Evidence for Direct Local Effect of Angiotensin in Vascular Hypertrophy

In Vivo Gene Transfer of Angiotensin Converting Enzyme

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Abstract

In vitro studies have demonstrated that angiotensin (Ang) II directly stimulates vascular smooth muscle cell (VSMC) growth. However, it is still unclear if Ang II exerts a direct effect on vascular hypertrophy in vivo independent of its effect on blood pressure. In vivo gene transfer provides the opportunity to assess the effects of increased activity of the vascular angiotensin system in the intact animal while avoiding an increase in circulating angiotensin or in blood pressure. Accordingly, we transfected the human angiotensin converting enzyme (ACE) vector into intact rat carotid arteries by the hemagglutinating virus of Japan–liposome method. 3 d after transfection, we detected increased ACE activity in the transfected artery. Immunohistochemistry localized immunoreactive ACE in the medial VSMC as well as in the intimal endothelial cells. The increase in vascular ACE activity was associated with a parallel increase in DNA synthesis as assessed by BrdU (bromo-deoxyuridine) index and vascular DNA content. This increase in DNA synthesis was abolished by the in vivo administration of an Ang II receptor–specific antagonist (DuP 753). Morphometry at 2 wk after transfection revealed an increase in the wall to lumen ratio of the ACE-transfected blood vessel as compared with control vector transfected vessels. This was accompanied by increases in protein and DNA contents without an increase in cell number. Local transfection of ACE vector did not result in systemic effects such as increased blood pressure, heart rate, or serum ACE activity. These morphological changes were abolished by the administration of the Ang II receptor antagonist. In this study, we used in vivo gene transfer to increase local expression of vascular angiotensin converting enzyme and provided proof that increased autocrine/paracrine angiotensin can directly cause vascular hypertrophy independent of systemic factors and hemodynamic effects. This approach has important potentials for defining the role of autocrine/paracrine substances in vascular biology and hypertension. (*J. Clin. Invest.* 1994. 94:978–984.) Key words: vascular angiotensin • vascular growth • in vivo gene transfer • hypertension • paracrine/autocrine

Introduction

The pathogenesis of vascular diseases such as hypertension, atherosclerosis, and restenosis involves a process of vascular remodeling associated with increased local expression of biologically active substances that are postulated to play pathophysiological roles. In hypertension, the arteries undergo a process of vascular hypertrophy that is associated with the activation of a local angiotensin system (1–3). The potential role of autocrine/paracrine mediators such as angiotensin in vascular pathobiology independent from systemic factors has been suggested from indirect evidence, that is, cell culture studies, morphologic analysis, and/or by systemic administration of antagonists and agonists (1–16). 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 a segment of the carotid artery in the intact rat. 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. In this study, we examined the role of autocrine/paracrine angiotensin as a mediator of vascular hypertrophy in vivo. Previous data have demonstrated that angiotensin II (Ang II)[1] can stimulate smooth muscle cell growth and modulate extracellular matrix (10–13). Ang II is generated via an enzymatic cascade in which tissue angiotensin converting enzyme (ACE) plays a key role (17–19). We postulate that increased vascular ACE expression induces vascular hypertrophy via increased local generation of Ang II within the vessel wall. Our results provide the first direct evidence that overexpression of an autocrine/paracrine factor (i.e., angiotensin) transfected into the intact vessel wall in vivo mediates the vascular remodeling process of hypertension independent of systemic factors or hemodynamic stimuli.

Our approach involves the use of the hemagglutinating virus of Japan (HVJ)–liposome gene transfer technique (20) to transfer an expression vector containing human ACE cDNA driven by a beta-actin promoter and cytomegalovirus enhancer into the intact uninjured rat carotid artery (21–23). In this study, we used this technique to test our hypothesis by (*a*) transfecting ACE vector locally into intact rat carotid arteries in vivo, and (*b*) studying the biochemical and physiological consequences of overexpression of ACE within vessel wall. Our data demonstrate that increased local expression of ACE within vessel wall

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Received for publication 1 December 1993 and in revised form 29 March 1994.

J. Clin. Invest.

0021-9738/94/09/0978/07 $2.00
Volume 94, September 1994, 978–984

1. *Abbreviations used in this paper:* ACE, angiotensin converting enzyme; Ang, angiotensin; BrdU, bromo-deoxyuridine; HVJ, hemagglutinating virus of Japan.

promotes autocrine/paracrine Ang II–mediated vascular hypertrophy in vivo.

Methods

Construction of plasmids. The pUC-CAGGS expression vector plasmid (kindly provided by Junichi Miyazaki, Tokyo University, Tokyo, Japan) was digested with EcoRI. The EcoRI fragment containing human truncated ACE vector of RB 35–15 including two putative active sites was inserted into the EcoRI site in this vector by filling EcoRI ends with T4 polymerase (21). CAGGS contains the entire envelope region open reading frame consisting of three translation initiation codons, which represent the amino termini of the large, middle, and major surface (S) polypeptides downstream of the cytomegalovirus enhancer and the chicken beta-actin promoter. The control vector used as a control was pUC-CAGGS, which did not contain the ACE cDNA.

Preparation of HVJ–liposomes. The preparation of HVJ–liposomes has been described previously (20–23). 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. Dried lipid was hydrated in 200 μl of balanced salt solution (137 mM NaCl, 5.4 mM KCl, 13 mM Tris-HCl, pH 7.6) containing DNA–high mobility group-1 complex (300 μg: 96 μg), which had been incubated previously at 20°C for 1 h. Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by ultraviolet irradiation (110 erg/mm^2/s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (20,000 hemagglutinating units) in a total volume of 4 ml of balanced salt solution. 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 was collected for use.

