Angiotensin-Converting Enzyme Expression in Human Carotid Artery Atherosclerosis

Masayo Fukuhara, Randolph L. Geary, Debra I. Diz, Patricia E. Gallagher, John A. Wilson, Steven S. Glazier, Richard H Dean, Carlos M. Ferrario

Abstract—Angiotensin-converting enzyme (ACE) inhibitors reduce the progression of atherosclerosis in animal models and reinfarction rates after myocardial infarction in humans. Although expression of components of the renin-angiotensin system has been reported in human coronary arteries, no data regarding their presence in carotid arteries, a frequent site for the occurrence of atherosclerosis plaques, are available. The following study sought to determine whether ACE mRNA and protein can be detected in human carotid atheromatous lesions. Twenty-four intact endarterectomy specimens were obtained from patients with severe carotid occlusive disease (17 males and 7 females, aged 68 ± 1 years) and fixed within 30 minutes. Carotid artery specimens contained advanced Stary type V and VI lesions, and human ACE mRNA expression and protein were localized in cross sections by the combination of in situ hybridization and immunohistochemistry. Cell type–specific antibodies were used to colocalize ACE to smooth muscle cells, endothelial cells, macrophages, or lymphocytes. ACE protein was localized in the intima, whereas the overlying media was largely free of ACE staining. In less complicated lesions, ACE staining was modest and could be visualized in scattered clusters of macrophages and on the luminal side of carotid artery vascular endothelium. Smooth muscle cells were largely negative. ACE staining increased as lesions became more complex and was most prominent in macrophage-rich regions. The shoulder regions of plaques contained numerous ACE-positive macrophage foam cells and lymphocytes. In these areas, microvessels were positive for endothelial cell and smooth muscle cell ACE expression. However, microvessels in plaques free of inflammatory cells were stained only faintly for ACE expression. Labeling for ACE mRNA mirrored the pattern of protein expression, localizing ACE mRNA to macrophages and microvessels within the intima. In conclusion, atherosclerosis alters carotid artery ACE production, increasing transcription and translation within regions of plaque inflammation. These data provide another important mechanism by which inflammation associated with increased ACE expression may contribute to the progression of atherosclerosis.

Key Words: carotid arteries ■ angiotensin-converting enzyme ■ macrophages ■ immunohistochemistry ■ hybridization

Angiotensin II (Ang II) has been implicated in the pathobiology of atherosclerosis and the arterial response to injury and restenosis via mechanisms that include vascular hypertrophy, extracellular matrix production, and induction of cytokines. In animal models of atherosclerosis, angiotensin-converting enzyme (ACE) inhibition reduces lesion progression, whereas blockade of the Ang II type 1 receptor (AT1) inhibits monocyte activation, and LDL oxidation, and fatty streak formation.

It has been reported that both ACE and immunoreactive Ang II may be found within the walls of atherosclerotic coronary arteries. In the present study, we extend these observations to the human carotid artery by using immunohistochemistry and in situ hybridization to localize ACE expression within atherosclerotic plaques of varying complexity.

Methods

Twenty-four atherosclerotic plaques were obtained from patients undergoing carotid endarterectomy for severe stenosis. Tissues were processed within 30 minutes after removal of the specimen, cut into 3 rings, and either fixed (formalin or methyl Carnoy’s fixative) or frozen in OCT within 30 minutes of removal. Fixed rings were then paraffin-embedded and sectioned at 5 μm for immunohistochemistry and in situ hybridization. Tissue collection and analysis were approved by the Human Subjects Committee of Wake Forest University School of Medicine, and procedures conformed to institutional and federal guidelines for research involving human tissue. Informed consent was not used as specimens were removed as a routine component of the surgical procedure, which was not altered in

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**Patient Demographics**

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tbody>
<tr>
<td>Sex, male/female</td>
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<tr>
<td>Age, y</td>
<td>68±1</td>
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<tr>
<td>Height, cm</td>
<td>169±2</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76.7±2.5</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.9±0.7</td>
</tr>
<tr>
<td>Smoker Current</td>
<td>15 (62.5%)</td>
</tr>
<tr>
<td>Past</td>
<td>3 (12.5%)</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>21 (67.5%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>8 (33.3%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>8 (33.3%)</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>15 (62.5%)</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>13 (54.2%)</td>
</tr>
<tr>
<td>Medications ACE inhibitor</td>
<td>4 (16.7%)</td>
</tr>
<tr>
<td>β-blocker</td>
<td>4 (16.7%)</td>
</tr>
<tr>
<td>ACE inhibitor + β-blocker</td>
<td>1 (4.2%)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM or number (percentage) of patients.

*Systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg or treatment with antihypertensive medication.

**Histology and Immunohistochemistry**

Formalin-fixed and methyl Carnoy–fixed specimens were stained with hematoxylin-eosin. Adjacent sections were deparaffinized in xylene and rehydrated in graded alcohols. Sections were incubated with anti-human ACE primary antibodies (clones 3A5 and 9B9, Chemicon International Inc) to detect the ACE protein. Antibodies specific for smooth muscle cells (α-actin, Boehringer-Mannheim), endothelial cells (von Willebrand factor, Dako), macrophages (CD68, and HAM56, Dako), and T lymphocytes (CD45RO, Becton Dickenson) were applied to either the same or adjacent sections by using a double-labeling strategy to identify cell types. Sections were incubated with primary antibodies overnight at 4°C and then rinsed and incubated with appropriate biotinylated secondary antibodies at 4°C for 3 hours, followed by tertiary avidin-biotin complex staining (Vectors Laboratory). Sections were then counterstained with hematoxylin and examined by light microscopy.

**In Situ Hybridization**

Formalin-fixed and paraffin-embedded specimens were sectioned, and in situ hybridization was performed by use of riboprobes, as described previously.13 For the ACE riboprobe, total RNA was isolated from cultured human neuroblastoma SK-N-SH cells by use of TRIZOL reagent (GIBCO-BRL Products). The RNA concentration was quantified by UV spectroscopy, and any degradation was assessed by ethidium bromide staining intensity of 28S and 18S ribosomal RNA after agarose gel electrophoresis. The RNA was incubated with BQ1 DNase (Promega Corp) to eliminate any residual DNA. Approximately 1 μg of total RNA was incubated with or without AMV reverse transcriptase (Promega Corp) in a mixture containing deoxynucleotides, random hexamers, and RNase inhibitor in reverse transcriptase buffer. Heating the mixture for 5 minutes at 95°C terminated the reaction.

For amplification of the resulting cDNA, 1 μmol/L gene-specific primers, 0.2 mmol/L deoxynucleotides, 1.5 mmol/L MgCl₂, and 1.5 IU Taq polymerase were added to 3 μL of the RNA sample in a final volume of 50 μL. Primer sequences, based on the sequence of the cloned human ACE gene,14 were as follows: 5'-(CACCTTTCAT-CAGTTCC-3′ (ACE forward primer) and 5'-(CCAGGAAGAG-CAGCAGCCAC-3′ (ACE reverse primer), yielding an amplification product of 407 bp. Amplification conditions were as follows: denaturation at 94°C for 60 seconds, annealing at 62°C for 60 seconds, and elongation at 72°C for 60 seconds for 35 cycles, with a final elongation step at 72°C for 7 minutes. After polymerase chain reaction, the amplification product was separated on a 2.5% agarose gel containing ethidium bromide and was visualized by UV light. The band was cut from the gel and eluted into sterile 10 mmol/L Tris-HCl, pH 8.0, containing 1 mmol/L EDTA, and the concentration was determined by UV absorbance at 260 nm.

Approximately 200 ng of the ACE cDNA fragment was incubated with 50 ng pGEM-T vector (Promega Corp) and T4 DNA ligase at 4°C overnight. A 2 μL aliquot of the ligation mixture was transfected into JM109 competent cells (Promega Corp). Bacteria-containing plasmids with an insert were detected as white colonies on antibiotic culture plates containing isopropyl β-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Plasmids containing the ACE insert were isolated from 500 mL cultures by using the Wizard Plus Maxiprep DNA purification system (Promega Corp). The orientation and DNA sequence of the insert were verified by the DNA Sequencing Facility at Wake Forest University School of Medicine.

Anti-sense and sense riboprobes were transcribed from the linearized cDNA by use of T7 and T3 polymerases (Promega Corp) and labeled with [35S]UTP. Slides were deparaffinized in xylene, rehydrated, and sequentially incubated with proteinase K for 15 minutes at 37°C (50 μg/mL) and hybridization buffer (0.3 mol/L NaCl, 20 mmol/L Tris [pH 8.0], 5 mmol/L EDTA, 1× Denhardt’s solution, 10% dextran sulfate, 10 mmol/L dithiothreitol, and 50% formamide) for 2 hours at 48°C. Riboprobes were then applied (300 000 cpm/μL) overnight in hybridization buffer at 55°C. Slides were washed and coated with autoradiographic emulsion (NTB2, Kodak), exposed for 10 and 17 days, and developed with D-19 (Kodak). Slides were counterstained with hematoxylin and examined under dark-field microscopy. Specificity of ACE mRNA signals was verified by using positive and negative control tissues, comparing sense- and antisense-labeled sections, and comparing an irrelevant antisense riboprobe for human α1-procollagen.

