In Vivo Evidence of the Importance of Cardiac Angiotensin-Converting Enzyme in the Pathogenesis of Cardiac Hypertrophy

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Abstract—Cardiac angiotensin-converting enzyme (ACE) may play an important role in regulating cardiac hypertrophy. Angiotensin II (Ang II) stimulates cardiac hypertrophy as well as the production of extracellular matrix. However, it is still unclear whether Ang II exerts a direct effect on cardiac hypertrophy independent of its effect on blood pressure or the circulating renin-angiotensin system. Although ACE inhibitors and/or Ang II receptor antagonists have regressed cardiac hypertrophy, classic pharmacological experiments cannot exclude the contribution of hemodynamics and the circulating renin-angiotensin system. In vivo gene transfer provides the opportunity of assessing the effects of increased cardiac angiotensin in the intact animal without circulating angiotensin or blood pressure. Therefore, we used a “gain of function” approach to obtain local overexpression of cardiac ACE. Transfection of the human ACE vector into rat myocardium resulted in a significant increase in cardiac ACE activity (**P**<0.01). More interestingly, morphometry at 2 weeks after transfection revealed a significant increase in the thickness and areas of cardiac myocytes in hearts transfected with the ACE vector (**P**<0.01). In addition, transfection of the ACE vector also resulted in a significant increase in collagen content (**P**<0.01). This increase in cardiac hypertrophy was abolished by the administration of perindopril. Local transfection of the ACE vector into the heart did not result in systemic effects such as increased blood pressure, heart rate, or serum ACE activity. In summary, we have demonstrated that increased autocrine/paracrine angiotensin can directly cause cardiac hypertrophy independent of systemic factors and hemodynamic effects. This approach has important potentials for defining the role of autocrine/paracrine substances in cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2000;20:428-434.)

Key Words: remodeling ■ gene transfer ■ hypertrophy ■ angiotensin-converting enzyme ■ renin-angiotensin system

The pathogenesis of cardiovascular diseases such as hypertension, atherosclerosis, and myocardial infarction involves a process of cardiac remodeling associated with increased local expression of biologically active substances that are postulated to play pathophysiological roles. In hypertension, the heart undergoes a process of cardiac hypertrophy that is associated with the activation of a local angiotensin system (the renin-angiotensin system [RAS]).1–3 The potential role of autocrine/paracrine mediators such as angiotensin in cardiovascular pathobiology independent from systemic factors has been suggested from indirect evidence: cell culture studies, morphological analysis, and/or systemic administration of antagonists and agonists. To elucidate the role of a specific autocrine/paracrine factor, we have developed an efficient in vivo gene transfer technique to examine the consequences of overexpression of the factor in local tissues in the intact rat. Using this approach, we had previously reported that transfection of human angiotensin-converting enzyme (ACE) into local segment of the carotid artery resulted in vascular hypertrophy independent of the circulating RAS and hemodynamics.4 This approach is particularly powerful because the locally transfected vessel segment can be compared with adjacent untransfected segments as well as the contralateral vessel. Furthermore, the transfected segment is exposed to the same blood pressure and circulating factors as the control vessel. However, the role of ACE within the heart still remains unclear, because it is very difficult to transfect efficiently into cardiac myocytes in vivo. To overcome these issues, we reported a high transfection efficiency with the hemagglutinating virus of Japan (HVJ, or Sendai virus) liposome method into intact rat hearts.5–7 For example, luciferase activity was significantly higher in intact noninfarcted hearts transfected with the luciferase vector by the HVJ liposome method than in intact noninfarcted hearts transfected by direct injection of “naked” plasmid DNA.6 Using this method, we further examined the role of autocrine/paracrine angiotensin as a mediator of cardiac hypertrophy in vivo in this study.
Previous data have demonstrated that angiotensin II (Ang II) can stimulate the protein contents of cardiac myocytes and modulate extracellular matrix.\textsuperscript{8–11} Ang II is generated via an enzymatic cascade in which tissue ACE plays a key role.\textsuperscript{12,13} We postulate that increased cardiac ACE expression induces cardiac hypertrophy via increased local generation of Ang II within the heart. Our results provide the first direct evidence that overexpression of an autocrine/paracrine factor (ie, angiotensin) transfectioned into the heart in vivo mediates the cardiac remodeling process of hypertension independent of systemic factors or hemodynamic stimuli. In this study, we tested our hypothesis by (1) transfecting the human ACE vector locally into intact rat hearts in vivo and (2) studying the biochemical and physiological consequences of overexpression of ACE within the hearts. Our data demonstrate that increased local expression of ACE within the heart promotes autocrine/paracrine Ang II–mediated cardiac hypertrophy in vivo.