In vivo gene transfer. Male Sprague-Dawley rats (400–500 g; Charles River Breeding Laboratories, Wilmington, MA) were anesthetized with ketamine, and the left common carotid artery was surgically exposed (20, 24). The distal half of the artery was temporarily isolated with ligatures. A cannula was inserted from external carotid artery into the common carotid artery. The HVJ–liposome complex (300 μl) containing either the ACE vector or control vector plasmid DNA (3 μg DNA within liposomes) was infused into the isolated carotid segment by means of a cannula in the external carotid, after a brief irrigation with PBS to wash out the blood. After 15 min of incubation, the temporary ligatures on the common carotid artery were released, and blood flow was restored. The neck wound was then closed. At 3 d and 2 wk after transfection, the animals were killed. After perfusion fixation with 4% paraformaldehyde under physiological pressure, carotid arteries were removed for morphologic studies. No adverse neurological or vascular effects were observed in any animals undergoing this procedure.

Administration of Ang II receptor antagonist (DuP 753). Before transfection, rats received alzet minipumps (Alza Corp., Palo Alto, CA) implanted intraperitoneally. Untreated animals received vehicle (1:1, saline/PEG 400) while the treated groups received the Ang II receptor antagonist (DuP 753) (generously donated by Parke-Davis/Warner Lambert, Ann Arbor, MI) at a dose of 20 mg/kg/d. This administration regimen has been demonstrated previously to block the effects of Ang II in vivo (25, 26). The drugs were administered for 1 wk before transfection of the left common carotid artery and continued until vessels were harvested for DNA synthesis analysis or morphometry.

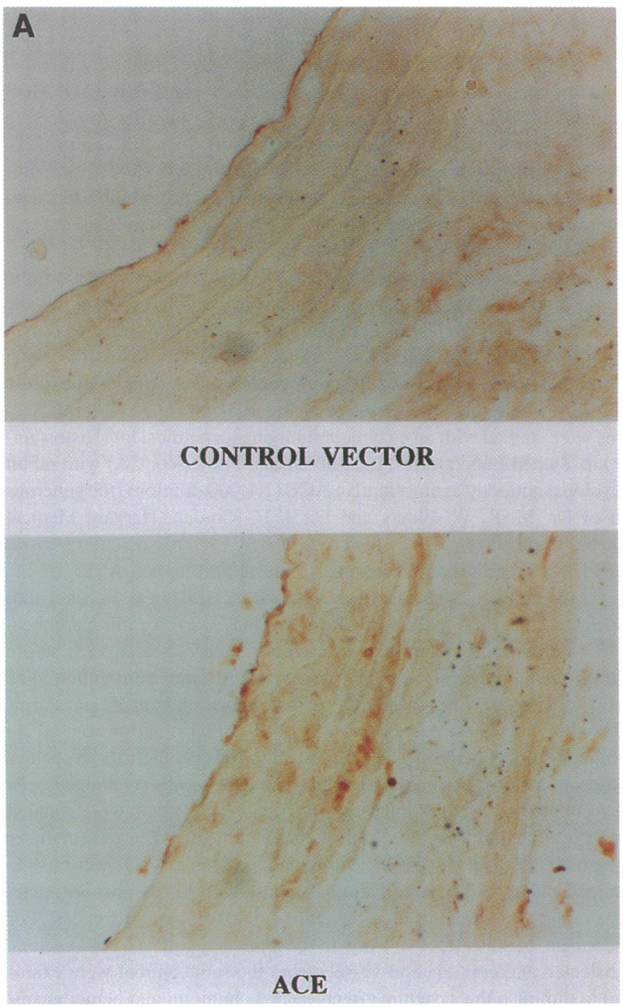
Measurement of ACE activity and DNA content. For the measurement of vascular ACE activity, rats were killed 3 d after transfection. After infusion of PBS, carotid arteries were removed and dissected free of periadventitial tissues and immediately frozen in liquid nitrogen. On the day of assay, the vessels were thawed, weighed, and homogenized in 50 mM KPO_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 (27). Vascular ACE level was expressed as enzymatic activity per milligram of protein. The specificity of ACE activity in the rat carotid artery was previously confirmed by its complete inhibition by either quinaprilat (a specific ACE inhibitor) or neutralizing antibodies to ACE as described previously (21). Measurement of DNA was performed at 3 d and 2 wk after transfection using the bisbenzimide trihydrochloride method (Pierce, Rockford, IL) (14). Blood pressure and heart rate were measured by direct measurement method. Briefly, a catheter was inserted from femoral artery into abdominal aorta under ether anesthesia. After 48 h, blood pressure and heart rate were measured in the conscious state for 30 min.

Histological and histochemical analyses. 3 d after transfection, the carotid arteries were stained for immunoreactive ACE protein. Tissue specimens from the midsection of the transfected region were sectioned (6 μM thick) at 60-μm intervals along the vessel in a proximal to distal fashion. Histological sections from untreated vessels, known to express ACE in the endothelial layer, were included as a positive control. Sections were stained with an enzyme immunohistochemical kit (Histostain-SP kit; Zymed Laboratories, Inc., South San Francisco, CA) with rabbit polyclonal antibody against human ACE (1:1,000 dilution) (the generous gift of Dr. M. R. W. Ehlers and Dr. J. F. Riordan, Harvard Medical School, Cambridge, MA) which was tested for crossreactivity to rat ACE (24). This antibody recognizes both rat and human ACE.

