Int J Physiol Pathophysiol Pharmacol 2010;2(2):111-124
www.ijppp.org /IJPPP1004001

Original Article

Increased expression and co-localization of ACE, angiotensin II AT_1 receptors and inducible nitric oxide synthase in atherosclerotic human coronary arteries

Mitsuru Ohishi[1,2], Gregory J. Dusting[2], Paul A. Fennessy[2], Frederick A.O. Mendelsohn[2], Xiao C. Li[3], Jia L. Zhuo[2,3]

[1]Department of Geriatric Medicine, Osaka University Medical School, Osaka, Japan; [2]Howard Florey Institute of Experimental Physiology and Medicine, The University of Melbourne, Victoria 3010, Australia; and [3]Division of Hypertension and Vascular Research, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

Received April 16, 2010, accepted April 22, 2010, available online April 30, 2010

Abstract: Using immunohistochemistry and quantitative *in vitro* autoradiography, the present study was undertaken to examine whether co-expression of pro-atherosclerotic factors, ACE, the AT_1 receptor, and iNOS, is increased in early and advanced atherosclerotic lesions of human coronary arteries. In normal coronary arteries, ACE and eNOS were strongly co-expressed in endothelial cells (ECs), whereas the AT_1 receptor was expressed in medial smooth muscle cells (SMCs). By contrast, iNOS was not expressed in ECs and SMCs. In early atherosclerotic lesions and atheromatous plaques, ACE, the AT_1 receptor and iNOS immunostaining were primarily co-localized in infiltrated macrophages and SMCs adjacent to macrophages. eNOS expression was lower in ECs than in normal arteries, and absent in accumulated macrophages and SMCs. In fibrosclerotic plaques, ACE, the AT_1 receptor, and iNOS immunostaining were still positive in macrophages as well as new microvessels within the plaques. Interestingly, SMCs in vasa vasorum of the adventitia in atheromatous and fibrosclerotic plaques were also strongly positive for AT_1 receptor and iNOS, while ECs of the vasa vasorum were positive for ACE and eNOS. The present study demonstrates that multiple pro-atherosclerotic factors ACE, AT_1 receptor and iNOS are co-localized almost exclusively in infiltrated macrophages and SMCs that have accumulated in or adjacent to early and advanced atherosclerotic plaques, while the anti-atherosclerotic enzyme eNOS is reduced in ECs. These data therefore suggest that increased formation of Ang II and iNOS in infiltrated macrophages and medial SMCs might well play important roles in the development and progression of human coronary atherosclerosis.

Keywords: Atherosclerosis, angiotensin converting enzyme, angiotensin II, human coronary artery, nitric oxide synthase, immunohistochemistry

Introduction

The development and progression of human coronary artery atherosclerosis are dependent on multiple factors, including genetics, life style, and the imbalance of pro-atherosclerotic and anti-atherosclerotic humoral influences. Many vasoactive hormones, growth factors and cytokines promote while others counteract the development and progression of coronary atherosclerosis. Angiotensin II (Ang II) is now well recognized as one of most important vasoactive pro-atherosclerotic factors [1-5]. Ang II, which is converted from Ang I by angiotensin converting enzyme (ACE), can induce vascular injury due to its potent vasoconstrictor action [6] and ability of generating reactive oxygen species, and cause vascular hypertrophy by increasing smooth muscle cell (SMC) proliferation via acting on the AT_1 receptor [7]. While ACE occurs predominantly in the endothelium and adventitia of healthy human blood vessels [8-10], increased ACE expression has been observed in cellular structures beyond the endothelium and adventitia, such as the neointima developed following balloon injury in rat aorta [11]. Moreover, early atherosclerotic lesions commonly involve infiltration and/or migration of macro-

phages into the vessel wall, where increased expression of ACE has been reported in macrophages and vascular smooth muscle cells in atherosclerotic plaques of human coronary arteries [12-15]. These studies therefore suggest that local formation of Ang II is increased at the injured sites and therefore may play an important role in the development of human coronary atherosclerosis. However, it is not known whether local expression of AT_1 receptors is also increased in migrated macrophages and/or proliferative vascular SMCs in human atherosclerotic coronary arteries.

In contrast to Ang II, nitric oxide (NO) is generally a vasorelaxant and anti-atherosclerotic factor [16-18]. NO inhibits angiogenesis by preventing SMC proliferation [19]. Three isoforms of NO synthase (NOS) have been identified and cloned: neural NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) [20]. eNOS is constitutively present in endothelial cells of blood vessels and plays an important and beneficial role in maintaining normal endothelial function [21]. By contrast, iNOS is normally absent or expresses only at very low levels in different layers of blood vessels, but may become cytotoxic if excessively induced in infiltrated macrophages, proliferated SMCs, and endothelial cells by influences of vasoactive agents such as Ang II, cytokines, and/or under diseased states [18,22]. Although previous immunohistochemical studies have demonstrated that iNOS is expressed in macrophages of atherosclerotic coronary specimens taken from patients with unstable angina [23] and eNOS, though present, is reduced in endothelial cells of atherosclerotic lesions [24], co-localization and redistribution of different NOS isoforms during development and progression of human coronary atherosclerosis remain to be further investigated.

Similarly, while there is strong evidence that the renin-angiotensin system (RAS) and NO systems counteract each other in the regulation of vascular structures and tone physiologically as well as during the development and progression of human coronary atherosclerosis [25-29], to our knowledge there is still a lack of a comprehensive cellular mapping of the major components of the RAS (ACE and AT_1 receptor) and NO systems (eNOS and iNOS) in normal and early and advanced atherosclerotic human coronary arteries. In the present study, we hypothesized that cellular expression of pro-atherosclerotic factors, ACE, AT_1 receptors and iNOS, is increased and co-localized in major cellular components of early atherosclerotic lesions and atheromatous plaques during the development and progression of human coronary disease. Increased co-expression or occurrence of pro-atherosclerotic factors ACE, which forms Ang II, the AT_1 receptor, which mediates actions of Ang II, and cytotoxic iNOS, while decreasing eNOS expression, in early and advanced atherosclerotic plaques may indeed promote human coronary atherosclerosis.

Methods

Tissue collection and American Heart Association (AHA) histological classification

The study was conducted on 88 coronary artery segments obtained at autopsy from 31 Caucasians patients who died from various causes including 24 males and 7 females with ages ranging from 21 to 83 years old (Table 1). 12 patients died from cardiovascular events including acute myocardial infarction, chronic heart failure and stroke, 11 from multiple organ injuries due to suicide or traffic accidents, and the remaining from lung or gastrointestinal diseases. The 88 coronary arterial samples consisted of 28 left main trunks, 22 left anterior descending arteries, 12 left circumflexes and 26 right coronary arteries according to American Heart Association classification. Collection of these autopsied coronary arteries for the current study was approved by the Human Ethics Committee of the Howard Florey Institute of Experimental Physiology and Medicine, The University of Melbourne.

All coronary arteries were obtained from autopsy within 24 hours after death, but most samples were collected between 8 to 12 hours. Samples were immediately frozen in liquid nitrogen and stored at - 70°C before sectioning. Serial frozen sections, ~10 μm thick, were cut on a cryostat at – 20°C for histological classification following haematoxylin and eosin staining, for immunohistochemical cellular localization of ACE, AT_1 receptors, iNOS or eNOS and cellular markers [12,13,30], and for autoradiographic quantification of ACE [8,9]. Classification of human coronary atherosclerosis was made according to American Heart Association Definitions with modifications [31-33]. Type I lesion

includes normal coronary artery with diffuse intimal thickening. Type II and/or type III are early atherosclerotic lesions, whereas type IV and/or type V shows advanced lesions. We further classified advanced lesions into atheromatous plaques and fibrosclerotic plaques. We had no type VI lesions designated complicated plaques.

