Novel and Effective Gene Transfer Technique for Study of Vascular Renin Angiotensin System

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Abstract

Vascular renin angiotensin system (RAS) has been reported to exist in vascular wall. However, there is no direct evidence whether the vascular RAS per se can modulate growth of vascular smooth muscle cells (VSMC), because there is no suitable method to investigate the effect of endogenously produced vasoactive substances on growth of these cells. In this study, we transferred angiotensin-converting enzyme (ACE) and/or renin cDNAs into cultured VSMC using the efficient Sendai virus (hemagglutinating virus of Japan) liposome-mediated gene transfer method, to examine their relative roles in VSMC growth in vitro. Within 35 min or 6 h, the transfection of ACE cDNA into VSMC by hemagglutinating virus of Japan method resulted in a twofold higher ACE activity than control vector, whereas a cationic liposome (LipofectinTM)-mediated method failed to show any effect. This in vitro system provided us with the opportunity to investigate the influence of endogenous vascular RAS on VSMC growth. Transfection of ACE or renin cDNA resulted in increased DNA and RNA synthesis, which was inhibited with the specific angiotensin II receptor antagonist (DuP 753; 10−8 M). Angiotensin I added to ACE-transfected VSMC increased RNA synthesis in a dose-dependent manner. Cotransfection of renin and ACE cDNAs stimulated further RNA synthesis as compared to ACE or renin cDNA alone. These results showed that transfected components of RAS can modulate VSMC growth through the endogenous production of vascular angiotensin II, and that ACE as well as renin are rate limiting in determining the VSMC RAS activity. We conclude that the hemagglutinating virus of Japan liposome-mediated gene transfer technique provides a new and useful tool for study of endogenous vascular modulators such as vascular RAS. (J. Clin. Invest. 1993. 91:2580–2585.) Key words: gene transfer • Sendai virus • angiotensin-converting enzyme • smooth muscle cells • vascular growth

Introduction

Recent data suggest that angiotensin (Ang) II may be generated locally in many tissues. Components of the renin angiotensi-
inhibition). Such an approach may increase our understanding of the biology and pathobiology of the vascular RAS.

Current methods of DNA transfection include DEAE dextran, calcium phosphate precipitation, electroporation, or Lipofectin™. All of these methods are associated with significant cell injury which frequently results in cell death. These methods are generally ineffective for gene transfer in the intact animal. Thus, the gene transfer methods to date have not been useful in the studies of VSMC RAS. Recently, we reported an efficient and nontoxic method of gene transfer using hemagglutinating virus of Japan (HVJ) liposome-mediated transfer (16–18). In this study, we (a) compared the efficiency of HVJ versus Lipofectin in transferring ACE and/or renin cDNAs into cultured rat aortic VSMC and (b) studied the biochemical and physiological effects of overexpression of ACE and/or renin on these cells. Our data demonstrate that increased ACE and/or renin expression in VSMC can result in enhanced VSMC DNA replication and RNA synthesis that are mediated by autocrine Ang II production and action.

Methods

Construction of plasmids

The pUC-CAGGS expression vector plasmid (kindly provided by Junichi Miyazaki, Tokyo University, Tokyo, Japan) was restricted with EcoRI. The EcoRI fragment containing human truncated ACE cDNA of RB 35-15 including two putative active sites (kindly provided by P. Corvol, INSERM, Paris, France) or full-length rat renin cDNA (kindly provided by Kevin Lynch, University of Virginia, Charlottesville, VA) was inserted into the EcoRI site in this vector by filling EcoRI ends with T4 polymerase. CAGGS contains the entire envelope region open reading frame consisting of three translation initiation codons, which represent the NH2 termini of the large, middle, and major surface (S) polypeptides downstream of the cytomagalovirus enhancer and the chicken beat-actin promoter.

Cell culture

Rat aortic VSMC (passages 4–10) were isolated and cultured according to the method of Owens et al. (19). They were maintained in Waymouth's medium (Gibco Laboratories, Grand island, NY) with 5% calf serum, penicillin (100 U/ml), streptomycin (1,000 μg/ml). Cells were incubated at 37°C in a humidified atmosphere of 95% air-5% CO2 with media changes every 2 d.