For bromo-deoxyuridine (BrdU) staining, BrdU was injected into transfected rats (100 mg/kg subcutaneous and 30 mg/kg intraperitoneal at 18 h before killing, and then 30 mg/kg intraperitoneal at 12 h before killing) (20). The rats were then killed at 3 d after transfection. The carotid arteries were removed after perfusion fixation with 4% paraformaldehyde under physiological pressure (110 mmHg) and processed for immunohistochemistry in a standard manner using anti-BrdU antibodies (Amersham Corp., Arlington Heights, IL). The positively stained cells were counted under light microscopy. Total cell numbers were counted similarly after light staining with hematoxylin (20). The total number of hematoxylin-stained nuclei in the medial layer of each section was summed and expressed as cell numbers per section. BrdU-positive nuclei were reported as a percentage of total hematoxylin-stained nuclei. For assessment of the effect of Ang II receptor antagonist, ACE vector–transfected and contralateral vessels from the same animal were examined to diminish the systemic effects such as humoral and hemodynamics. At least three individual sections were evaluated per vessel. The contralateral artery in ACE vector–transfected animals was used as untreated sham control in all studies.

For morphological analyses, rat carotid arteries were removed at 2 wk after transfection, after perfusion fixation with 4% paraformaldehyde under physiological pressure (110 mmHg). Medial and lumen area were measured on a digitizing tablet (model 2200; South Micro Instruments, Atlanta, GA) after staining with hematoxylin. The medial area was readily demarcated as the vessel area between the internal and external elastic laminae. At least three individual sections from the middle of the transfected arterial segments were analyzed. Animals were coded so that the analysis was performed without the knowledge of which treatment each individual animal received. Cell counting analysis was performed using a computerized morphometry system (NEXUS 6400; Kashiwagi Research Co., Tokyo, Japan) by individuals unaware of the treatment each animal received, as described previously (28). The reproducibility of the results was assessed. Intraobserver variability was determined from triplicate measurements performed by one observer for all sections. The mean variation between 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 two 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. If the internal elastic lamina or media showed any evidence of crenation or compression, the section was excluded from analysis.

Statistical analysis. All values are expressed as mean±SEM. Analysis of variance with Dunnett's test was used to determine the significance



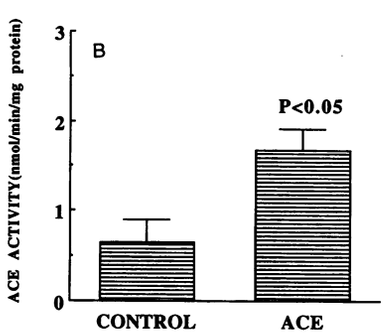


Figure 1. (*A*) Representative immunohistochemical staining of ACE in ACE- and control vector–transfected vessels 3 d after transfection. (*B*) Vascular ACE activity in carotid arteries dissected free of periadventitial tissues 3 d after transfection. *CONTROL,* control vector–transfected arteries (n = 5); *ACE,* ACE vector–transfected arteries (n = 10).

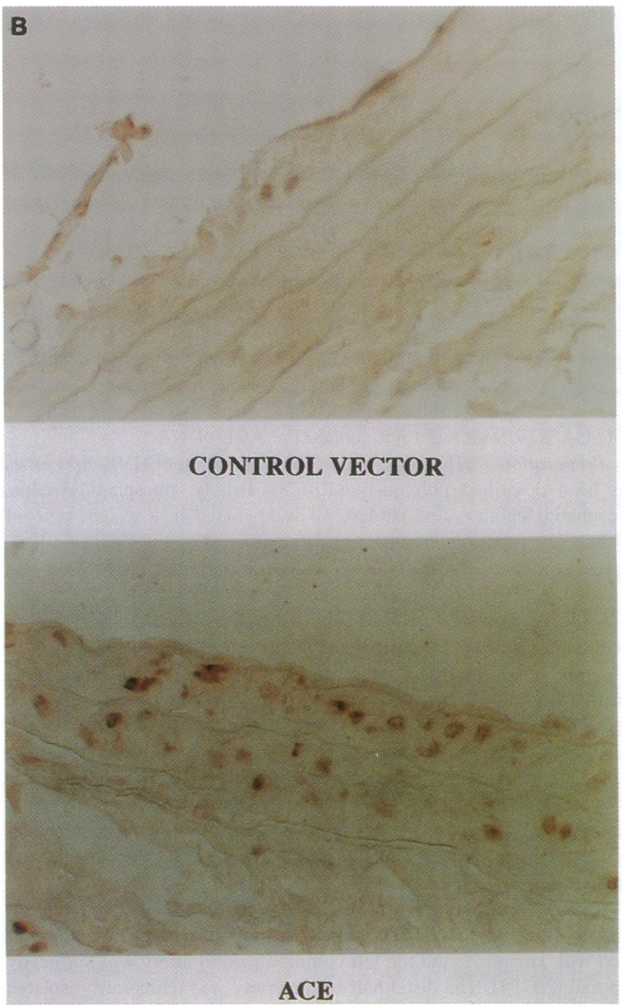


Figure 2. (*A, opposite page*) The effect of Ang II receptor antagonist on BrdU index in ACE- or control vector–transfected vessels at 3 d after transfection. *$P < 0.05$, **$P < 0.01$ vs control. *CONTROL (VEHICLE),* vehicle-treated control vector–transfected arteries (n = 6); *ACE (VEHICLE),* vehicle-treated ACE vector–transfected arteries (n = 5); *CONTROL (DuP 753),* DuP 753–treated control vector–transfected arteries (n = 6); *ACE (DuP 753),* DuP 753–treated ACE vector–transfected arteries (n = 6). (*B, above*) Representative immunohistochemical staining of BrdU in ACE- and control vector–transfected vessels. (*C, opposite page*) The effect of Ang II receptor antagonist on DNA content in ACE- or control vector–transfected vessels at 3 d after transfection. *$P < 0.05$ vs control. *CONTROL (VEHICLE),* vehicle-treated control vector–transfected arteries (n = 6); *ACE (VEHICLE),* vehicle-treated ACE vector–transfected arteries (n = 5); *CONTROL (DuP 753),* DuP 753–treated control vector–transfected arteries (n = 6); *ACE (DuP 753),* DuP 753–treated ACE vector–transfected arteries (n = 6).

of differences in multiple comparisons. $P < 0.05$ was considered to be statistically significant.