Methods

Construction of Plasmids

The pUC-CAGGS expression vector plasmid (kindly provided by Junichi Miyazaki, Tokyo University, Tokyo, Japan)\textsuperscript{4,15} was digested with EcoRI. The EcoRI fragment containing the human truncated ACE vector of RB 35-15, including 2 putative active sites, was inserted into the EcoRI site in this vector by filling EcoRI ends with T4 polymerase.\textsuperscript{4,15} CAGGS contains the entire envelope region open reading frame consisting of 3 translation initiation codons, which represent the N-termini of the large, middle, and major surface (S) polypeptides downstream of the cytomegalovirus enhancer and the chicken β-actin promoter. The vector used as a control was pUC-CAGGS, which did not contain the ACE cDNA. The luciferase gene expression vector is driven by the Epstein-Barr virus (EBV) promoter or β-actin promoter.\textsuperscript{16}

Preparation of HVJ Liposomes

The preparation of conventional HVJ liposomes has been described previously.\textsuperscript{4,6,17,18} In brief, phosphatidylinerine, 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 the sucrose gradient. The top layer of the sucrose gradient was HVJ removed from the HVJ liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient was collected for use. Cationic HVJ liposome were made by phosphatidylcholine, cholesterol, and cholesteryl N-(dimethylaminoethyl)-carbamate mixed in a weight ratio of 8:4:1.\textsuperscript{16,19}

In Vivo Gene Transfer Into the Heart by the Direct Injection Approach

Male Sprague-Dawley rats (400 to 500 g; Charles River Breeding Laboratories, Boston, MA) were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.1 mL/100 mg body weight). Rats were intubated and connected to a respirator. HVJ-liposome complex containing human ACE or the control vector was carefully injected directly into the heart with a 30-gauge needle through a left lateral thoracotomy into multiple sites. One injection volume of HVJ-liposome complex was 10 μL (0.2 μg of plasmid).\textsuperscript{4–7} These experiments were approved by the Osaka University Animal Use Committee.

Administration of ACE Inhibitor (Perindopril)

Before transfection, rats received Alzet minipumps (Alza Inc) implanted intraperitoneally. Untreated animals received vehicle (1:1, vol/vol, of saline/polyethylene glycol 400), whereas the treated groups received the ACE inhibitor (perindopril, generously donated by Daiichi Pharmaceutical Company, Tokyo, Japan) at a dose of 10 mg · kg\textsuperscript{-1} · d\textsuperscript{-1}. This administration regimen had previously been demonstrated to block the effects of Ang II in vivo.\textsuperscript{7,21} The drugs were administered immediately after transfection with human ACE or the control vector and continued until the hearts were harvested for morphometry.

Measurement of ACE Activity

For the measurement of cardiac ACE activity, rats were killed 3 days after transfection. After infusion of PBS, the hearts were removed and immediately frozen in LN\textsubscript{2}. On the day of assay, the hearts were thawed, weighed, and homogenized in 50 mmol/L KPO\textsubscript{4} (pH 7.5). ACE activity, expressed as hippuryl-l-histidyl-l-leucine hydrolyzing activity of the homogenate, was determined by the modified method of Cushman and Cheung.\textsuperscript{22} Cardiac ACE activity was normalized by expressing activity per milligram of tissue. The specificity of ACE activity was previously confirmed by its complete inhibition by either quinaprilat (a specific ACE inhibitor) or neutralizing antibodies to ACE, as previously described.\textsuperscript{4,23}