Preparation of HVJ liposomes

The preparation of HVJ liposomes has been previously described (16–18). Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4:8:2. The lipid mixture (10 mg) was deposited on the sides of a flask by removal of tetrahydrofuran in a rotary evaporator. High mobility group (HMG) 1 nuclear protein was purified from calf thymus. A DNA-HMG 1 complex (200 μg:64 μg) was formed by incubation at 20°C for 1 h (16–18). Dried lipid was hydrated in 200 μl of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing the DNA-HMG 1 complex. Liposome-DNA–HMG 1 complex suspension was prepared by vortex, sonication for 3 s, and shaking for 30 min. Purified HVJ (Z strain) was inactivated by ultraviolet irradiation (110 erg/mm² s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (64,000 hemagglutinating units) in a total volume of 2 ml of BSS. The mixture was incubated at 4°C for 10 min and then for 30 min with gentle shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient containing the HVJ liposome complex was collected for use (16–18).

Measurement of ACE activity

For measurement of ACE activity, VSMC were maintained in Waymouth's media with 5% calf serum, which had previously been inactivated at 60°C for 1 h and 58°C for 1 h. This protocol of heat inactivation abolishes serum ACE activity; data not shown. 1 × 10⁴ cells were seeded onto 60-mm petri dishes and grown to confluence.

HVJ-liposome method. Confluent cells were washed three times with BSS containing 2 mM CaCl₂, followed by incubation with 1 ml of HVJ liposomes in BSS (2.5 mg of lipids and 2.5–10 μg of encapsulated DNA) for 35 min or 6 h using the following protocols. (a) 35-min transfaction: The cells were then incubated at 4°C for 5 min and then at 37°C for 30 min. (b) 6-h transfaction: The cells were incubated at 4°C for 5 min and then 37°C for 6 h. To maintain cell viability, 4 ml of serum-free medium was added to the 35-min transfaction dishes.

Lipofectin method. The DNA (30 μg)-HMG complex, or DNA alone was dissolved in 50 μl culture media (Opti-mem; BRL Life Technologies, Gaithersburg, MD) and mixed with 50 μl of Lipofectin reagent DOTMA (1:2,3,4-triolsnyl)-propyl-n,n,n-trimethy lammonium chloride (BRL Life Technologies) dissolved in the same volume of water in a ratio of 1:3 (wt/wt; DNA 30 μg and Lipofectin 90 μg/4 ml media). Mixtures were incubated for 30 min at room temperature. 100 μl of DNA–Lipofectin complex was added dropwise to each dish, and incubated for 35 min or 6 h at 37°C (20). Cell ACE activity was normalized by expressing activity per milligram homogenate protein. Using this protocol, measured ACE activity is completely abolished by either quinaprilat (a specific ACE inhibitor) or neutralizing antibodies to ACE (9).

After incubation, the medium with 5% calf serum was changed to fresh medium and the cells were incubated overnight in a CO2 incubator. The day after transfaction, the medium was replaced by fresh medium with 5% calf serum and the cells were maintained for 2 d. On day 3 after transfaction, cells were washed twice with PBS to remove residual medium and cell ACE activity were measured. ACE activity, expressed as hippuryl-l-histidyl-l-leucine (HHL) hydrolyzing activity, in cell homogenates, was determined by the modified method of Cushman and Cheung (21). To examine cell injury by the transfaction, cells were inoculated to 24-well plates. After 6-h incubation with Lipofectin or HVJ complex including control-vector DNA, media with 5% calf serum were replaced and incubated overnight in a CO2 incubator. Cells were trypsinized and counted by counter.

Determination of DNA and RNA synthesis

VSMC were seeded onto 12-well culture dishes (Costar Corp., Cambridge, MA) and maintained with Waymouth's medium with 5% calf serum. After confluence, cells were washed three times with BSS containing 2 mM CaCl₂. Then, 500 μl of HVJ liposomes (1.3 mg of lipids and 1.3–5 μg of encapsulated DNA) was added to the wells. The cells were incubated at 4°C for 5 min and then at 37°C for 30 min, and after changing to fresh medium with 5% calf serum, they were incubated overnight in a CO2 incubator. In preparation of experiments for determination of DNA and RNA synthesis, the cells were made quiescent by placing them for 48 h in a defined serum-free (DSF) medium containing insulin (5 × 10⁻⁷ M), transferrin (5 μg/ml), and ascorbate (0.2 mM), as previously reported (19). Relative rates of DNA and RNA synthesis were assessed by determination of tritiated thymidine and uridine incorporations into TCA-precipitable material. Quiescent rat VSMC cells grown in 12-well culture dishes (Costar Corp.) were pulsed for 24 h (12–36 h after the stimulation) with tritiated thymidine (2 μCi/ml) and 4 h (15–19 h after the stimulation) with tritiated uridine (2 μCi/ml), washed twice with cold PBS, twice with 10% (wt/vol) cold TCA, and incubated with 10% TCA at 4°C for 30 min. Cells were rinsed in ethanol (95%) and dissolved in 0.25 N NaOH at 4°C for 3 h, neutralized, the radioactivity determined by liquid scintillation spectrometry (22).

