of ACE cDNA using the cationic liposome method (30 µg DNA) resulted in a significant increase in ACE activity in VSMCs to a level comparable to that of the 35-minute incubation with the HVJ method (approximately 20 µg encapsulated DNA). ACE activity in control vector–transfected VSMCs by both cationic lipid (30 µg DNA) and HVJ (approximately 20 µg DNA) methods showed no difference from that in untreated cells (data not shown). The viability of cells was not affected by the transfection with the HVJ method as documented by trypan blue exclusion or protein synthesis.

Table 1 shows the influence of HVJ-transfected human ACE cDNA on VSMC DNA synthesis. DNA synthesis in the ACE-transfected VSMCs increased significantly compared with that in control vector–transfected VSMCs. The increase in DNA synthesis was completely blocked by incubation with the specific Ang II receptor antagonist DuP 753 (10-6 M) for 36 hours. Neither transfection with control vector–HVJ complex nor with ACE cDNA in “naked” liposomes without HVJ particles resulted in an increase in DNA synthesis (data not shown).

<table>
<thead>
<tr>
<th>TABLE 1. Effect of Transfected Human Angiotensin Converting Enzyme Complementary DNA on DNA Synthesis of Vascular Smooth Muscle Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vector</td>
</tr>
<tr>
<td>DuP 753 (10-6 M)</td>
</tr>
<tr>
<td>ACE</td>
</tr>
<tr>
<td>ACE+DuP 753</td>
</tr>
</tbody>
</table>

ACE, angiotensin converting enzyme. Values are mean±SEM. Each group contains five samples. *p<0.05 vs. control vector–transfected vascular smooth muscle cells. **p<0.01 vs. ACE-transfected vascular smooth muscle cells.
FiguRe 2. Bar graphs show angiotensin converting enzyme (ACE) activity in ACE complementary DNA (cDNA)-transfected and control vector-transfected cultured COS cells and vascular smooth muscle cells. Control vector and human ACE cDNAs were transfected to COS cells (panel a) and vascular smooth muscle cells (panel b). CONTROL, control vector-transfected cells; ACE, ACE cDNA-transfected cells with lipofection or HVJ (hemagglutinating virus of Japan) method. Lipofectin-DNA complex (DNA, 20 μg; Lipofectin, 90 μg) was incubated for 24 hours; HVJ-DNA complex (DNA, 20 μg) was incubated for 35 minutes. Each group contains four to six samples.

Finally, we examined the effectiveness of transfection and expression of human ACE cDNA in cultured balloon-injured rat carotid arteries maintained in organ culture. Immunohistochemistry demonstrated positive staining within the medial layer (Figure 3), whereas there was no positive staining in control vector-transfected and untreated vessels. Three of four ACE-transfected vessels exhibited positive staining (more than 20% of total medial layer), but none of the control vector-transfected (n=4) and none of the untreated (n=4) vessels showed positive immunostaining. In the absence of primary antibodies against human ACE, there was no positive staining in any of the injured blood vessels (data not shown).

Discussion

Current in vitro gene transfer methods, including lipofection, calcium phosphate, and electroporation, cannot be used to study the effect of transfected genes on cell growth, because these methods also cause significant cell injury or death. In vascular biology research, in vivo studies on the growth regulatory role of endogenous vascular modulators (e.g., renin-angiotensin) are limited by the methodological difficulties of studying the function of a locally produced modulator in the absence of any contribution by the circulatory system. The development of a nontoxic and highly efficient gene transfer method for vascular cells in vitro, ex vivo, or in vivo should represent an important advancement in vascular research and possibly in vascular gene therapy. Our current study shows that the HVJ method is a highly efficient and nontoxic gene transfer method for cultured VSMCs and the intact blood vessel maintained in organ culture. Within 35 minutes of incubation with the SV40 T-antigen gene, the HVJ method demonstrated 10-fold higher transfection efficiency than the lipofection method. Moreover, our studies of ACE cDNA transfer and expression revealed that the brief 35-minute incubation with the HVJ method is as effective in transfection and expression as a prolonged 24-hour incubation with the lipofection method. These results demonstrate that the HVJ method requires only a brief incubation period (35 minutes) and therefore may be useful in vitro and especially in vivo compared with the lipofection method, which requires 24 hours of incubation.

