Evidence That Macrophages in Atherosclerotic Lesions Contain Angiotensin II

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Background—We have reported that human mononuclear leukocytes contain large amounts of angiotensin II (Ang II). The goal of the present study was to test the hypothesis that Ang II is present in monocyte/macrophages in atherosclerotic lesions.

Methods and Results—Segments of thoracic aorta and left circumflex coronary artery were obtained from 3 groups of cynomolgus monkeys: normal, atherosclerotic, and regression. Samples of human coronary arterial atherosclerotic lesions were obtained from directional atherectomy. Sections were stained for Ang II with 3 different polyclonal rabbit anti–human Ang II antisera. In aorta and coronary arteries from normal monkeys, there was no or minimal anti–Ang II staining in endothelial cells. All sections from atherosclerotic monkeys displayed discrete, localized regions of staining for Ang II in intima-media. Macrophages were present throughout the atherosclerotic intima-media, and anti–Ang II staining appeared to colocalize with macrophages. All human coronary atherectomy samples stained positive for Ang II and macrophages. Staining for both Ang II and macrophages was observed in vascular lesions from all 5 monkeys after regression of atherosclerosis, but staining was less extensive than in atherosclerotic blood vessels from monkeys.

Conclusions—These findings suggest that Ang II is present in atherosclerotic lesions in monkeys and humans, colocalizes with macrophages in intima-media of atherosclerotic vessels from monkeys, and decreases in lesions in monkeys with regression of atherosclerosis. (Circulation. 1998;98:800-807.)

Key Words: angiotensin ■ cells ■ immunohistochemistry ■ atherosclerosis

When circulating mononuclear leukocytes enter a tissue, they become macrophages. Macrophages appear to play a pivotal role in development and progression of atherosclerotic lesions.1 Mononuclear leukocytes synthesize angiotensinogen,2 and when mononuclear leukocytes become macrophages, ACE is upregulated.3 Circulating mononuclear leukocytes in humans contain angiotensin I and a large amount of angiotensin II (Ang II).4 Thus, it seems likely that macrophages in atherosclerotic lesions might contain Ang II. This finding would be of interest, in part because the renin-angiotensin system may contribute to development of atherosclerotic lesions.5,6

The first goal of this study was to determine whether monocyte/macrophages in atherosclerotic lesions contain Ang II. We adapted an immunocytochemical technique that was used previously to study circulating monocytes.7 The validity of the immunocytochemical technique was supported by measurements of Ang II in monocytes with radioimmunoassay.4 In this study, we examined aorta and coronary arteries from monkeys with diet-induced atherosclerosis and atherectomy samples from human coronary arteries.

Regression of atherosclerosis in primates results in reabsorption of lipids from the intima and reduction of intimal thickening.7,8 During regression of atherosclerosis, many monocyte/macrophages disappear from the vessel wall.9 The second goal of this study was to determine whether regression of atherosclerosis in monkeys is accompanied by a decrease in Ang II–containing cells in intimal lesions.

Methods

Three groups of cynomolgus monkeys were studied. Five monkeys were fed commercial laboratory diet (Purina monkey chow, Ralston Purina; normal group). Five monkeys were fed an atherogenic diet containing cholesterol (1 mg/calorie) and fat (43% total calories) for 23±1.9 months (mean±SEM; atherosclerotic group). Five monkeys were fed an atherogenic diet for 27±3.6 months followed by commercial laboratory diet for 14±2.8 months (regression group). Monkeys were sedated with ketamine (15 mg/kg IM) and euthanized with intravenous KCl. Segments of thoracic aorta and left circumflex coronary artery were removed and cleared of adherent fat, fixed in 10% formaldehyde, and embedded in paraffin. Serial sections were stained for the presence of Ang II, macrophages, and smooth muscle cells (SMCs).