Immunohistochemical localization

Antibodies: For cellular localization of ACE, a polyclonal antibody generated against a 25-amino-acid peptide located near the COOH terminus of human ACE was used (a gift from Professor Kunio Hiwada, Ehime University School of Medicine, Ehime, Japan). Its potency and specificity for human kidney ACE has been described previously [12,13,34]. The cellular localization of the AT_1 receptors was determined using a polyclonal antibody generated against the amino acid sequence corresponding to residues 15-24 of the human AT_1 receptor, Ac-QDDCPKAGRHC-NH_2 a hydrophilic portion from the amino-terminal extracellular domain coupled to an additional COOH-terminal cysteine (a gift from Professor Toshio Ogihara, Osaka University School of Medicine, Suita, Japan). Its specificity for the human AT_1 receptor has been reported previously [30,35,36].

To co-localize two isoforms of NO synthase with ACE and the AT_1 receptor in the same coronary section, we used a polyclonal anti-iNOS antibody (Transduction Laboratory Ltd.) and an anti-eNOS antibody (Transduction Laboratory Ltd.), respectively. We also identified cellular structures in each coronary artery using cell-specific antibodies with a monoclonal anti-smooth muscle antibody (1A4, DAKO Co Ltd.) for smooth muscle cells, a monoclonal anti-macrophage antibody (HAM56, DAKO Co Ltd.) for macrophages, and a monoclonal anti- von Willebrand factor antibody (DAKO Co Ltd.) for endothelial cells.

Immunohistochemistry: Immunohistochemical localization of ACE, AT_1 receptor, eNOS and iNOS was performed using an avidin-biotin complex method as described previously [12,13,36,37]. 10 μm-thick frozen sections were first fixed with acetone, endogenous peroxidase was blocked by 3% hydrogen peroxidase, and non-specific binding was blocked by incubation of sections in 10% normal goat serum, respectively. Sections were then incubated with the primary antibodies for overnight at 4° C. After washes with phosphate buffered saline, sections were sequentially incubated with biotinylated secondary antibody and avidin-biotin complex (Vector Laboratories Inc.), and developed with 3-amino-9-ethylcarbazole (DAKO). Sections were then counter-stained with haematoxylin for histological identification. For negative controls, non-immune rabbit serum (DAKO) instead of the primary antibodies was used on adjacent sections.

In vitro autoradiographic localization and quantification of ACE: To further examine whether enhanced ACE protein expression in early and advanced atherosclerotic plaques was correlated with ACE activity in human coronary arteries, we determined ACE binding using quantitative *in vitro* autoradiography as described previously [8,9]. Briefly, ^{125}I-351A, a tyrosyl derivative of the ACE inhibitor, lisinopril (Merck Institute of Therapeutic Research) was used to label ACE, which binds to the active site of ACE and serves as an index of ACE activity. Four slide-mounted frozen sections of each coronary sample were pre-incubated in 10 mmol/L sodium phosphate buffer, pH 7.4, for 15 min, and then incubated in the same fresh buffer containing 0.2% bovine serum albumin (BSA) and ~0.3 mCi/mL of the radioligand, ^{125}I-351A, for 1 hour at 22°C. Non-specific binding was determined in the presence of 1 mmol/L EDTA, which inactivates ACE. After incubation, sections were washed with fresh buffer by 4 x 1 min, air-dried, and directly exposed to Agfa-Scopix CR3 X-ray film together with a set of radioactivity standards (Agfa-Gevaert) for 48 hours. ACE binding in the adventitia, early atherosclerotic lesions, atheromatous plaques, and fibrosclerotic plaques was quantitated by a computerized densitometry (MCID, Imaging Research Institute) compared with hematoxylin eosin-stained adjacent sections. Statistical analysis on ACE binding was performed using unpaired t test between normal and early or advanced atherosclerotic plaques. A value of $p<0.05$ was considered significant.

Results

Patient characteristics

Patients were classified into two broad groups, one died from known cardiovascular events and the other from non-cardiovascular causes. The former group of patients were generally older

(59.6 ± 4 vs. 42.6 ± 4 years old, $p < 0.05$), had higher body weight (71.6 ± 3.5 vs. 64 ± 3.5 kg, $p < 0.05$) and heart weight (464.5 ± 25 vs. 368.5 ± 11.3 g, $p < 0.01$) than the latter. However, body mass index (BMI) was similar between two groups.

Histological AHA classification

Of all coronary arterial samples, 33 were classified as normal coronary arteries with diffuse intimal thickening (see **Figure 1A**), 18 showed early atherosclerotic lesions (**Figure 2A**) and 37 had advanced lesions (**Figure 3A**). In advanced lesions, we identified 17 samples as atheromatous plaques and 10 as fibrous plaques. Normal coronary arteries with diffuse intimal thickening were identified primarily from patients died from non-cardiac events such as multiple organ injuries due to traffic accidents, suicide or other non-cardiovascular diseases, whereas advanced coronary lesions were identified predominantly in patients died from cardiovascular disorders ranging from myocardial infarction, hypertension and chronic heart failure.

Co-localization of ACE, AT_1 receptor, eNOS and iNOS by immunohistochemistry

Normal coronary arteries: **Figure 1** shows co-localization of ACE and eNOs, AT_1 receptor and iNOS in a representative normal coronary artery with diffuse intimal thickening. Intense ACE (**Figure 1B**) and eNOS immunostaining (**Figure 1C**) was co-localized predominantly in endothelial cells (**Figure 1D**), where only weak signals for AT_1 receptors (**Figure 1E**) and iNOS expression (**Figure 1F**) were present. By contrast, AT_1 receptors and iNOS were strongly expressed in vascular SMCs of thickened intima and medial SMCs. ACE and eNOS expression were absent in intimal and medial SMCs (**Figure 1B** and **Figure 1C**).

Atherosclerotic lesions

Early atherosclerotic lesions: In most early atherosclerotic lesions examined, ACE, iNOS and AT_1 receptors were co-localized in accumulated macrophages and SMCs in the intima, where eNOS expression is weak (data not shown).

Atheromatous plaques: **Figure 2** shows a typical representative atheromatous plaque, which consists of a lipid core surrounded by a thickened intima and accumulated macrophages (**Figure 2D**). Macrophages adjacent to the lipid core were positive for ACE (**Figure 2B**), AT_1 receptors (**Figure 2E**) and iNOS (**Figure 2F**), but not for eNOS (**Figure 2C**). eNOS staining was only seen in endothelial cells and its expression level was much weaker than in normal coronary arteries (**Figure 1**). In addition to accumulated macrophages, AT_1 receptors were localized in SMCs around accumulated macrophages or adjacent to lipid cores in the intima, as well as in the media (**Figure 2E**).

Fibrosclerotic plaques: Infiltration of macrophages was still evident when atheromatous plaques advanced to fibrosclerotic plaques (**Figure 3D**). In addition, neovascularization occurs within the fibrous plaques. Co-localization of intensive ACE (**Figure 3B**), AT_1 receptor (**Figure 3E**) and iNOS immunostaining (**Figure 3F**) still occurred in infiltrated macrophages of fibrous plaques. ACE and eNOS immunostaining were weak in endothelial cells of coronary arteries, but appeared in endothelial cells of neovascularization within the plaque (**Figure 3B** and **Figure 3C**). Strong AT_1 receptor and iNOS expression was observed in medial SMCs (**Figure 3E** and **Figure 3F**).

Adventitia of *advanced plaques:* In the adventitia, ACE (**Figure 4B**) and eNOS (**Figure 4C**) were strongly expressed in, or co-localized with, endothelial cells (**Figure 4A**) of large and small vasa vasorum, whereas strong AT_1 receptor (**Figure 4E**) and iNOS expression (**Figure 4F**) was observed in the medial SMCs (**Figure 4D**) of vasa vasorum.