Results

Histological and biological analyses at 3 d after transfection. To evaluate the success of gene transfer and to localize the transgene product, we performed immunohistochemical analysis of ACE 3 d after transfection. Cellular staining for ACE was observed diffusely within the medial and endothelial layers of ACE vector–transfected vessels, while in the control vector–transfected vessels and intact untransfected vessels the positive staining was limited to the endothelial layer (Fig. 1 *A*). No staining was observed in control sections treated with nonimmune serum. The biological activity of the transgene product was documented by a threefold increase in ACE activity within

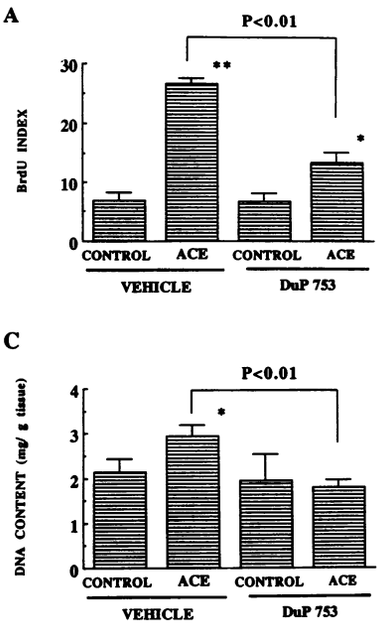


Figure 2 (Continued)

ACE-transfected arteries (Fig. 1 *B*). The increase in vascular ACE activity was associated with local growth stimulation as indicated by increases in DNA synthesis (BrdU index: stained cells/total cells in the medial layer) and vascular DNA content in the ACE vector–transfected vessels as compared with control vector–transfected vessels (Fig. 2, *A–C*). A typical example of immunohistochemistry for BrdU is shown in Fig. 2 *A*. Positive staining for BrdU colocalized with ACE staining in the medial and adventitial cells of ACE-transfected carotid arteries. This growth stimulatory effect was primarily Ang II–mediated as documented by inhibition of the growth response by the specific Ang II antagonist, DuP 753. Ang II receptor blockade had no effect on BrdU labeling or DNA content in control vessels (Fig. 2, *A–C*).

We also observed an increase in BrdU index in control vector–transfected vessel compared with the unmanipulated contralateral vessel. This appears to reflect minor injury induced by the transfection and/or operation procedures. This effect appears to be transient and minor insofar as the endothelium remains intact as defined histologically (Fig. 1 *A*), and there was no sustained effect on vessel structure as determined by DNA content (control vector–transfected arteries: 1.9±0.6 mg/g tissue versus intact arteries: 2.6±0.5 mg/g tissue) and vessel morphometry as compared with the contralateral vessel. Overall, these findings suggest that the local overexpression of ACE at pathophysiological levels results in increased DNA synthesis via the local production and action of vascular Ang II. Importantly, these vascular changes are independent of the circulating renin angiotensin system or hemodynamic changes such as blood pressure, heart rate, and serum ACE activity (Table I).

Histological and biological analyses at 2 wk after transfection. Given that overexpression of ACE in blood vessels increases DNA synthesis at 3 d after transfection, we hypothesized that increased local production of ACE would modulate vascular structure if the growth stimulatory effect is sustained. Evidence of local vascular hypertrophy after ACE gene transfer

Table I. The Effect of Local Carotid ACE Gene Transfer on Systemic Blood Pressure, Heart Rate, and Serum ACE Activity

	Control vector*	ACE‡
Blood pressure (*mmHg*)	126±2	129±2
Heart rate (*beats/min*)	403±6	400±5
Serum ACE activity (*nmol/min/ml*)	13.1±0.7	14.6±1.6

* Animals transfected with control vector. ‡ Animals transfected with ACE expression vector. Each group contains seven animals.

persisted for at least 2 wk after transfection as reflected by increased vascular DNA content (Fig. 3, *A–D*) and increased vascular protein content (ACE vector–transfected arteries: 30.2±2.1 mg protein/g tissue versus contralateral arteries: 19.4±4.0 mg protein/g tissue, $P < 0.01$) in ACE vector–transfected arteries compared with controls. Parallel to these biochemical changes, morphometric analysis documented that the medial thickness of the ACE-transfected vessels was significantly increased as compared with control vector–transfected and contralateral control vessels (Fig. 3, *B* and *C*). Interestingly, the luminal diameters of ACE-transfected, control vector–transfected, and contralateral vessels did not differ (Fig. 3 *B*). Consequently, the wall to lumen ratio in ACE-transfected vessels was significantly greater than that of control vector–transfected vessels (Fig. 3 *C*). However, there were no discernible differences in cell numbers counted in serial sections of the medial layer between ACE-transfected and control vector–transfected vessels (control vector: 889±58, ACE: 803±64 cells/medial section area: $P > 0.05$). The morphometric changes of vascular hypertrophy induced by ACE overexpression were also abolished by Ang II receptor blockade (DuP 753) (Fig. 3 *D*). These data demonstrate that overexpression of vascular ACE results in increased vascular DNA synthesis, protein content, and medial wall hypertrophy via local autocrine/paracrine Ang II production.