Samples of human atherosclerotic coronary artery were obtained during clinically indicated directional atherectomy in 5 patients (AtheroCath, Devices for Vascular Intervention, Inc). All procedures were approved by the Animal Care and Human Use Committee at the University of Iowa.
Immunohistochemical Staining of Ang II

Sections 3 µm thick were cut and mounted on microscope slides, dried in room air for at least 4 hours, deparaffinized (Amiclear, Baxter), and rehydrated in ethyl alcohol. Slides were incubated in PBS, pH 7.0, washed with PBS, and covered with 5% normal goat serum (Vector Laboratories) and 0.05% Triton X-100 for 60 minutes to block nonspecific protein binding sites.

The immunoreaction was performed by an alkaline phosphatase method with 3 different polyclonal rabbit anti–human Ang II antisera. The immunoreaction, performed with antiserum from Peninsula Laboratories Inc, was similar to that used for staining of circulating leukocytes, except that the primary antibody was diluted 1:500 in 1% normal goat serum and 0.1% BSA and tissue sections were incubated for 60 minutes at 37°C. The second antiserum (“Denise”) was obtained from Martin Cassell (University of Iowa, a gift from D. Ganten), and the third antiserum (“RAB 82”) was a gift from Donna Farley (University of Iowa). Each antiserum was diluted 1:500 in 1% normal goat serum, and tissue sections were incubated for 60 minutes at 37°C. The Denise antiserum has been previously tested for cross-reactivity and sensitivity.10

After further washing, slides were incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratories) and conjugated with avidin. Vector Blue was used as a chromogenic substrate (0.1 mol/L Tris buffer, pH 8.2). Sections were counterstained with nuclear fast red. As positive controls for Ang II, we used juxtaglomerular cells of normal monkey kidney and sections of normal monkey aorta incubated with 10⁻¹¹ to 10⁻⁹ mol/L human Ang II (Peninsula Laboratories Inc) for 60 minutes.11,12 Negative controls were performed by substituting normal rabbit serum for primary antiserum or omitting the primary antiserum and omitting the primary antiserum from the 1% normal goat serum.

The specificity of the antiserum for Ang II was tested by solid-phase preabsorption with Ang II bound to Sepharose beads, similar to the procedure of Swaab and Poole13 and Imboden et al.14 Conjugation of preabsorption with Ang II bound to Sepharose beads, similar to the goat serum. Antiserum and omitting the primary antiserum from the 1% normal goat serum.

Results

Plasma Lipids

Plasma total cholesterol concentration was 123±13 mg/dL (mean±SEM) in normal monkeys and 648±41 mg/dL in atherosclerotic monkeys. Plasma cholesterol in regression monkeys was 682±62 mg/dL when they received the atherogenic diet and 121±4.6 mg/dL after they were returned to the normal diet.

Ang II in Monkey Arteries

In aorta and coronary arteries from normal monkeys, there was faint, diffuse staining in the media of each vessel, with darker staining in the adventitia (Figure 1A). Cell-associated anti–Ang II staining was very sparse and restricted to a few cells of the endothelium and adventitia (Figure 1A).

In atherosclerotic aortas and coronary arteries, Ang II was localized in the fibrofatty region of the thickened intima and immediately beneath the endothelium. Anti–Ang II staining was observed both within cells and extracellularly throughout the lesion and adventitia (Figure 1B). Areas with cell-associated staining had an intense blue color, in contrast to extracellular staining, which was a diffuse, light blue. Similar anti–Ang II staining was noted in adjacent sections stained with the Peninsula, Denise (Figure 2), and RAB 82 antisera.

Anti–Ang II staining in monkeys was similar in coronary arteries and aortas. Normal rabbit serum was used as a negative control and produced no anti–Ang II immunostaining.

We used liquid-phase preabsorption to determine whether excess Ang II would prevent binding of the antiserum for Ang II to tissue. We were not able to prevent binding of the Ang II antiserum to tissue using 10 µg/mL to 10 mg/mL of unconjugated Ang II. This finding is similar to that of Imboden et al.14 for Ang II antiserum and Swaab and Poole13 for other antisera (antioxytocin and antivasopressin), who also were unable to successfully preabsorb the antisera with excess peptide (Ang II, oxytocin, or vasopressin).