Quantification of ACE activity

Although ACE binding was not significantly different in the endothelia of various stages of coronary atherosclerosis (**Figure 5A**), ACE binding in early atherosclerotic lesions (44.3 ± 6.0 dpm/mm^2) was significantly higher than that of normal coronary arteries (25.9 ± 2.1 dpm/mm^2) ($p<0.001$) or that in non-plaque samples ($p<0.01$) (**Figure 5B**). ACE binding in atheromatous plaques (68.8 ± 5.5 dpm/mm^2) was also significantly higher than that of early atherosclerotic lesion ($p<0.001$), or that of fibrous plaque (33.3 ± 3.5 dpm/mm^2) (**Figure 5B**) ($p<0.001$). Examination of hematoxylin and eosin-stained adjacent sections confirmed that increased ACE

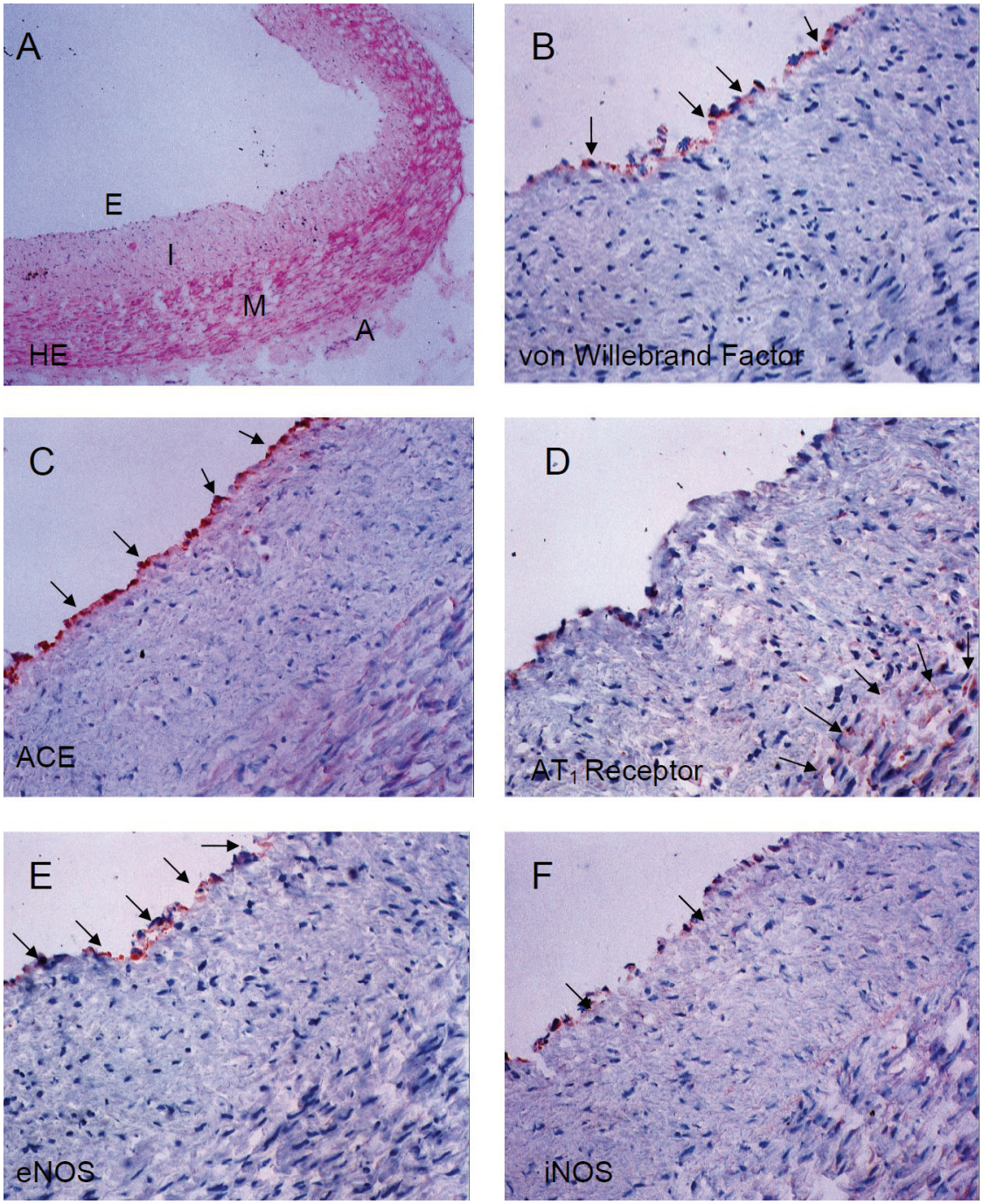

Figure 1. Cellular co-localization of ACE, AT_1 receptor, eNOS and iNOS expression in a representative normal human coronary artery with diffuse intimal thickening from a patient who died from a non-cardiovascular event. Panel A: a section stained with hematoxylin and eosin showing a mono-layer of endothelial cells (E), the thickened intima (I), medial smooth muscle cells (M), and adventitia (A). Panel B: a consecutive section of A stained with anti-von Willebrand factor antibody showing endothelial cells. Panel C: a consecutive section of B stained with anti-human ACE antibody showing ACE immunostaining co-localized with endothelial cells. Panel D: a consecutive section of C staining with anti-human AT_1 receptor antibody showing strong AT_1 receptor immunostaining mainly in vascular smooth muscle cells. Panel E: a consecutive section of D staining with anti-eNOS antibody showing eNOS immunostaining co-localized with endothelial cell marker and ACE. Panel F: a consecutive section of E stained with anti-iNOS antibody showing very weak iNOS immunostaining in endothelial cells and smooth muscle cells. Positive immunostaining is indicated by arrows. Magnification: Panel A, x 50; Panel B-F, x 200.