Discussion

This study addresses two important questions in hypertension and vascular research. First, can the renin angiotensin system directly mediate vascular hypertrophy independent of its blood pressure effect? Second, is local tissue ACE important in regulating local vascular function and in contributing to pathophysiology? To date, 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 (1–15). However, none of these data are completely convincing due to the contribution of confounding variables such as hemodynamic effects, cell culture conditions, etc. To circumvent the limitation of the above experimental approaches, we used in this study in vivo gene transfer technology which has the following advantages: (*a*) the target gene can be transfected into a local segment of a blood vessel, thereby avoiding a systemic effect; (*b*) this transfected vascular segment can be compared with adjacent untransfected segments or to the contralateral control blood vessel which is subject to the same hemodynamics and circulating humoral factors; and (*c*) the consequences of local overexpression within the physiological/pathophysiological range of the target gene may be studied.

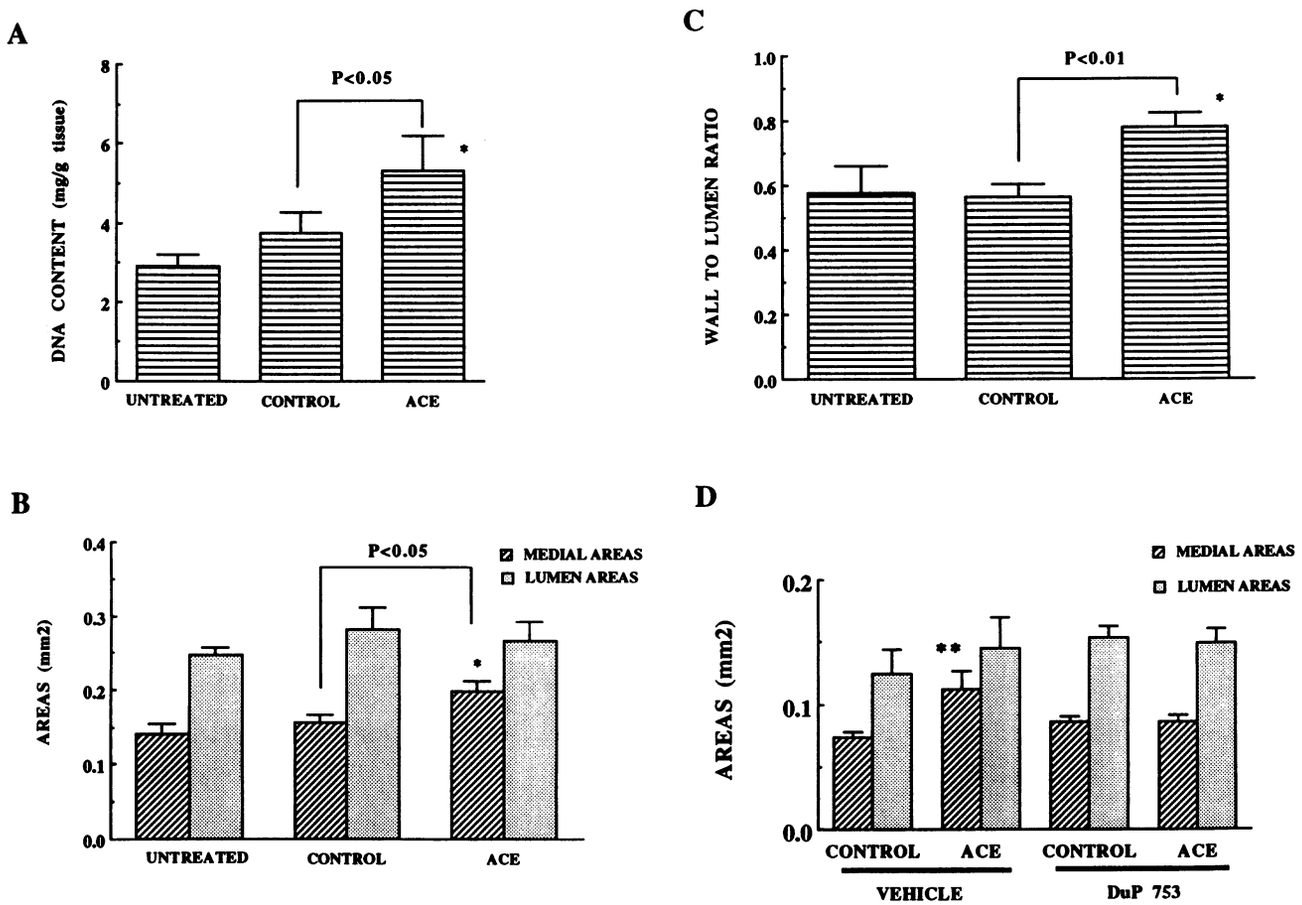


Figure 3. (*A*) The effect of ACE vector transfection on DNA content at 2 wk after transfection. *UNTREATED,* sham operated vessels (n = 5); *CONTROL,* control vector–transfected arteries (n = 5); *ACE,* ACE vector–transfected arteries (n = 10). *$P < 0.05$ vs untreated. Each group contains 5–10 animals. (*B*) The effect of ACE vector transfection on the medial and luminal areas at 2 wk after transfection. *$P < 0.05$ vs untreated. (*C*) The effect of ACE vector transfection on the wall to lumen ratio at 2 wk after transfection. *UNTREATED,* sham operated vessel (n = 5); *CONTROL,* control vector–transfected arteries (n = 5); *ACE,* ACE vector–transfected arteries (n = 10). *$P < 0.05$ vs untreated. (*D*) Effect of administration of Ang II receptor antagonist on medial and luminal areas in ACE vector–transfected and contralateral vessels at 2 wk after transfection. *CONTROL (VEHICLE),* vehicle-treated control vector–transfected arteries (n = 6); *ACE (VEHICLE),* vehicle-treated ACE vector–transfected arteries (n = 6); *CONTROL (DuP 753),* DuP 753–treated control vector–transfected arteries (n = 6); *ACE (DuP 753),* DuP 753–treated ACE vector–transfected arteries (n = 6). **$P < 0.01$ vs control. To assess the effect of Ang II receptor antagonist, ACE vector–transfected and contralateral vessels from the same animal were examined to minimize the influence of systemic effects on humoral factors or hemodynamics.

In this study, we were able to study the long-term vascular effects of increased tissue ACE activity.

Vascular hypertrophy is thought to be an adaptive response to hypertension. Although it is well accepted that the mechanical effects of increased blood pressure stimulate vascular hypertrophy, recent indirect evidence suggests that humoral factors may also play an important role. Ang II can stimulate vascular smooth muscle cell growth in vitro and influence extracellular matrix (7–13). In vivo data from experimental animals have provided additional support for a direct Ang II contribution (4–6, 14, 15). A key enzyme in the renin angiotensin biochemical cascade is ACE which is expressed diffusely in blood vessels. 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 vascular or cardiac ACE activity in experimental animals result in parallel increases in tissue Ang I conversion to Ang II and changes in local function (1–3, 28). Indeed, we observed that pressure overload cardiac hypertrophy induced by aortic banding induced cardiac ACE gene expression and increased cardiac Ang II production (29). Vascular ACE expression can be activated by experimental hypertension (1, 2), in response to vascular injury (24), and during blood vessel growth and development (30). These changes in vascular ACE expression are associated with structural changes of the blood vessel which can be blocked by ACE inhibition (4–6, 31–33). Indeed, our recent work using HVJ–liposome—mediated transfer of ACE vector into vascular smooth muscle cells in vitro demonstrated that ACE per se modulates vascular growth via the autocrine/paracrine production of Ang II (21). These data would suggest that local tissue ACE is rate limiting in regulating local Ang II production, which may have physiological consequences.