Solid-phase preabsorption was used to examine specificity of the Peninsula antiserum for Ang II. Antiserum for Ang II developed by 3,3′-diaminobenzidine (Vector Laboratories). Tryptsin incubation and antigen retrieval were not used in sections that were stained with HHF-35. Sections stained with HAM-56 were counterstained with nuclear fast red. Sections stained with HHF-35 were not counterstained. For both stains, the primary antibody was excluded from 1% normal goat serum as a negative control. Each section was viewed under a light microscope and photographed.

To quantify Ang II–containing cells in atherosclerotic lesions, total cells and cells that stained positively to the anti–Ang II antisera were counted in the thickened intima-media of atherosclerotic and regressive arteries. Endothelial cells were not counted. There were no cells between the endothelium and internal elastic lamina of any arteries from normal monkeys. Four high-power fields (×400) were chosen at evenly spaced radial locations within lesions of atherosclerotic and regression monkey vessels. If the lesion was sufficiently large, another field was counted. On average, 500 to 600 cells were counted in each section. Only 1 high-power field was counted in each of 4 to 5 sections from each human coronary atherosclerotic sample.

Values for normal, atherosclerotic, and regressive arteries were compared by a 1-way ANOVA, followed by Bonferroni’s corrected t test. A value of P<0.05 was considered statistically significant.
Figure 1. Immunohistochemical staining of monkey aorta for angiotensin II (Ang II) with Peninsula antiserum and counterstained with nuclear fast red. A, Normal monkey. There is diffuse, faint staining in adventitia and minimal staining in endothelium (magnification ×130). B, Atherosclerotic monkey. There is dark cell-associated staining in intima-media and diffuse extracellular staining in intima-media and adventitia (magnification ×85). C, Regression monkey. There is faint diffuse staining in intima-media and adventitia (magnification ×85).
that was preabsorbed once by solid-phase preabsorption with Ang II bound to Sepharose beads did not produce cell-associated staining in the intima-media. Some diffuse blue staining was observed in the adventitia, media, and intima-media with antiserum that was preabsorbed once. Antiserum that was preabsorbed 3 times did not produce any immunostaining, either cell-associated or diffuse. Incubation of antiserum with the negative control Sepharose beads (with no conjugated Ang II) resulted in staining that was similar to staining observed in sections with primary Ang II antiserum.

After regression of atherosclerosis, cell-associated anti-Ang II staining appeared to decrease compared with atherosclerotic arteries, and extracellular stain was also reduced (Figure 1C).

**Macrophages and SMCs**

To determine the cellular localization of Ang II, adjacent serial sections were stained for the presence of Ang II, macrophages (with HAM-56), and SMCs (with HHF-35). Normal blood vessels did not stain for macrophages in the endothelium or media, but staining of macrophages was occasionally observed in the adventitia. In normal vessels, no correlation was detected between the diffuse anti–Ang II staining and macrophages or SMCs.

Intima-media of atherosclerotic lesions contained cells with Ang II (Figure 3A) that appeared, with HAM-56, to be macrophages (Figure 3B). Discrete areas stained very strongly with both Ang II antibody and HAM-56, suggesting clusters of macrophages. The media and intima-media stained for SMCs with HHF-35 (Figure 3C). Staining of cell-associated Ang II in atherosclerotic lesions was not associated with anti–smooth muscle cell staining (Figure 3).

**Human Coronary Atherectomy Samples**

Anti–Ang II staining was observed in all vessels and appeared to be both cell-associated and extracellular (Figure 4A). There was positive staining for macrophages (Figure 4B) and SMCs (Figure 4C). Because tissue samples were small and orientation was difficult, reliable assessment of correlation between Ang II and a specific cell type was not possible.