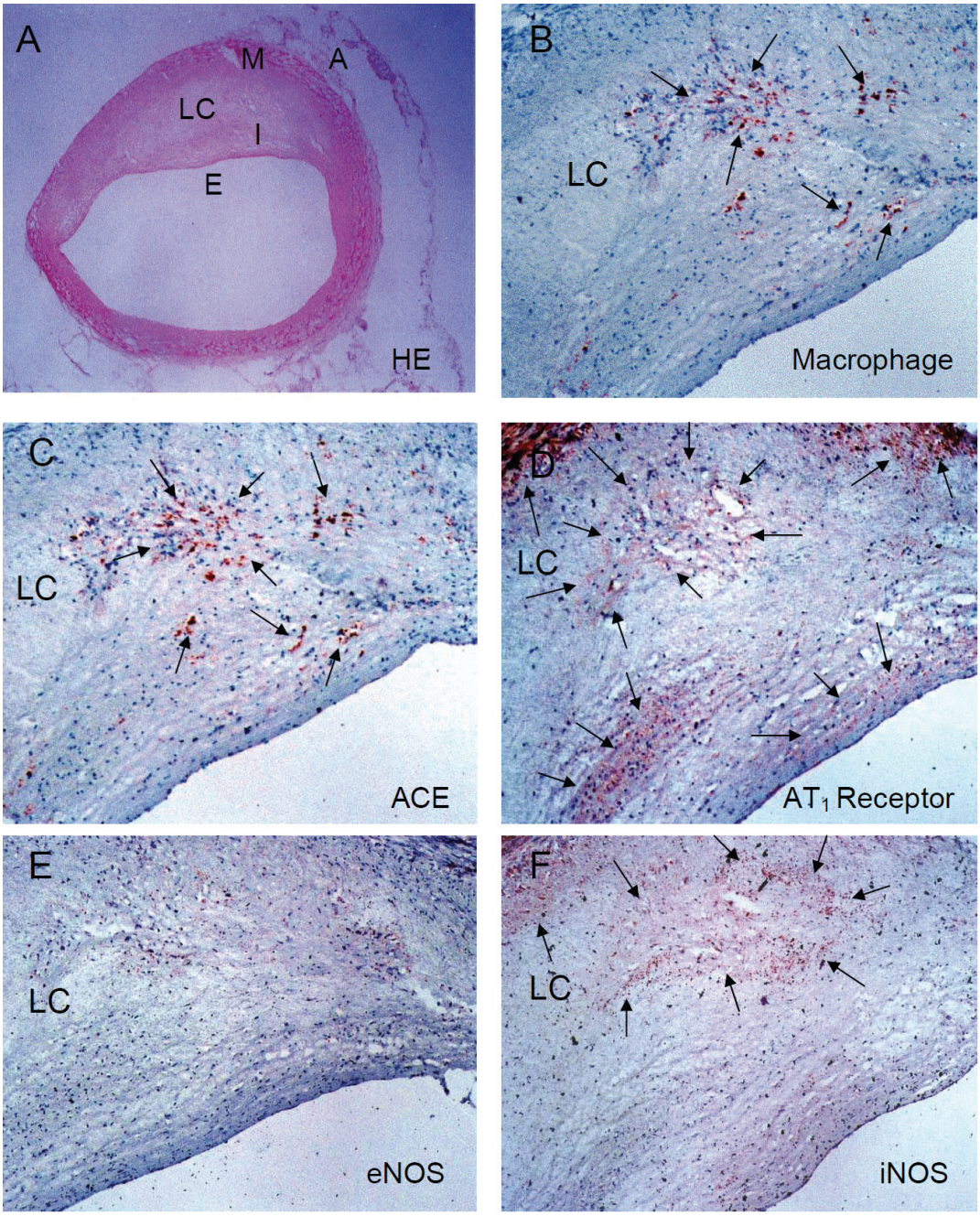

Figure 2. Cellular co-localization of ACE, AT_1 receptor, eNOS and iNOS expression in a representative atheromatous plaque of human coronary artery from a patient who died from a myocardial infarction. Panel A: a section stained with hematoxylin eosin showing an atheromatous plaque which has a lipid core (LC) surrounded by accumulated or infiltrated macrophages. Panel B: a consecutive section of A stained with anti-macrophage antibody (HAM56) showing intense immunostaining in the shoulder region of the plaque adjacent to the lipid core. Panel C: a consecutive section of B staining with anti-human ACE antibody showing ACE immunostaining co-localized with macrophage marker adjacent to the lipid core. Panel D: a consecutive section of C stained with anti-human AT_1 receptor showing strong AT_1 receptor immunostaining co-localized with thickening intima (I), infiltrated macrophages and in smooth muscle cells (M). Panel E: a consecutive section of D stained with anti-eNOS antibody showing reduced eNOS immunostaining in endothelial cells (E) and absent from atheromatous plaque. Panel F: a consecutive section of E stained with anti-iNOS antibody showing strong iNOS immunostaining co-localized with macrophage marker adjacent to the lipid core, and in smooth muscle cells. Positive staining is indicated by arrows. Magnification: Panel A, x 50; B-F, x 200.

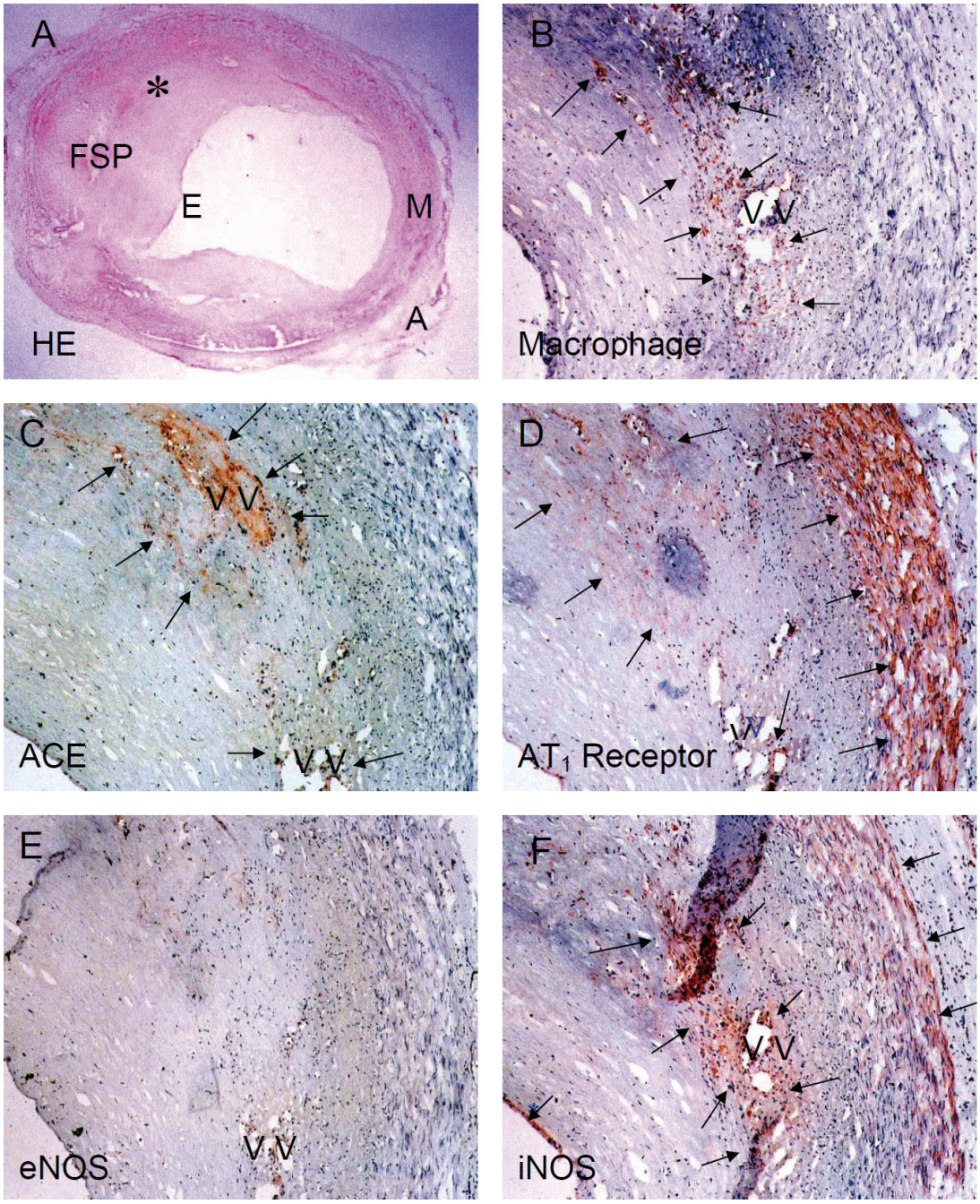

Figure 3. Cellular co-localization of ACE, AT_1 receptor, eNOS and iNOS expression in a representative fibrosclerotic plaque of human coronary artery from a patient who died from myocardial infarction. Panel A: a section stained with hematoxylin and eosin showing a fibrosclerotic plaque (FSP), which consists of dense connective tissues in atherosclerotic lesions and neovascularization. Vasa vasorum (VV) is occasionally found within the plaque. Panel B: a consecutive section of A showing macrophage marker immunostaining surrounding a vasa vasorum within the plaque. Panel C: a consecutive section of B showing strong ACE immunostaining co-localized with macrophages and neovascularization, but reduced in endothelial cells. Panel D: a consecutive section of C showing strong AT_1 receptor immunostaining localized in macrophages, thickening intima and medial smooth muscle cells. Panel E: a consecutive section of D showing reduced eNOS immunostaining in endothelial cells as well as neovascularization. Panel F: a consecutive section of E showing strong iNOS immunostaining co-localized with accumulated macrophages surrounding vasa vasorum and medial smooth muscle cells. Positive staining is indicated by arrows in macrophage-rich lesions around vasa vasorum and medial smooth muscle cells. Magnification: Panel A, x 50; Panel B-F, x 200.