In this study, we have extended our investigation to ACE gene transfer in vivo. HVJ–liposome—mediated gene transfer is an efficient in vivo method that has minimal or no toxicity and provides sustained gene expression (20–23). This local gene transfer approach provides the advantage that the local transfected carotid segment can be compared with adjacent non-

transfected segments as well as the contralateral carotid artery. Furthermore, the transfected segment is subject to the same blood pressure and circulating factors as control vessels. Although transgenic technology also provides the opportunity to study specific gene function, this technology has several disadvantages: (*a*) it is time consuming and costly; (*b*) the effect of the overexpressed transgene is exerted throughout development; (*c*) it is impossible to target the transgenic expression to only a local segment of a blood vessel; and (*d*) it is difficult to exclude the potential contribution of the systemic effect of transgene expression. Thus, local gene transfer approach may be more effective for studying the role of autocrine/paracrine mediators.

Previous studies have documented the roles of hemodynamic stimuli and systemic neurohumoral factors in vascular hypertrophy in hypertension (34–36). The present study documents for the first time that the hypertensive vascular structural response (cellular hypertrophy with endoreplication or polyploidy) can be mediated by locally generated factors within the vessel wall. Although we cannot exclude the possible contribution of bradykinin degradation related to ACE overexpression, the marked inhibition of the vascular hypertrophy by DuP 753 would suggest that Ang II is at least a primary mechanism. Our study also supports the notion that ACE is a key rate-limiting enzyme governing Ang II production in the vasculature. The increased local expression of ACE is sufficient to induce Ang II–mediated vascular hypertrophy independent of changes in the circulating renin angiotensin system. It is intriguing that we observed a growth stimulatory effect of Ang II within the intact vessel wall in vivo that was strikingly similar to responses previously observed in vitro as well as in response to Ang II infusion in vivo (8, 9, 14, 15, 37). In both cases, Ang II appears to stimulate DNA synthesis and vascular cell growth by hypertrophy without discernible changes in cell number. The increases in DNA synthesis and DNA content without demonstrable increases in cell number are suggestive of Ang II–induced polyploidy as observed previously (8, 9, 14, 15, 37).

This observation is consistent with previous morphometric analyses of hypertensive vessels documenting hypertrophy (increased vascular protein content) and polyploidy, but not hyperplasia (3–6, 35–40). Why increased DNA synthesis did not lead to hyperplasia might be explained by the bifunctional growth action of Ang II and the role of the endothelium. In vitro studies of vascular smooth muscle cells indicate that Ang II induces the expression of proliferative factors such as fibroblast growth factor and PDGF and also antiproliferative factors such as TGF-beta (7, 11, 12). Therefore, smooth muscle–derived TGF-beta induced by increased Ang II within vascular wall may promote vascular hypertrophy and polyploidy, but not hyperplasia. Future studies are needed to further define the role of the autocrine/paracrine growth factors as mediators of the response to vascular angiotensin in vivo.

How can our present findings that increased vascular ACE induces vascular hypertrophy be reconciled with the data suggesting that vascular angiotensin may promote neointimal lesion development after vascular injury (24, 31, 33)? We speculate that the presence of endothelial cells plays an important role here. We have hypothesized that the endothelial cells regulate angiotensin's action of vascular growth via the constitutive expression of growth inhibitory substances such as nitric oxide, prostacyclin, and TGF-beta 1 (41). We postulate that increased vascular ACE expression in the presence of an intact endothelium results in vascular hypertrophy, whereas increased vascular ACE promotes neointimal formation in the absence of the endothelium.