**Quantification of Cells**

There were minimal macrophages in normal vessels. Atherosclerotic aortas and coronary arteries contained a total of 157±10 and 132±9 cells per high-power field, respectively (both P<0.05 versus normal vessels). Aortas and coronary arteries from regression monkeys contained a total of 83±7 and 73±6 cells per high-power field, respectively (aortas, P>0.05 versus normal vessels; coronary arteries, P<0.05 versus normal vessels; both P<0.05 versus atherosclerotic vessels).

There were virtually no Ang II–containing cells in normal vessels (Figure 5). There were a large number of Ang II–containing cells in intima-media of aortas and coronary arteries of atherosclerotic monkeys (54±3% of total cells; both P<0.05 versus normal vessels) (Figure 5). After regression of atherosclerosis, the number of Ang II–containing cells decreased significantly in intima-media of aortas and coronary arteries (both P<0.05 versus normal and atherosclerotic vessels) (Figure 5).

Atherectomy samples from human coronary arteries contained a large number of Ang II–containing cells (57±15% of total cells) (Figure 5).

**Discussion**

The major finding of the present study is that the intima-media of atherosclerotic blood vessels of monkeys and humans contains substantial amounts of Ang II, mainly colocalized with macrophages in atherosclerotic vessels from monkeys. During regression of atherosclerosis in monkeys, cell-associated Ang II and macrophages decreased in the intima-media.

In a previous study in which immunocytochemistry was used to demonstrate Ang II in circulating monocytes, we also used radioimmunoassay to quantitate the amount of Ang II. With radioimmunoassay, we demonstrated that there is indeed a large amount of Ang II in monocytes (about 2300 fmol/mg protein). In the present study, it was not possible to quantify the amount of Ang II in lesions, but dense staining in >50% of macrophages in vessels of atherosclerotic monkeys and >50% of cells in lesions of atherosclerotic humans suggests that atherosclerotic lesions contain a large amount of Ang II.

Three approaches were used to examine specificity of the antisera for Ang II. First, we used 3 different antisera. All antisera demonstrated a similar pattern of staining. Second, we used liquid-phase preabsorption with excess Ang II. Excess Ang II did not prevent staining in tissue sections by Ang II antisera, as discussed below. Third, we performed solid-phase preabsorption of antiserum with Ang II conjugated to Sepharose beads. Cell-associated staining in intima-
Figure 3. Immunohistochemical staining in serial sections of atherosclerotic monkey aorta. A, Angiotensin II (Ang II) with Peninsula antiserum. B, Macrophages with HAM-56. C, Smooth muscle cells with HHF-35. Magnification ×217. Anti–Ang II staining is observed in intima-media and adventitia. Anti–Ang II staining in intima-media appears to be associated with macrophages and not associated with smooth muscle cells.
Figure 4. Immunohistochemical staining in serial sections of atherosclerotic human coronary artery. A, Angiotensin II (Ang II) with Peninsula antiserum. B, Macrophages with HAM-56. C, Smooth muscle cells with HHF-35. Magnification ×425. Blue anti–Ang II staining appeared to be both associated with cells and extracellular. In some cells (arrows), blue stain appeared to be perinuclear. It was not possible to determine whether anti–Ang II staining was associated with macrophages or smooth muscle cells.
Angiotensin II in Atherosclerotic Lesions

Macrophage-associated staining for Ang II was widely distributed throughout the intima-media of atherosclerotic blood vessels in monkeys. Extracellular anti–Ang II stain, which was less intense than the cell-associated stain, was observed in the adventitia and media of each group.

Cellular and extracellular Ang II was also identified in samples from human atherosclerotic coronary arteries. It was not possible to determine in atherectomy samples whether Ang II–containing cells colocalized with specific types of cells, because features of adjacent sections were difficult to identify and orientation within a vessel was not feasible.