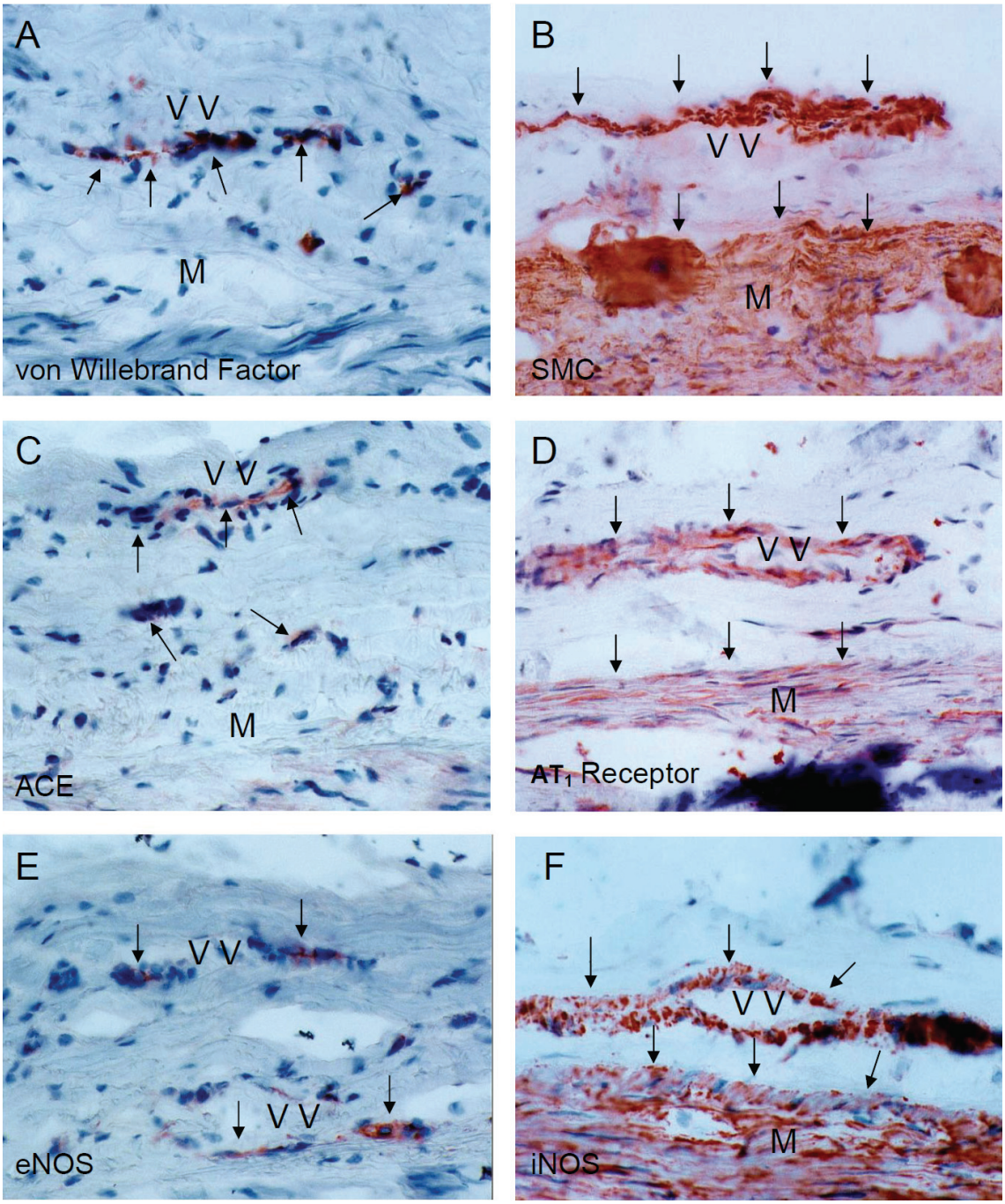

Figure 4. Cellular co-localization of ACE, AT_1 receptor, eNOS and iNOS expression in the adventitia of a representative advanced atherosclerotic plaque of human coronary artery from a patient who died from myocardial infarction. Panel A: a section stained with anti-von Willebrand factor antibody showing that the endothelium of vasa vasorums (VV) in the adventitia (A) of this advanced plaque were positive for the endothelial marker. Panel B: a consecutive section of A stained with the smooth muscle cell marker, HAM56. Panel C: a consecutive section of B showing strong ACE immunostaining co-localized with the endothelial marker in vasa vasorum. Panel D: a consecutive section of C showing strong AT_1 receptor immunostaining in smooth muscle cells of large vasa vasorum and medial smooth muscle cells. Panel E: a consecutive section of D showing eNOS immunostaining localized in endothelial cells of vasa vasorum. Panel F: a consecutive section of E showing strong iNOS immunostaining co-localized with the AT_1 receptor in smooth muscle cells of large vasa vasorum and medial smooth muscle cells. Positive staining is indicated by arrows in endothelial cells of vasa vasorum and medial smooth muscle cells. Magnification: Panels A-F, x 200.

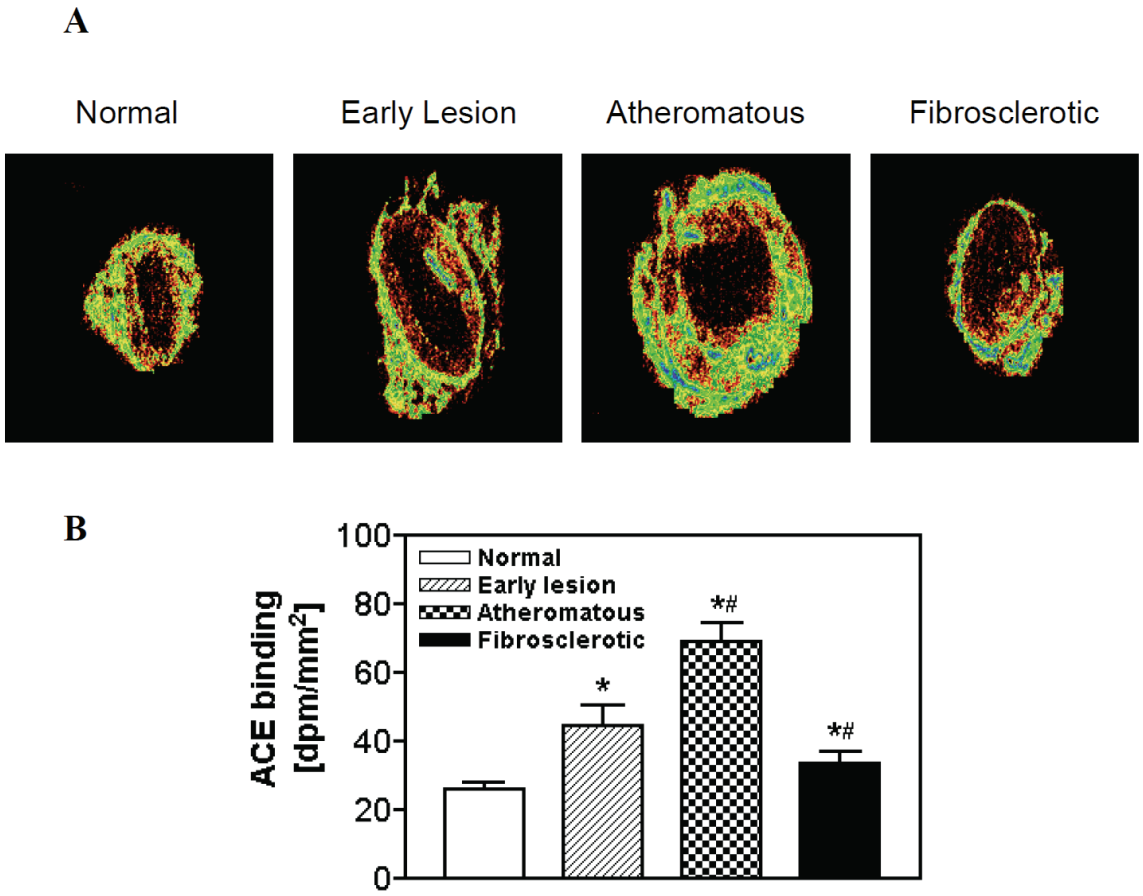

Figure 5. *In vitro* autoradiography showing increased ACE binding in early atherosclerotic lesions and advanced plaques of human coronary arteries (A) with the quantitated data shown in Panel B. Yellow shows the highest level of ACE binding, while green and black the moderate and background levels, respectively. *, $p < 0.05$ *vs.* normal; #, $p < 0.05$, atheromatous *vs*. early lesions; or atheromatous *vs.* fibrosclerotic.

binding at these coronary arteries was closely associated with accumulated macrophages, adventitial vasa vasorum, and endothelial cells of neovascularization in advanced plaques.