Next, we examined whether this method can be used to study the effect of autocrine-paracrine vascular modulators on cell growth. The presence of components of the renin-angiotensin system in VSMCs has been reported by many investigators.9-11 Ang II can induce vascular hypertrophy as well as hyperplasia both in vitro and in vivo.12-15 Therefore, vascular Ang II is thought to play an important role in vascular growth. However, there are many unresolved questions; i.e., what are the rate-limiting steps in Ang II production within the vasculature? Does autocrine Ang II regulate VSMC growth? What are the cellular mechanisms of vascular Ang II production? Although we previously reported the existence of renin,16 ACE activity,17 and angiotensigen18 in VSMCs, it is not clear whether changes in the level of expression of these components can regulate VSMC growth. In this study, we successfully transfected and overexpressed human ACE cDNA in VSMCs. The increased ACE expressed resulted in an increase in DNA synthesis that was completely abolished by the specific Ang II receptor antagonist DuP 753. The complete blockade of DNA synthesis by an Ang II receptor antagonist suggests that the increased expression of the ACE gene can modulate VSMC growth through the production of vascular Ang II but not of another factor (e.g., bradykinin). Indeed, our preliminary results also show an increase in Ang II production in these ACE-transfected cells. The present study has demonstrated that the HVJ gene transfer method is a useful and powerful tool for studying the effect of autocrine-paracrine vascular modulators (such as the vascular renin-angiotensin system) on vascular biology.

In this study, we also examined the effectiveness of this HVJ method for gene transfer into whole blood vessels in culture. At 3 days after transfection with ACE cDNA, positive staining for ACE could be readily detected in the medial layer of cultured vessels, whereas...
FIGURE 3. Photomicrographs show expression of angiotensin converting enzyme (ACE) in balloon-injured cultured rat carotid arteries. Three days after incubation with hemagglutinating virus of Japan (HVJ) liposomes containing human ACE complementary DNA, the vessel was perfused with 4% paraformaldehyde solution. After fixation, the tissue was sectioned at 6 μm thickness in a cryostat and stained. ACE was stained red by enzyme immunohistochemistry (×320 optical magnification). Panel A: ACE-transfected artery; panel B: control vector-transfected artery. L, lumen; M, media; A, adventitia.

no immunostaining was seen in control vector-transfected and untreated vessels. The successful gene transfer into the intact vessel in organ culture infers that it will be feasible to investigate the effects of autocrine-paracrine vascular factors on vascular growth, i.e., neointimal formation after angioplasty in vivo. The successful gene transfer in the intact blood vessel also suggests that this technique may be an effective method for in vivo gene transfer and therapy for vascular disease. We anticipate that other efficient transfection
methods such as retroviral and adenoviral infection will be useful for in vivo gene transfer. The advantages of the HVJ method are that the virus is incapable of replication, does not integrate into the genome, and exploits the flexibility of plasmid expression vector systems. The HVJ method provides us with the opportunity to investigate the autocrine-paracrine role of vascular modulators on vascular growth. Specifically, this method may be useful for the study of the biology of the vascular renin-angiotensin system, an important area of hypertension research.

Acknowledgments

We thank Drs. Naruya Tomita and Jistuo Higaki for technical advice and Wendy Lee for excellent technical assistance.

References

R Morishita, G H Gibbons, Y Kaneda, T Ogihara and V J Dzau

Hypertension. 1993;21:894-899
doi: 10.1161/01.HYP.21.6.894

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/21/6_Pt_2/894

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at: http://hyper.ahajournals.org//subscriptions/