Bioassay of Ang II activity

Quiescent VSMC were transfected with control vector or ACE-transfected cDNA. The conditioned medium (CM) was collected from
these cells 24 h later and Ang II activity was bioassayed using quiescent naive VSMC. CM of transfected VSMC was added to these naive cells in a 1:1 (vol/vol) and the resultant rates of RNA synthesis of the naive VSMC were measured as above.

**Materials**

Ang I and II were obtained from Sigma Chemical Co. (St. Louis, MO). Ang II type I (AT 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. P < 0.05 was considered significant.

**Results**

**ACE activity.** Fig. 1 shows ACE activity in the transfected and untreated cultured VSMC. ACE activity in CAGGS (control vector)–transfected VSMC by both cationic lipid and HVJ methods showed no difference from that in untreated VSMC. Under the condition of the transfection (35 min), ACE-transfected VSMC using the HVJ method showed twofold higher ACE activity than control vector-transfected VSMC, as shown in Fig. 1 A. In contrast, the cationic liposome method of transfection (35 min incubation) resulted in no difference in ACE activity between control vector and ACE-transfected VSMC. Control vector–transfected VSMC using either methods showed no significant change in total protein content as compared to untreated VSMC (data not shown). However, ACE-transfected VSMC using the HVJ method showed a significant increase in cellular protein content as compared to control vector–transfected VSMC (6.85±0.044 mg/well vs ACE-transfected VSMC: 1.035±0.156 mg/well; P < 0.05). Fig. 1 B shows the effect of 6 h of transfection. ACE-transfected cells using the HVJ method showed a significant increase in ACE activity as compared to untreated and control vector–transfected VSMC. In contrast, no change in ACE activity could be seen in the ACE-transfected VSMC using the Lipofectin method. On the other hand, 6 h of incubation with Lipofectin resulted in cell injury and loss as compared to untreated VSMC (8.57±0.18 x 10^3 cells/well vs Lipofectin-treated VSMC: 7.85±0.28 x 10^3 cells/well; P < 0.05), while there was no difference in cell numbers between HVJ-treated and untreated cells.

**Effects of transfected ACE cDNA on DNA and RNA synthesis of postconfluent VSMC.** The effects of HVJ-transfected ACE or renin cDNA on VSMC DNA and RNA synthesis are shown in Figs. 2 and 3. Transfection of control vector did not show any change in DNA and RNA synthesis from the basal rate of untreated VSMC (DNA synthesis; untreated VSMC: 4.370±1.067 cpm/well vs control vector–transfected VSMC: 4.313±0.720 cpm/well, RNA synthesis; untreated VSMC: 21.818±1.345 cpm/well vs control vector–transfected VSMC: 21.949±1.295 cpm/well). DNA and RNA synthesis in the ACE or renin-transfected VSMC increased significantly as compared with that in untreated and control vector–transfected VSMC. These increases in DNA and RNA synthesis was completely blocked by the specific Ang II receptor antagonist DuP753 (10^-6 M). Moreover, addition of exogenous Ang I stimulated RNA synthesis in a dose-dependent manner in both the control vector– and the ACE-transfected cells, as shown in Fig. 4. At any concentration of Ang I, higher uridine incorporation rate was observed in the ACE-transfected cells than in control vector–transfected VSMC. The Ang I (10^-7 M)–induced increase in RNA synthesis in the ACE-transfected VSMC was also inhibited by DuP 753 (10^-6 M) (addition of Ang I to ACE-transfected cells: 41.184±335 cpm/well, vs addition of Ang I and Ang II antagonist to ACE-transfected VSMC: 27.916±4.737 cpm/well, P < 0.01). On the other hand, transfecting with increasing amounts of ACE cDNA (from 0.2 to 0.6 to 2.5 to 10 μg enwrapped in liposome) stimulated RNA synthesis dose dependently, as shown in Fig. 5.