Discussion

The present study demonstrates that during the development and progression of human coronary atherosclerosis, cellular expression of pro-atherosclerotic factors, ACE, AT_1 receptor and iNOS, is markedly increased at cellular sites with early and advanced atherosclerotic lesions or plaques. This enhanced expression of ACE, AT_1 receptor and iNOS at the protein level occurs almost exclusively in accumulated or infiltrated macrophages and vascular smooth muscle cells within or adjacent to the shoulder of atherosclerotic lesions or lipid cores. By contrast, eNOS expression is markedly reduced in endothelial cells and no eNOS expression occurs in accumulated macrophages and smooth muscle cells within and around the plaques. To our knowledge our study represents the first comprehensive immunohistochemical investigation showing increased expression and co-localization of ACE, AT_1 receptor and iNOS in the same early and advanced atherosclerotic lesions during the development and progression of human coronary artery atherosclerosis.

It should be acknowledged that the current study has strengths as well as limitations. Because all normal and atherosclerotic coronary artery segments were obtained from autopsied patients several hours after death, only immu-

nohistochemistry was performed to co-localize these vasoactive factors with the exception for ACE activity, which can be determined by quantitative *in vitro* autoradiography. For the same reason, we were also unable to measure Ang II or NO production in these autopsied coronary arteries ex vivo. However, collection of normal and atherosclerotic coronary arteries from patients died from known cardiovascular events and those from other non-cardiac causes allowed us to classify all coronary tissue sections from these patients into four different groups according to the AHA classification before immunohistochemistry was performed. This approach made it possible to co-localize ACE, AT_1 receptor and two isoforms of NOS, eNOS and iNOS, together with cellular markers including antibodies against von Willenbrand factor (endothelial cells), 1A4 (smooth muscle cells) and HAM56 (macrophages) in consecutive sections of the same coronary artery.

The results of the present study on ACE and AT_1 receptors are consistent with previous studies in experimentally injured vessels of animals or in early and advanced atherosclerotic plaques of human coronary arteries [11-13,38,39]. Rakugi et al have demonstrated that increased expression of ACE occurs in the developing neointima two weeks following induction of aorta balloon injury in rats [11]. In monkeys with experimental atherosclerosis induced by high cholesterol diet, both ACE and AT_1 receptor binding significantly increase in atherosclerotic vessels [38]. Increased AT_1 receptor expression has also been reported in a rabbit model of hypercholesterolemic atherosclerosis [39]. In human studies, we [12,13,40] and other investigators [14,15] further showed increased ACE expression in accumulated macrophages of early atherosclerotic lesions and atheromatous plaque, as well as at sites of injury following percutaneous transluminal coronary angioplasty in humans [13]. However, AT_1 receptor was not co-localized with ACE in accumulated macrophages and smooth muscle cells within and around early and advanced atherosclerotic plaques in afore-mentioned studies. Because ACE is commonly expressed in the endothelium and adventitia but not in medial smooth muscle cells of normal coronary artery, increased ACE expression in accumulated macrophages and smooth muscle cells adjacent to atherosclerotic plaques strongly suggests an increase in local formation of Ang II at these cellular sites. Indeed, in a study in patients with acute coronary syndrome ACE activity increased 4-fold in coronary artery specimens [41]. Using quantitative *in vitro* autoradiography the present study was able to demonstrate increased ACE binding as a marker of ACE activity in early and advanced atherosclerotic plaques, where ACE co-localized with macrophages and smooth muscle cells (**Figure 5**). These data are consistent with our previous studies in which Ang II immunostaining was identified in macrophages and smooth muscle cells of human coronary hypercellular lesions and atheromatous plaques [40], thus providing further support to the concept that Ang II is produced locally in infiltrated macrophages and smooth muscle cells during the development and progression of human coronary atherosclerosis.

While AT_1 receptor expression occurs in medial smooth muscle cells as expected, it is highly significant for the present study that AT_1 receptor expression is increased and co-localized with ACE and iNOS (see below) in accumulated macrophages and smooth muscle cells within and around atherosclerotic lesions. This enhanced expression was observed in most coronary sections classified as early atherosclerotic lesions, atheromatous plaques and fibrosclerotic plaques examined. It is also of particular interest that AT_1 receptor immunostaining occurs in accumulated macrophages and smooth muscle cells adjacent to lipid cores in addition to medial smooth muscle cells (**Figure 2E**). These findings have not been reported previously in human coronary atherosclerotic lesions, though AT_1 receptors mRNA has been found in human macrophages [30].

The significance of co-localization of ACE, Ang II and AT_1 receptors at these atherosclerotic lesions is still poorly understood, but may imply important implications, which warrant further investigations. It has been suggested that AT_1 receptors may mediate vasoconstriction in the contractile phenotype [6] and cell proliferation in the synthetic phenotype of vascular smooth muscle cells [7]. Although we were not able to differentiate whether smooth muscle cells adjacent to the lipid core or to the shoulder of atherosclerotic lesions are of the synthetic phenotype, AT_1 receptors may play dual roles in mediating both cell contraction and proliferation at these sites. Thus, it is conceivable that Ang II-induced vascular smooth muscle cell contrac-

tion and proliferation, together with Ang II-induced iNOS expression (see discussion below), may be one of most important contributors to the development of human coronary atherosclerosis. Additionally, expression of ACE and AT_1 receptors in inflammatory macrophages accumulated at early and advanced atherosclerotic lesions also implies an unspecified but important role for interactions between Ang II and macrophages. Ang II has been shown to increase macrophage-induced oxidation of low-density lipoprotein or peroxide production via the AT_1 receptor [42] or via a lipoxygenase-dependent pathway [43]. Furthermore, stimulation of peroxide production or induction of iNOS expression (see discussion below) in accumulated macrophages by Ang II via the AT_1 receptor has also been reported. In keeping with this context, it is not surprising to note that Ang II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficiency mice [1,2]. Taken together, Ang II and macrophages may interact to promote atherosclerosis via multiple mechanisms related to cell contraction and proliferation, and synthesis and/or release of cytokines, elastase, collagenase and free radicals.

Other equally important findings in the present study are that while eNOS expression was reduced (**Figure 2C** and **Figure 3C**) in endothelial cells comparing with normal coronary artery (**Figure 1C**) and was absent at cellular sites with early atherosclerotic lesions and advanced atherosclerotic plaques, enhanced iNOS expression was localized in accumulated macrophages and smooth muscle cells of the shoulder lesions adjacent to the lipid core (**Figure 2F** and **Figure 3F**). This cellular distribution of iNOS expression coincides with those of enhanced expression of ACE (**Figure 2B** and **Figure 3B**) and AT_1 receptors (**Figure 2E** and **Figure 3E**) in early and advanced atherosclerotic lesions. Reduced eNOS expression and production has been reported previously in atherosclerotic human carotid arteries compared with normal internal mammary arteries [24]. However, it is not known whether this applies to human coronary atherosclerotic arteries. In this context, our results provide morphological evidence that eNOS expression and production may be reduced during the progression of human coronary atherosclerosis. Indeed, accelerated atherosclerosis and ischemic heart disease has been reported in apolipoprotein E/eNOS double knockout mice [44].