Overall, the present findings demonstrate a direct role for the vascular angiotensin system as a mediator of the vascular hypertrophy process in hypertension, independent of changes in blood pressure or the endocrine renin angiotensin system. Moreover, this study of the vascular angiotensin system serves as a paradigm for the elucidation of the role of other autocrine/paracrine mediators of vascular remodeling in the pathogenesis of disease, such as atherosclerosis graft occlusion and restenosis after angioplasty. Our data demonstrate that localized in vivo gene transfer technique is a useful experimental tool for the study of autocrine/paracrine factors in complex pathophysiologic 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. In addition, it may foster the development of new therapeutic approaches.

Acknowledgments

We wish to thank Dr. Naruya Tomita and Dr. Jitsuo Higaki for their technical advice, and Melinda Hing for preparing this manuscript. The human ACE cDNA was a kind gift from Dr. F. Soubrier (INSERM, Paris, France) (42).

This work was supported by National Institutes of Health grants HL-35610, HL-35252, and HL-042663 and the American Heart Association Bugher Foundation Center for Molecular Biology. Dr. Morishita is the recipient of a fellowship from the American Heart Association. Dr. Gary H. Gibbons is a recipient of a Robert Wood Johnson Foundation Minority Faculty Development Award.

References

1. Okamura, T., M. Miyazaki, T. Inagami, and N. Toda. 1986. Vascular renin angiotensin system in two-kidney, one clip hypertensive rats. *Hypertension (Dallas).* 8:560–565.

2. Shiota, N., M. Miyazaki, and H. Okunishi. 1992. Increase of angiotensin converting enzyme gene expression in the hypertensive aorta. *Hypertension (Dallas).* 20:168–174.

3. Morishita, R., J. Higaki, M. Miyazaki, and T. Ogihara. 1992. Possible role of the vascular renin angiotensin system in hypertension and vascular hypertrophy. *Hypertension (Dallas).* 19:II-62–II-67.

4. Wang, D.-H., and R. L. Prewitt. 1990. Captopril reduces aortic and microvascular growth in hypertensive and normotensive rats. *Hypertension (Dallas).* 15:68–77.

5. Lee, R. M., K. H. Berecek, J. Tsoporis, R. McKenzie, and C. R. Triggle. 1991. Prevention of hypertension and vascular changes by captopril treatment. *Hypertension (Dallas).* 17:141–150.

6. Owens, G. K. 1987. Influence of blood pressure on development of aortic medial smooth muscle hypertrophy in spontaneously hypertensive rats. *Hypertension (Dallas).* 9:178–187.

7. Naftilan, A. J., R. E. Pratt, and V. J. Dzau. 1989. Induction of platelet-derived growth factor A-chain and c-*myc* gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J. Clin. Invest.* 83:1419–1424.

8. Geisterfer, A. A. T., M. J. Peach, and G. K. Owens. 1988. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ. Res.* 62:749–756.

9. Berk, B. C., V. Vekshtein, H. M. Gordon, and T. Tsuda. 1989. Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension (Dallas).* 13:305–314.

10. Kato, H., H. Suzuki, S. Tajima, Y. Ogata, T. Tominaga, A. Sato, and T. Saruta. 1991. Angiotensin II stimulates collagen synthesis in cultured vascular smooth muscle cells. *J. Hypertens.* 9:17–22.

11. Gibbons, G. H., R. E. Pratt, and V. J. Dzau. 1992. Vascular smooth muscle cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor β_1 expression determines growth response to angiotensin II. *J. Clin. Invest.* 90:456–461.

12. Owens, G. K., A. A. T. Geisterfer, Y. W.-H. Yang, and A. Komoriya. 1988. Transforming growth factor-β–induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. *J. Cell Biol.* 107:771–780.

13. Scott-Burden, T., A. W. Hahn, T. J. Resink, and F. R. Buhler. 1990. Modulation of extracellular matrix by angiotensin II stimulated glycoconjugate synthesis and growth in vascular smooth muscle cells. *J. Cardiovasc. Pharmacol.* 16(Suppl. 4):S36–41.

14. Griffin, S. A., W. C. B. Brown, F. MacPherson, J. C. McGrath, V. G. Wilson, N. Korsgaard, M. J. Mulvany, and A. F. Lever. 1991. Angiotensin II causes vascular hypertrophy in part by a non-pressor mechanism. *Hypertension (Dallas).* 17:626–635.

15. Lever, A. F. 1992. Angiotensin II, angiotensin-converting enzyme inhibitors, and blood vessel structure. *Am. J. Med.* 92:35S–38S.

16. Schelling, P., H. Fischer, and D. Ganten. 1991. Angiotensin and cell growth: a link to cardiovascular hypertrophy? *J. Hypertens.* 9:3–15.

17. Campbell, D. J. 1987. Circulating and tissue angiotensin systems. *J. Clin. Invest.* 79:1–6.

18. Paul, M., and D. Ganten. 1992. The molecular basis of cardiovascular hypertrophy: the role of the renin-angiotensin system. *J. Cardiovasc. Pharmacol.* 19(Suppl. 5):S51–58.

19. Dzau, V. J., and R. E. Pratt. 1993. Molecular and cellular biology of angiotensin-mediated growth of the cardiovascular system. *In* Cellular and Molecular Biology of the Renin Angiotensin System. M. K. Raizaida, M. I. Phillips, and C. Sumners, editors. CRC Press, Inc., Boca Raton, FL. 471–484.