Histological Analyses

For morphological analyses, rat hearts were removed 2 weeks after transfection, after perfusion-fixation with 10% formaldehyde under physiological pressure (110 mm Hg). The thickness and area of cardiac myocytes were measured on a digitizing tablet (model 2200, South Micro Instruments) after the tissue was stained with hematoxylin. At least 5 individual sections from the hearts were analyzed to avoid the effects of needle injection, as the area of transfection could be detected throughout the myocardium owing to the multiple injection sites for the HVJ liposome method.\textsuperscript{6,7} Animals were coded so that the analysis was performed without the investigator’s knowledge of which treatment each animal had received. Histological analysis was performed by using a computerized morphometry system (Nexus 6400, Kashiwagi Research Co) by individuals who were unaware of the treatment each animal had received. The reproducibility of the results was assessed. Intraobserver variability was determined from repeated measurements performed by 1 observer for all sections. The mean±SD differences among measurements made by the same observer was 2.2±0.4%. Interobserver variability was determined from measurements of 10 randomly selected sections performed by a second observer in addition to the first observer. The difference between measurements made by the 2 observers was 3.3±0.4%. These observers were blinded to other data concerning the rats, as well as to the results of the other observer.

Sirius Red Method for Collagen Staining

Sirius red microscopy detects interstitial collagen, including types I and III.\textsuperscript{24} Fresh-frozen sections (6 μm) were rinsed with distilled water and incubated with 0.1% Sirius red F3BA (Polysciences Inc) in saturated picric acid for 90 minutes. Sections were rinsed twice with 0.01N HCl for 1 minute and then immersed in distilled water. After dehydration with 70% ethanol for 30 seconds, sections were cover-slipped. The stained sections were observed under polarized light and photographed with the same exposure time for each section. Analysis of Sirius red staining was performed with a computer-based quantitative color image analysis system.\textsuperscript{25} Photographs were scanned into a 1K×1K image buffer of the Optimas 5.2 image analysis system (Optimas Co). A color threshold mask for immunostaining was defined to detect the red color by sampling, and the same threshold was applied to all specimens. The percentage of the total area with positive color for each section was recorded.
Analysis of Luciferase Activity

Firefly luciferase activity was measured by using a luciferase assay system (PicaGene, Toyo-Inki). Rats were killed 4 days after transfection with the luciferase gene either by direct transfection of “naked” plasmid or by the HVJ-liposome method via direct injection into the apex, as described below. The tissue samples (200 mg around the injection site) were rapidly frozen in LN2 and homogenized in a lysis buffer. The tissue lysates were briefly centrifuged (3000 rpm, 10 minutes), and 20 μL of supernatant was mixed with 100 μL of luciferase assay reagents. Measurements of the luminescent reaction were started 5 seconds after addition of the sample. The counting lasted for 10 seconds, and the number of counts in 10 seconds was used for calculation of luciferase activity.6

Statistical Analysis

All values are expressed as mean±SEM. ANOVA with Duncan’s test was used to determine the significance of differences in multiple comparisons. P<0.05 was considered to be statistically significant.

Results

Overexpression of Cardiac ACE

To evaluate the success of gene transfer, we initially measured cardiac ACE activity. The biological activity of the transgene product was documented by a 2-fold increase in ACE activity within ACE-transfected hearts (Figure 1). Given that the administration of ACE inhibitors or Ang II receptor antagonists resulted in the improvement of cardiac hypertrophy in hypertension, we hypothesized that increased local production of ACE would modulate cardiac structure. Therefore, we performed morphological studies 2 weeks after transfection. Evidence of local cardiac hypertrophy after ACE gene transfer persisted for at least 2 weeks after transfection, as reflected by the increasing thickness and area of cardiac myocytes (Figure 2) and the increasing cardiac protein content in ACE vector–transfected hearts compared with control.

Figure 1. Cardiac ACE activity in hearts 3 days after transfection. Control indicates control vector–transfected hearts (n=8); ACE, ACE vector–transfected hearts (n=10). **P<0.01 vs control.