**Results**

The Table shows the clinical characteristics of patients from whom specimens were taken during the surgical procedure. As expected, carotid endarterectomies were performed in hypertensive males with a positive history for cigarette smoking. Ischemic heart disease was present in 54% of the subjects. At the time of the procedure, 9 of the 24 subjects were medicated with ACE inhibitors or β-blockers alone or in combination.

Endarterectomy specimens were characterized histologically according to the classification proposed by the Nomenclature Committee of the American Heart Association (Stary et al15). Analyzed sections consisted of intimal plaque and a majority of the tunica media. The plane of dissection did not extend through the external elastic lamina; thus, the adventitia was excluded. All 24 carotid artery segments contained advanced atheromatous lesions classified as type V (n=15) or VI (n=9) lesions.15 Adjacent segments showed less complicated features (Stary type III or IV lesions). Specimens with type V lesions showed a necrotic lipid core and an intact fibrous connective tissue cap. Type VI lesions were complicated by surface defects, cap rupture, hematoma/hemorrhage, or thrombus. Type III and IV lesions found in adjacent areas were uncomplicated fibrous plaques with nests of foam cells and early extracellular lipid accumulations, respectively.
Staining for ACE protein was localized to the intima of all 24 specimens and was only occasionally visualized in the media. In uncomplicated plaque segments (Stary types III and IV), luminal endothelial cells and scattered macrophages exhibited faint cytoplasmic staining for ACE protein, whereas α-actin–positive smooth muscle cells were consistently negative for ACE protein.

Figure 1 shows the staining of ACE protein at the level of the plaque shoulder. ACE staining was particularly prominent in macrophages surrounding the necrotic lipid core and within the shoulder regions of atherosclerotic caps. Figure 2 shows an image from the inner region of a carotid artery fibrous plaque overlying the necrotic lipid core. Strong cytoplasmic staining for ACE protein was found with regularity in cells that did not stain positively for α-actin (Figure 2A). Double immunolabeling with CD68 showed that the ACE protein was localized to macrophages (Figure 2B).

Figures 3 and 4 show the expression of ACE mRNA and protein on atheromatous plaques. In panel A of Figure 3, the lipid core is surrounded by an inflammatory reaction with significant cellularity in the shoulder regions of the plaque. In situ hybridization with an ACE riboprobe showed intense labeling within the cellular regions surrounding the lipid core. The intensity of the signal is greater in cells found in the shoulder region outside the lipid core. Scattered labeling of cells is present in the fibrous cap (Figure 3B). Consistently and in keeping with the results obtained with immunocytochemistry, ACE expression was localized in macrophages and T lymphocytes, as demonstrated by correlative staining with the macrophage-specific antibody HAM56 (Figure 3C), CD 68 (Figure 4C), and the T-lymphocyte–specific antibody CD45 (Figure 4D). In contrast, there was little expression of ACE in α-acting smooth muscle cells (Figure 3D).

A small number of ACE-positive cells were negative for leukocyte markers. These cells were primarily located within or adjacent to large collections of macrophage foam cells. By use of double-label immunohistochemistry, these cells were occasionally found to be actin-positive smooth muscle cells within the intima or associated with plaque microvessels (Figures 3 and 4).

Microvessels within plaques were stained positively for ACE protein and expressed the ACE mRNA (Figure 4). Examination under higher magnification illustrated that the ACE staining in endothelial cells of microvessels showed a
stronger signal compared with the staining in the endothelial layer of the carotid artery (Figure 4A and 4F). Although plaque vasa are common within complicated plaques, those staining for ACE were usually associated with surrounding inflammatory infiltrates, as shown by labeling with macrophage (CD68, Figure 4C) and T-lymphocytes (CD45, Figure 4D) antibodies in shoulder regions of plaque caps. Vasa outside the area of inflammatory response were stained only faintly for ACE.