As for iNOS expression, our results are consistent with previous studies on iNOS expression during vascular injury and human coronary atherosclerosis. For example, induction of vascular iNOS expression has been reported in animal models of vascular injury, such as those induced by periarterial collar [45] or by endothelial denudation [46]. In human coronary disease, iNOS expression was increased in macrophages as well as smooth muscle cells of transplanted human coronary arteries [17], and in human coronary atherosclerotic plaques of patients [23,47,48]. However, it is not clear whether induction of iNOS is associated with, or accompanied by, enhanced ACE and AT_1 receptor expression and reduced eNOS expression in these vascular lesions. The roles of NO in the development of human coronary atherosclerosis are currently under intensive investigations. It has been suggested that NO, produced by eNOS and iNOS, may serve dual roles in the development and progression of human coronary atherosclerosis [16]. Endothelial NOS is located predominantly in endothelial cells and produces small amounts of NO in response to physiological shear stress from raised arterial pressure or to stimulation by kinins via the B_2 receptor [18]. NO produced by eNOS is beneficial, anti-atherosclerotic, and important in maintaining normal endothelial function as it induces vasorelaxation, inhibits leukocyte adhesion and platelet aggregation, decreases expression of adhesion molecules and chemotactic factors, and inhibits smooth muscle cell proliferation [16,18]. By contrast, iNOS induced locally in macrophages and smooth muscle cells by cytokines or during the inflammatory processes including Ang II can produce large quantity of NO at sites of its expression; this NO can be cytotoxic and pro-atherosclerotic [18,49]. The pro-atherosclerotic properties of iNOS as a result of producing large amount of NO include: a) interaction with the free radical superoxide to form peroxynitrate, which is known to damage cellular proteins; b) induction of vascular hyperreactivity; and c) promotion of leukocyte adhesion and platelet aggregation [16-18]. Thus, increased expression of pro-atherosclerotic iNOS and reduced expression of anti-atherosclerotic eNOS in early and advanced atherosclerotic lesions or plaques as shown in the present study may play an important role in human coronary atherosclerosis.

In summary, the present study demonstrates for

the first time that expression of ACE, AT_1 receptor and iNOS at the protein level are co-localized almost exclusively in infiltrated macrophages and vascular smooth muscle cells adjacent to the lipid core of early and advanced atherosclerotic lesions or plaques, while eNOS expression is reduced in endothelial cells of atherosclerotic human coronary artery. These data therefore suggest that over-expression or formation of Ang II and iNOS in infiltrated microphages and migrated smooth muscle cells plays an important role in the development and progression of human coronary atherosclerosis.

Acknowledgements

This work was supported by an Institute Block Grant to the Howard Florey Institute of Experimental Physiology and Medicine from the National Health and Medical Research Council of Australia (No. 983001) and a Grant-in-Aid from National Heart Foundation of Australia to Dr. Zhuo. Dr. Mitsuru Ohishi was a recipient of an International Fellowship from the High Blood Pressure Research Council of Australia. Dr. J Zhuo's current address is the Division of Hypertension and Vascular Research, Department of Internal Medicine, Henry Ford Hospital, Detroit, Michigan, USA 48202-2689, and his current research is supported by grants from the National Institute of Diabetes, Digestive and Kidney Diseases (5R01DK067299, 2R56DK067299, and 2R01DK067299), American Society of Nephrology, and Henry Ford Health System.

Please address correspondence to: Jia L. Zhuo, MD, PhD, Division of Hypertension and Vascular Research Department of Internal Medicine Henry Ford Hospital 2799 West Grand Blvd, Detroit, MI 48202. Tel: (313) 916-4115, Fax: (313) 916-9055, E-mail: jzhuo1@hfhs.org

References

[1] Daugherty A, Manning MW and Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest 2000; 105(11):1605-1612.

[2] Berk BC, Haendeler J and Sottile J. Angiotensin II, atherosclerosis, and aortic aneurysms. J Clin Invest 2000; 105(11):1525-1526.

[3] Weiss D, Kools JJ and Taylor WR. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. Circulation 2001; 103(3):448-454.

[4] van Kleef EM, Fingerle J and Daemen MJ. Angiotensin II-induced progression of neointimal thickening in the balloon-injured rat carotid artery is AT_1 receptor mediated. Arterioscler Thromb Vasc Biol 1996; 16(7):857-863.

[5] The Heart Outcomes Prevention Evaluation (HOPE) Study Investigators. Effects of an angiotensin-converting enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. N Engl J Med 2000; 342(3):145-153.

[6] Holmgren A, Pantev E, Erlinge D and Edvinsson L. Inhibition of angiotensin II-induced contraction by losartan in human coronary arteries. J Cardiovasc Pharmacol 1998; 32(4):662-664.

[7] Hafizi S, Chester AH, Allen SP, Morgan K and Yacoub MH. Growth response of human coronary smooth muscle cells to angiotensin II and influence of angiotensin AT_1 receptor blockade. Coron Artery Dis 1998; 9(4):167-175.

[8] Zhuo JL, Froomes P, Casley D, Liu JJ, Murone C, Chai SY, Buxton B and Mendelsohn FA. Perindopril chronically inhibits angiotensin-converting enzyme in both the endothelium and adventitia of the internal mammary artery in patients with ischemic heart disease. Circulation 1997; 96 (1):174-182.

[9] Zhuo JL, Casley D, Murone C and Mendelsohn FA. Acute and chronic in vivo inhibition of angiotensin-converting enzyme by perindopril in the endothelium and adventitia of large arteries and organs of the rabbit. J Cardiovasc Pharmacol 1997; 29(3):297-310.

[10] Zhuo JL, Moeller I, Jenkins T, Chai SY, Allen AM, Ohishi M and Mendelsohn FA. Mapping tissue angiotensin-converting enzyme and angiotensin AT_1, AT_2 and AT_4 receptors. J Hypertens 1998; 16(12 Pt 2):2027-2037.

[11] Rakugi H, Kim DK, Krieger JE, Wang DS, Dzau VJ and Pratt RE. Induction of angiotensin converting enzyme in the neointima after vascular injury. Possible role in restenosis. J Clin Invest 1994; 93(1):339-346.

[12] Ohishi M, Ueda M, Rakugi H, Naruko T, Kojima A, Okamura A, Higaki J and Ogihara T. Enhanced expression of angiotensin-converting enzyme is associated with progression of coronary atherosclerosis in humans. J Hypertens 1997; 15 (11):1295-1302.

[13] Ohishi M, Ueda M, Rakugi H, Okamura A, Naruko T, Becker AE, Hiwada K, Kamitani A, Kamide K, Higaki J and Ogihara T. Upregulation of angiotensin-converting enzyme during the healing process after injury at the site of percutaneous transluminal coronary angioplasty in humans. Circulation 1997; 96(10):3328-3337.

[14] Haberbosch W, Bohle RM, Franke FE, Danilov S, Alhenc-Gelas F, Braun-Dullaeus R, Holschermann H, Waas W, Tillmanns H and Gardemann A. The expression of angiotensin-I converting enzyme in human atherosclerotic plaques is not related to the deletion/insertion polymorphism but to the risk of restenosis after coronary interventions. Atherosclerosis 1997; 130(1-2):203-213.