The origin of Ang II in macrophages in blood vessels is not clear. It is not known whether Ang II is produced from angiotensinogen within monocyte/macrophages or whether it is taken up from plasma by monocytes or monocyte/macrophages. Monocytes contain cathepsin G,16–18 which cleaves angiotensinogen directly to Ang II. Also, macrophages synthesize angiotensin45 and contain ACE20,21 and thus may generate intracellular Ang II. In addition, monocyte/macrophages have receptors for Ang II,6,22,23 which take up Ang II by endocytosis.23 Thus, monocyte/macrophages may be capable of either taking up Ang II from plasma or synthesizing Ang II intracellularly.

Effect of Regression on Ang II in Lesions

There was less Ang II in vessels after regression of atherosclerosis than in atherosclerotic vessels. Cell-associated staining for Ang II decreased significantly after regression, although diffuse extracellular staining was still observed in the intima-media. The finding that there are fewer macrophages in the intima-media after regression of atherosclerosis9 was confirmed in the present study. The marked decrease in macrophages after regression of atherosclerosis parallels the decrease of Ang II–containing cells in intima-media after regression of atherosclerosis.

Limitations of Method for Immunohistochemical Staining for Ang II

Because Ang II is a small, diffusible peptide, it is likely that Ang II leaked out of cells during fixation, accounting for the diffuse staining. Because this artifact may contribute to diffuse Ang II staining, cell-associated localization of Ang II was more convincing than diffuse staining, especially because Ang II in intima-media appeared to colocalize with macrophages in monkeys. The dark blue staining with 3 different antisera was similar to staining of juxtaglomerular cells of monkey kidney (positive controls).11,12 Cell-associated localization of Ang II was similar with 3 different antisera and was prevented by solid-phase preabsorption. Both findings strongly suggest that the dark blue staining was specific for Ang II. We also noted perinuclear anti–Ang II staining in some samples (eg, Figure 4A), which has been reported in cardiac myocytes and fibroblasts.24

In contrast to the cell-associated staining, it is difficult to know whether diffuse blue staining is specific for Ang II. After incubation of monkey coronary artery with purified human Ang II, there was an increase in diffuse blue staining in the adventitia and a less pronounced increase in diffuse blue staining in the intima-media and media. Antiserum preabsorbed 3 times with conjugated Ang II prevented anti–Ang II staining. Thus, it seems likely that the diffuse blue extracellular stain was specific for Ang II.

Anti–Ang II staining was patchy in endothelium of normal monkey blood vessels. Endothelial cells contain ACE and several components of the renin-angiotensin system,25 which suggests that the staining in normal monkey blood vessels is Ang II. Moreover, there was no staining in endothelial cells after solid-phase preabsorption and in experiments in which normal rabbit serum was substituted for the primary antibody. Thus, staining of endothelium in normal monkey blood vessels probably indicates Ang II.

Because each Ang II antiserum reacts completely with Ang III, we cannot exclude the possibility that Ang III is also present in atherosclerotic aortas and coronary arteries from monkeys and humans.
Possible Role of Ang II in Atherosclerotic Lesions

Ang II has been identified in circulating human mononuclear leukocytes, macrophages in granulomas in mice, and now in macrophages in atherosclerotic lesions. Vascular proliferation and remodeling are major events in formation of atherosclerotic lesions. Because Ang II appears to be important in modulation of proliferation of vascular SMCs and production of extracellular matrix, we speculate that Ang II in macrophages may play an important role during vascular proliferation and remodeling in atherosclerosis. Ang II appears to be chemotactic for adherent monocytes. Thus, Ang II may also stimulate infiltration and accumulation of macrophages in atherosclerotic lesions. Furthermore, Ang II-modified LDL may be taken up by macrophages via the scavenger receptor at an enhanced rate. Thus, Ang II may contribute to vascular dysfunction in atherosclerosis not only through stimulation of proliferation of SMCs but also by increasing accumulation of cholesterol and formation of foam cells.

In conclusion, these findings demonstrate a potentially important and previously unrecognized source of Ang II in atherosclerotic lesions. The presence of Ang II in cells within the intima-media of atherosclerotic blood vessels and the decrease during regression may have important implications for the role of Ang II in the pathophysiology of atherosclerosis.

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