**Cotransfection of ACE and renin cDNAs into VSMC.** Transfection of renin cDNA also stimulated RNA synthesis, as shown in Table I. Moreover, cotransfection of renin and ACE cDNAs showed twofold further higher RNA synthesis than ACE or renin cDNA alone. The increase in RNA synthesis in cotransfected VSMC was also inhibited by Ang II receptor antagonist DuP 753 (10^-6 M).

Incubation with CM collected from ACE-transfected VSMC also resulted in significant increase in RNA synthesis of quiescent VSMC (as bioassay) as compared to incubation with CM from control vector–transfected VSMC, as shown in Table II. This increase in RNA synthesis was significantly reduced by DuP 753 (10^-6 M).
**Discussion**

The presence of components of RAS in VSMC have been reported by many investigators (15–18). Ang II, the final product of RAS, can induce vascular hypertrophy as well as hyperplasia both in vitro and in vivo (13–14, 23–25). Accordingly, it has been hypothesized that vascular Ang II plays an important role on vascular growth. However, knowledge of the cellular mechanisms of vascular Ang II production is very limited. For example, what are the rate-limiting steps in endogenous Ang II production by VSMC? Does autocrine Ang II regulate VSMC growth? What controls the VSMC RAS? These questions cannot be addressed easily by in vivo studies. Therefore, we developed an in vitro model of VSMC RAS using gene transfer technology.

In vitro gene transfer using calcium phosphate precipitation, DEAE dextran, electroporation, and cationic liposome methods have been reported. As these methods can result in substantial cell injury and death, they pose significant problems to the investigation of the role of potential autocrine mediators (e.g., angiotensin) in the regulation of cell growth using thymidine and uridine incorporation methodology. We reported previously the utility of cationic liposome for in vitro transfection and cell growth studies, but this method requires long incubation time (24 h) and has a low efficiency of transfection (20). In this study, we used the HVJ liposome method.

![Figure 2](image1.png)

*Figure 2. Stimulatory effect of transfected ACE (A) or renin (B) cDNA on the incorporations of $[^3]$H]thymidine in cultured VSMC. CONTROL, control vector–transfected cells by HVJ method; ACE, ACE-transfected cells by HVJ method; ACE + DuP753, Ang II receptor antagonist DuP 753 (10$^{-6}$ M) added to ACE-transfected cells; RENIN, renin-transfected cells by HVJ method; RENIN + DuP753, Ang II receptor antagonist DuP 753 (10$^{-6}$ M) added to ACE-transfected cells; Ang II, Ang II (10$^{-6}$ M) added to control vector–transfected cells. Each group contains five samples. *P < 0.01 vs control vector–transfected cells.*

![Figure 3](image2.png)

*Figure 3. Stimulatory effect of transfected ACE (A) or renin (B) cDNA on the incorporations of $[^3]$H]uridine in cultured VSMC. CONTROL, control vector–transfected cells by HVJ method; ACE, ACE-transfected cells by HVJ method; ACE + DuP753, Ang II receptor antagonist DuP 753 (10$^{-6}$ M) added to ACE-transfected cells; RENIN, renin-transfected cells by HVJ method; RENIN + DuP753, Ang II receptor antagonist DuP 753 (10$^{-6}$ M) added to ACE-transfected cells; Ang II, Ang II (10$^{-6}$ M) added to control vector–transfected cells. Each group contains five samples. *P < 0.01 vs control vector–transfected cells.*
Figure 4. Dose-dependent effect of Ang I on the incorporations of \(^{3}H\)uridine in control vector- and ACE-transfected VSMC. Open column shows control vector-transfected VSMC, and shaded column shows ACE-transfected VSMC. CONTROL, no addition of Ang I; Ang (-9), added Ang I (10\(^{-9}\) M); Ang (-8), added Ang I (10\(^{-8}\) M); Ang (-7), added Ang I (10\(^{-7}\) M). Each group contains five samples. \(* P < 0.01 vs control VSMC without Ang I; \* P < 0.01 vs transfected VSMC with Ang I (10\(^{-8}\) M).