**Discussion**

We show the expression of ACE within the carotid artery wall of human endarterectomy specimens in association with infiltrating macrophages and lymphocytes. Inflammatory cells account for the bulk of ACE expression, a finding implicating inflammation as a mechanism inducing ACE expression in adjacent smooth muscle cells and microvessels. The pattern of ACE protein by immunohistochemistry was consistent with ACE mRNA expression in both macrophage-rich regions and neighboring microvessels. These data, documenting for the first time an increased expression of ACE in plaques contained in human carotid arteries, provide further evidence for a local contribution of vasoactive peptides in the progression of human atherosclerosis. The findings establish a mechanism of commonality, in view of the fact that the pattern of distribution of ACE protein and expression is comparable to that reported in diseased human coronary arteries.\(^\text{16,17}\)

Previous immunohistochemistry studies visualized ACE immunoreactivity in human atherosclerotic coronary arteries.\(^\text{1,16,17}\) However, reverse transcription–polymerase chain reaction and Northern blot methods used on tissue from whole vessels did not allow a precise localization of the ACE mRNA. The present study is the first report to demonstrate the expression of ACE mRNA by in situ hybridization in areas of complicated atherosclerotic plaques. Using this technique, we identified that the cells expressing mRNA for ACE appear to be localized in macrophages and T lymphocytes, as verified by selective staining.

Extensive neovascularization was observed in the “shoulder” regions of advanced human carotid plaques. The shoulder of the plaque is known to contain higher concentrations of macrophages, mast cells, and T cells.\(^\text{18}\) In addition, it contains the areas of weakness associated with plaque disruption and...
The observation that microvessels within the shoulder region and even the fibrous cap showed greater staining for ACE than did carotid luminal endothelial cells or microvessels remote to clusters of leukocytes is important and new. Microvessels of normal artery were seen only in the adventitia and outer media. Neovascularization of the intima is an important feature in the development of plaques, because the number of microvessels in plaques correlates with stenosis of human coronary arteries (R.L. Geary, unpublished observations, 1998). An increased density of microvessels expressing ACE may contribute to angiogenesis, chronic focal vasoconstriction within the artery wall, remodeling, and stenosis. Moreover, the presence of ACE in the endothelium of microvessels provides an alternative explanation to account for the mechanism contributing to the detection of Ang II in plaques. Although there is evidence that Ang II is found in atherosclerotic lesions, whether the peptide is produced within cellular elements or is transported from the blood side remains to be determined. Our findings suggest that Ang II could be produced locally within the extracellular milieu of the plaque through the action of ACE on blood-borne angiotensin I. In keeping with this interpretation, Ohishi et al. showed that Ang II was found in ACE-positive cells of human coronary artery atheromatous plaques.

Previous studies have shown that ACE inhibitors and Ang II antagonists may retard the formation of fatty streaks. Recent data from our laboratory has revealed that losartan reduces fatty streak formation in a nonhuman primate model of atherosclerosis, thus implicating Ang-II in lesion initiation. Although chymase has been detected in human atherosclerotic plaques, further investigation will be required to ascertain whether it is a primary source for local Ang II formation, because chymase of endothelial origin was expressed in low levels and Ang II immunoreactivity was associated only with ACE expression.

Atherosclerosis is associated with an inflammatory response produced by the accumulation of macrophages, monocytes, and lymphocytes. Macrophages are a source of foam cell formation and participate in the stimulation of adhesion-cell surface glycoproteins and smooth muscle cell—derived cytokines. Ang II increases the secretion of cytokines from leukocytes and smooth muscle cells, which favors destabilization and rupture of plaques by the induction of matrix-degrading enzymes.
In summary, the present study demonstrates that ACE is associated with inflammatory areas and areas of neovascularization within human carotid artery atheromatous plaques. Increased ACE in the inflammatory area and microvessels might provide a mechanism for increased contributions of Ang II to atherosclerotic progression and vascular remodeling.

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References

Figure 4. Photomicrographs of shoulder region of advanced carotid lesion (A through E) and inner region of fibrous cap under luminal endothelium (F). A, Immunostaining with anti-human ACE antibody (red reaction product) is shown. A number of cells surrounding plaque microvessels are stained positive. The endothelium of the microvessel shows intense staining for ACE protein (see arrow). B, In situ hybridization with an ACE riboprobe localizes the ACE mRNA to microvessels. C and D, ACE-positive microvessels were surrounded by clusters of CD68-positive (C) or CD45-positive (D) cells, many of which were also ACE positive (A and B). E, Specific staining for von Willebrand factor (VWF) confirms the existence of endothelial lining in plaque microvessels. F, A comparative weaker staining for ACE protein is documented in luminal endothelial cells. Original magnification ×400.


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