20. Morishita, R., G. H. Gibbons, K. E. Ellison, M. Nakajima, L. Zhang, Y. Kaneda, T. Ogihara, and V. J. Dzau. 1993. Single intraluminal delivery of antisense cdc 2 kinase and PCNA oligonucleotides results in chronic inhibition of neointimal hyperplasia. *Proc. Natl. Acad. Sci. USA.* 90:8474–8479.

21. Morishita, R., G. H. Gibbons, Y. Kaneda, T. Ogihara, and V. J. Dzau. 1993. Novel and effective gene transfer technique for study of vascular renin angiotensin system. *J. Clin. Invest.* 91:2580–2585.

22. Kaneda, Y., K. Iwai, and T. Uchida. 1989. Introduction and expression of the human insulin gene in adult rat liver. *J. Biol. Chem.* 264:12126–12129.

23. Kaneda, Y., K. Iwai, and T. Uchida. 1989. Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science (Wash. DC).* 243:375–378.

24. Rakugi, H., D.-K. Kim, J. E. Krieger, D. S. Wang, V. J. Dzau, and R. E. Pratt. 1994. Induction of angiotensin converting enzyme in the neointima after vascular injury. Possible role in restenosis. *J. Clin. Invest.* 93:339–346.

25. Kauffman, R. F., J. S. Bean, K. M. Zimmerman, R. F. Brown, and M. I. Steinberg. 1991. Losartan, a nonpeptide angiotensin II (Ang II) receptor antagonist, inhibits neointima formation following balloon injury to rat carotid arteries. *Life Sci.* 49:PL223–228.

26. Soltis, E. E., A. L. Jewell, L. P. Dwonskin, and L. A. Cassis. 1993. Acute and chronic effects of losartan (DuP 753) on blood pressure and vascular reactivity in normotensive rats. *Clin. Exp. Hypertens.* 15:171–184.

27. Cushman, D. W., and H. S. Cheung. 1970. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* 20:11637–11648.

28. Yu, H., H. Rakugi, J. Higaki, R. Morishita, H. Mikami, and T. Ogihara. 1993. The role of activated vascular angiotensin II generation in vascular hypertrophy in one-kidney, one clip hypertensive rats. *J. Hypertens.* 12:1347–1356.

29. Schunkert, H., V. J. Dzau, S. S. Tang, A. T. Hirsh, C. S. Apstein, and B. H. Lorell. 1990. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J. Clin. Invest.* 86:1913–1920.

30. Pratt, R. E., V. J. Dzau, W. S. Lee, and D. K. Kim. 1993. Developmental regulation of angiotensin converting enzyme in the rat aorta. *Circulation.* 88:I-325*a* (Abstr.).

31. Powell, J. S., J. P. Clozel, R. K. M. Muller, H. Kuhn, F. Hefti, M. Hosang, and H. R. Baumgartner. 1989. Inhibitors of angiotensin converting enzyme prevent myointimal proliferation after vascular injury. *Science (Wash. DC).* 245:186–188.

32. Levy, B. I., J. B. Michel, J. L. Salzmann, M. Azizi, P. O. Poiiteven, M. Safar, and J. P. Camilleri. 1988. Effects of chronic inhibition on converting enzyme on mechanical and structural properties of arteries in rat renovascular hypertension. *Circ. Res.* 63:227–239.

33. Dzau, V. J., G. H. Gibbons, and R. E. Pratt. 1991. Molecular mechanisms of vascular renin-angiotensin system in myointimal hyperplasia. *Hypertension (Dallas).* 18(Suppl. 4):II100–105.

34. Dzau, V. J. 1993. The role of mechanical and humoral factors in growth regulation of vascular smooth muscle and cardiac myocytes. *Curr. Opin. Nephrol. Hypertens.* 2:27–32.

35. Mulvany, M. J. 1991. Are vascular abnormalities a primary cause or secondary consequence of hypertension? *Hypertension (Dallas).* 18:I-52–57.

36. Silvertsson, R. 1970. The hemodynamic importance of structural vascular changes in essential hypertension. *Acta Physiol. Scand.* 343:I-56.

37. Kleef, E. M. V., J. F. M. Smits, J. G. R. DeMey, J. P. M. Cleutjens, D. M. Lombardi, S. M. Schwartz, and M. J. A. P. Daemen. 1992. Alpha 1-adrenoreceptor blockade reduces the angiotensin II-induced vascular smooth muscle cell DNA synthesis in the rat thoracic aorta and carotid artery. *Circ. Res.* 70:1122–1127.

38. Owens, G. K. 1985. Differential effects of antihypertensive drug therapy on vascular smooth muscle cell hypertrophy, hyperploidy, and hyperplasia in the spontaneously hypertensive rat. *Circ. Res.* 56:525–536.

39. Korsgaared, N., and M. J. Mulvany. 1988. Cellular hypertrophy in mesenteric resistance vessels from renal hypertensive rats. *Hypertension (Dallas).* 12:162–167.

40. Owens, G. K., and S. M. Schwartz. 1983. Alterations in vascular smooth muscle mass in the spontaneously hypertensive rat. *Circ. Res.* 51:280–289.

41. Dzau, V. J., and G. H. Gibbons. 1991. Endothelium and growth factors in vascular remodeling of hypertension. *Hypertension (Dallas).* 18:III-115–121.

42. Soubrier, F., F. Alhenc-Gelas, C. Hubert, J. Allegrini, M. John, G. Tregear, and P. Corvol. 1988. Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc. Natl. Acad. Sci. USA.* 85:9386–9390.