[15] Diet F, Pratt RE, Berry GJ, Momose N, Gibbons GH and Dzau VJ. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. Circulation 1996; 94(11):2756-2767.
[16] Wever RM, Luscher TF, Cosentino F and Rabelink TJ. Atherosclerosis and the two faces of endothelial nitric oxide synthase. Circulation 1998; 97(1):108-112.
[17] Ravalli S, Albala A, Ming M, Szabolcs M, Barbone A, Michler RE and Cannon PJ. Inducible nitric oxide synthase expression in smooth muscle cells and macrophages of human transplant coronary artery disease. Circulation 1998; 97 (23):2338-2345.
[18] Moncada S. Nitric oxide in the vasculature: physiology and pathophysiology. Ann N Y Acad Sci 1997; 811:60-67.
[19] Groves PH, Banning AP, Penny WJ, Newby AC, Cheadle HA and Lewis MJ. The effects of exogenous nitric oxide on smooth muscle cell proliferation following porcine carotid angioplasty. Cardiovasc Res 1995; 30(1):87-96.
[20] Moncada S, Higgs A and Furchgott R. International Union of Pharmacology Nomenclature in Nitric Oxide Research. Pharmacol Rev 1997; 49 (2):137-142.
[21] Topper JN, Cai J, Falb D and Gimbrone MAJr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. Proc Natl Acad Sci U S A.1996; 93(19):10417-10422.
[22] Oemar BS, Yang Z and Luscher TF. Molecular and cellular mechanisms of atherosclerosis. Curr Opin Nephrol Hypertens 1995; 4(1):82-91.
[23] Depre C, Havaux X, Renkin J, Vanoverschelde JL and Wijns W. Expression of inducible nitric oxide synthase in human coronary atherosclerotic plaque. Cardiovasc Res 1999; 41(2):465-472.
[24] Oemar BS, Tschudi MR, Godoy N, Brovkovich V, Malinski T and Luscher TF. Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. Circulation 1998; 97 (25):2494-2498.
[25] Thorup C, Kornfeld M, Winaver JM, Goligorsky MS and Moore LC. Angiotensin-II stimulates nitric oxide release in isolated perfused renal resistance arteries. Pflugers Arch 1998; 435 (3):432-434.
[26] Olson SC, Dowds TA, Pino PA, Barry MT and Burke-Wolin T. ANG II stimulates endothelial nitric oxide synthase expression in bovine pulmonary artery endothelium. Am J Physiol 1997; 273 (2 Pt 1):L315-L321.
[27] Dubey RK, Jackson EK and Luscher TF. Nitric oxide inhibits angiotensin II-induced migration of rat aortic smooth muscle cell. Role of cyclic-nucleotides and angiotensin1 receptors. J Clin Invest 1995; 96(1):141-149.
[28] Ichiki T, Usui M, Kato M, Funakoshi Y, Ito K, Egashira K and Takeshita A. Downregulation of angiotensin II type 1 receptor gene transcription by nitric oxide. Hypertension 1998; 31(1 Pt 2):342-348.
[29] Pollman MJ, Yamada T, Horiuchi M and Gibbons GH. Vasoactive substances regulate vascular smooth muscle cell apoptosis. Countervailing influences of nitric oxide and angiotensin II. Circ Res 1996; 79(4):748-756.
[30] Okamura A, Rakugi H, Ohishi M, Yanagitani Y, Takiuchi S, Moriguchi K, Fennessy PA, Higaki J and Ogihara T. Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages. J Hypertens 1999; 17(4):537-545.
[31] [31] Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull WJr, Rosenfeld ME, Schwartz CJ, Wagner WD and Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995; 92(5):1355-1374.
[32] Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Jr., Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD and Wissler RW. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1994; 89(5):2462-2478.
[33] Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull WJr, Richardson M, Rosenfeld ME, Schaffer SA, Schwartz CJ and Wagner WD. A definition of the intima of human arteries and of its atherosclerosis- prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1992; 85(1):391-405.
[34] Takada Y, Kitami Y, Inoue Y, Hiwada K and Kokubu T. Immunohistologic characterization of angiotensin converting enzyme in the human kidney using monoclonal and polyclonal antibodies. Adv Exp Med Biol 1989; 247A:175-180.
[35] Rakugi H, Okamura A, Kamide K, Ohishi M, Sasamura H, Morishita R, Higaki J and Ogihara T. Recognition of tissue- and subtype-specific modulation of angiotensin II receptors using antibodies against AT_1 and AT_2 receptors. Hypertens Res 1997; 20(1):51-55.
[36] Zhuo JL, Ohishi M and Mendelsohn FA. Roles of AT_1 and AT_2 receptors in the hypertensive Ren-2 gene transgenic rat kidney. Hypertension 1999; 33(1 Pt 2):347-353.
[37] Ohishi M, Ueda M, Tanabe S, Han YS, Mendelsohn FAO and Zhuo JL. Increased angiotensin-converting enzyme and type 1 angiotensin receptors in cortical vasculature and tubulointerstitium of chronically rejected human kidney allografts. Nephrology 2001; 6(5):223-232.

[38] Song K, Shiota N, Takai S, Takashima H, Iwasaki H, Kim S and Miyazaki M. Induction of angiotensin converting enzyme and angiotensin II receptors in the atherosclerotic aorta of high-cholesterol fed Cynomolgus monkeys. Atherosclerosis 1998; 138(1):171-182.
[39] Yang BC, Phillips MI, Mohuczy D, Meng H, Shen L, Mehta P and Mehta JL. Increased angiotensin II type 1 receptor expression in hypercholesterolemic atherosclerosis in rabbits. Arterioscler Thromb Vasc Biol 1998; 18(9):1433-1439.
[40] Ohishi M, Ueda M, Rakugi H, Naruko T, Kojima A, Okamura A, Higaki J and Ogihara T. Relative localization of angiotensin-converting enzyme, chymase and angiotensin II in human coronary atherosclerotic lesions. J Hypertens 1999; 17 (4):547-553.
[41] Hoshida S, Kato J, Nishino M, Egami Y, Takeda T, Kawabata M, Tanouchi J, Yamada Y and Kamada T. Increased angiotensin-converting enzyme activity in coronary artery specimens from patients with acute coronary syndrome. Circulation 2001; 103(5):630-633.
[42] Keidar S, Kaplan M, Hoffman A and Aviram M. Angiotensin II stimulates macrophage-mediated oxidation of low-density lipoproteins. Atherosclerosis 1995; 115(2):201-215.
[43] Scheidegger KJ and Wood JM. Local application of angiotensin II to the rat carotid artery induces adventitial thickening. J Vasc Res 1997; 34 (6):436-446.
[44] Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH and Huang PL. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. Circulation 2001; 104(4):448-454.
[45] Arthur JF, Yin ZL, Young HM and Dusting GJ. Induction of nitric oxide synthase in the neointima induced by a periarterial collar in rabbits. Arterioscler Thromb Vasc Biol 1997; 17(4):737-740.
[46] Gonzalez-Fernandez F, Lopez-Farre A, Rodriguez-Feo JA, Farre J, Guerra J, Fortes J, Millas I, Garcia -Duran M, Rico L, Mata P, de Miguel LS and Casado S. Expression of inducible nitric oxide synthase after endothelial denudation of the rat carotid artery: role of platelets. Circ Res 1998; 83(11):1080-1087.
[47] Buttery LD, Springall DR, Chester AH, Evans TJ, Standfield EN, Parums DV, Yacoub MH and Polak JM. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. Lab Invest 1996; 75(1):77-85.
[48] Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG and Marsden PA. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. Arterioscler Thromb Vasc Biol 1997; 17 (11):2479-2488.
[49]Orsi A, Beltran B, Clementi E, Hallen K, Feelisch M and Moncada S. Continuous exposure to high concentrations of nitric oxide leads to persistent inhibition of oxygen consumption by J774 cells as well as extraction of oxygen by the extracellular medium. Biochem J 2000; 346 Pt 2:407-412.