Figure 2. a. Representative cross sections of cardiac myocytes in ACE- and control vector–transfected hearts 2 weeks after transfection. Control vector indicates control vector–transfected hearts (×200); ACE vector, ACE vector–transfected hearts (×200); ACE vector + perindopril, ACE vector–transfected hearts from rats that received an ACE inhibitor (perindopril) (×200). b. Effect of ACE vector transfection on the thickness of cardiac myocytes 2 weeks after transfection Untreated indicates sham-operated hearts (n=6); Control, control vector transected hearts (n=7); ACE=ACE vector–transfected hearts (n=10). **P<0.01 vs untreated, ###P<0.01 vs control. c. Effect of ACE vector transfection on the area of cardiac myocytes 2 weeks after transfection. Untreated indicates sham-operated hearts (n=6); control, control vector–transfected hearts (n=7); ACE, ACE vector–transfected hearts (n=10). **P<0.01 vs untreated, ###P<0.01 vs control.
controls. Interestingly, administration of the ACE inhibitor perindopril inhibited the cardiac hypertrophy induced by the local overexpression of ACE (Figure 2a). Morphometric analysis documented that the thickness of ACE transfected–hearts was significantly increased compared with control vector–transfected and untransfected hearts (Figure 2b). Consequently, the area of cardiac myocytes in ACE-transfected hearts was significantly greater than that of control vector–transfected hearts (Figure 2c). However, there were no discernible differences in cell numbers counted in serial sections of the hearts between ACE-transfected and control vector–transfected hearts (data not shown). Furthermore, we evaluated the effects of ACE overexpression on collagen synthesis, as Ang II is well known to stimulate collagen synthesis, and the accumulation of collagen matrix deposition is a typical feature of hypertensive cardiac hypertrophy. We performed Sirius red staining for collagen, as Sirius red staining when viewed under polarized light identifies collagen, including types I and III.24 Hearts transfected with control vector showed positive Sirius red staining near the injection sites only, demonstrating a low content of interstitial collagen at baseline (Figure 3). In contrast, hearts transfected with ACE vector exhibited substantial accumulation of interstitial collagen around the injection site (Figure 3). Of importance, transfection of the human ACE vector into intact hearts resulted in a significant increase in collagen matrix compared with control vector, as assessed by quantitative color image analysis (P<0.01, Figure 3).

Importantly, these cardiac changes are independent of the circulating RAS or hemodynamic changes such as blood pressure, heart rate, and serum ACE activity (Table 1). Moreover, the morphometric changes typical of cardiac hypertrophy induced by ACE overexpression were also abolished by administration of the ACE inhibitor perindopril. These results revealed that cardiac-specific transfection produced a novel animal model overexpressing cardiac ACE without interference from the circulating RAS or hemodynamic changes, such as blood pressure, heart rate, and serum ACE activity.

Comparison of Transfection Efficiency

Given the successful production of a novel cardiac hypertrophy model, we further modified the gene transfer techniques with the HVJ liposome method. First, comparison of the expression of different kinds of promoter was evaluated. We
constructed a luciferase expression vector driven by the β-actin promoter or the EBV promoter, as the EBV promoter prolongs transgene expression. Consistent with previous findings, luciferase activity was markedly increased in hearts transfected with the luciferase vector driven by β-actin and using the HVJ liposome method compared with that produced by direct injection (P < 0.01, Figure 4). Consistent with the previous observation that the EBV promoter increases the amount of expressed protein, our study showed a significant increase in luciferase activity in hearts transfected with the luciferase construct driven by EBV promoter and using the HVJ liposome complex with that produced by direct injection (P < 0.01, Figure 4). Consistent with the previous observation that the EBV promoter increases the amount of expressed protein, our study showed a significant increase in luciferase activity in hearts transfected with the luciferase construct driven by EBV promoter and using the HVJ liposome method compared with that produced by direct injection (P < 0.01, Figure 4). Consistent with the previous observation that the EBV promoter increases the amount of expressed protein, our study showed a significant increase in luciferase activity in hearts transfected with the luciferase construct driven by EBV promoter and using the HVJ liposome method compared with that produced by direct injection (P < 0.01, Figure 4). Consistent with the previous observation that the EBV promoter increases the amount of expressed protein, our study showed a significant increase in luciferase activity in hearts transfected with the luciferase construct driven by EBV promoter and using the HVJ liposome method compared with that produced by direct injection (P < 0.01, Figure 4).