Figure 5. Variable amount of transfected ACE cDNA (from 0.2-0.6 to 2.5-10 \(\mu\)g DNA enwrapped by liposome) on the percent change in \(^{3}H\)uridine incorporation. Each group contains five samples. CONTROL, control vector-transfected VSMC; \(* P < 0.01 vs control group. Statistical analysis was done by Kruskal-Wallis analysis.

Table I. Effect of Transfected cDNA on RNA Synthesis of VSMC

<table>
<thead>
<tr>
<th>DNA construct</th>
<th>[^{3}H]uridine incorporation</th>
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<tr>
<td>Control vector</td>
<td>7,941±138</td>
</tr>
<tr>
<td>ACE</td>
<td>12,878±1,077*</td>
</tr>
<tr>
<td>Renin</td>
<td>12,182±864*</td>
</tr>
<tr>
<td>ACE + renin</td>
<td>21,428±1,233*</td>
</tr>
<tr>
<td>ACE + renin + AIIRA</td>
<td>13,657±1,184*</td>
</tr>
</tbody>
</table>

AIIRA, Ang II receptor antagonist DuP 753 (10\(^{-8}\) M). Values are expressed as mean±SEM. Each group contains five samples. \* \(P < 0.01\) vs control vector; \(\dagger P < 0.01\) vs ACE- or renin-transfected VSMC; \(\ddagger P < 0.01\) vs cotransfected ACE and renin into VSMC.

cell toxicity. We also used both methods for 6 h of incubation. HVJ-mediated transfection resulted in a significant increase in VSMC ACE activity, while cationic liposome method did not result in any changes in ACE activity. Furthermore, 6 h of incubation with Lipolectin caused a significant decrease in cell number as compared to untreated VSMC, but the HVJ method did not affect cell viability. These observations demonstrate that the HVJ method is ideal for studies of autocrine regulation of vascular growth in vitro. Based on the short incubation time and high in vitro efficiency of the HVJ method, we anticipate that this may be an effective technique for in vivo gene transfer.

Using the HVJ method, we demonstrated that cells transfected with ACE and renin cDNAs exhibited increased RNA and DNA synthesis that could be abolished by specific Ang II receptor antagonist DuP 753, suggesting that the expression of VSMC ACE or renin can modulate cell growth through the autocrine production and action of Ang II. This hypothesis was supported further by our demonstration that conditioned media collected from ACE-transfected VSMC contained biologically active angiotensin. The observation that stimulation of RNA synthesis by CM was not completely inhibited by Ang II receptor antagonist could be explained by the presence of Ang II-stimulated PDGF or other growth factors in the CM (26).

In this study, increasing amounts of transfected ACE cDNA dose-dependently stimulated RNA synthesis. Moreover, the addition of exogenous Ang I resulted in a dose-dependent stimulation of RNA synthesis in control vector-transfected VSMC, consistent with previous reports that ACE activity is present in low quantity in VSMC (15). However, this effect was significantly greater in ACE-transfected VSMC, suggesting that ACE in VSMC is rate-limiting in Ang II produc-

Table II. Effect of Conditioned Media on RNA Synthesis

<table>
<thead>
<tr>
<th>Conditioned media</th>
<th>[^{3}H]uridine incorporation</th>
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<tbody>
<tr>
<td>CM</td>
<td>10,573±1,314</td>
</tr>
<tr>
<td>ACE</td>
<td>16,674±460*</td>
</tr>
<tr>
<td>ACE + AIIRA</td>
<td>13,033±678*</td>
</tr>
</tbody>
</table>

AIIRA, angiotensin II receptor antagonist DuP 753 (10\(^{-6}\) M). Values are expressed as mean±SEM. Each group contains four samples. \* \(P < 0.01\) vs CM; \(\dagger P < 0.01\) vs ACE.
tion and consequently RNA synthesis. Similarly, our data suggest that renin is rate-limiting in Ang II generation in these cells. Interestingly, cotransfection of ACE and renin cDNAs resulted in further increase in RNA synthesis than transfection with ACE or renin cDNA alone. We have previously reported that both angiotensinogen and ACE are induced in neointimal VSMC-like cells after balloon injury (9, 27). Our study provides additional insight into the role of RAS expression in VSMC in the autocrine regulation of VSMC growth.

Acknowledgments

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References