**Discussion**

Cardiovascular hypertrophy is thought to be an adaptive response to hypertension. Although it is well accepted that the mechanical effects of increased blood pressure stimulate cardiac and/or vascular hypertrophy, recent indirect evidence suggests that humoral factors may also play an important role. Ang II can stimulate the protein contents of cardiac myocytes and influence extracellular matrix. It has been stated that ACE is not rate limiting in Ang II production, since its total quantity in the body is high. However, several investigators have reported that in vivo increases in local cardiac ACE activity in experimental animals result in parallel increases in tissue Ang I conversion to Ang II and changes in local function. Indeed, it is reported that pressure-overload cardiac hypertrophy induced by aortic banding induces cardiac ACE gene expression and increased cardiac Ang II production. Nevertheless, 2 important questions have not yet been resolved in hypertension and cardiovascular research. First, can the RAS directly mediate cardiac hypertrophy, independent of its blood pressure effect? Second, is local tissue ACE important in regulating local cardiovascular function and in contributing to pathophysiology? To answer these questions, we previously employed in vivo gene transfer technology, since these questions have only been addressed indirectly by evidence derived from in vitro cell culture studies, in vivo systemic infusion of Ang II, and/or administration of ACE inhibitors. In vivo gene transfer technology has the following advantages: (1) the target gene can be transfected into a
local segment, thereby avoiding a systemic effect, and (2) the
consequences of local overexpression within the physiological/pathophysiological range of the target gene may be
studied. Indeed, luciferase activity was detected in the myo-
cardium only by transfection of the luciferase vector by the
HVJ liposome method, whereas luciferase activity was not
detected in other tissues (brain, lung, liver, kidney, and testis;
M.A. et al, unpublished observations, 1996). In contrast, none
of these data are completely convincing owing to the contri-
bution of confounding variables such as hemodynamic ef-
facts, cell culture conditions, etc. Indeed, our previous study
could demonstrate direct evidence of the contribution of
vascular ACE in the pathogenesis of vascular hypertrophy.4
However, none of the other reports provided direct evidence
of the involvement of cardiac ACE in the pathogenesis of
cardiac hypertrophy.

In this study, we have extended our investigation to ACE
gene transfer in vivo into rat hearts. We were able to study the
long-term cardiac effects of increased tissue ACE activity. As
previously reported, HVJ liposome-mediated gene transfer is
an efficient in vivo method that has minimal or no toxicity
and provides sustained gene expression for transfection into
hearts.5–7 Moreover, the present study confirmed the high
transfection efficiency of the HVJ liposome method by using
2 different kinds of luciferase construct. Although transgenic
technology also provides the opportunity to study specific
gene function, this technology has several disadvantages:
(1) it is time consuming and costly, (2) the effect of the
overexpressed transgene is exerted throughout development,
and (3) it is difficult to exclude a potential contribution of the
systemic effect of transgene expression. Previous studies
have documented the roles of hemodynamic stimuli and
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hypertrophy process in hypertension, independent of changes in blood pressure or the endocrine RAS. Moreover, this study of the cardiac angiotensin system serves as a paradigm for the elucidation of the role of other autocrine/paracrine mediators of cardiac remodeling in the pathogenesis of diseases such as myocardial infarction and cardiomyopathy. Our data demonstrate that the localized, in vivo gene transfer technique is a useful experimental tool for the study of autocrine/paracrine factors in complex pathophysiological states in vivo. This approach has broad applicability and is complementary to other methods, such as transgenic technology, in elucidating the biological roles of candidate genes in vivo.

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References
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