Aging Increases Aortic MMP-2 Activity and Angiotensin II in Nonhuman Primates

Mingyi Wang, Gen Takagi, Kuniya Asai, Ranilo G. Resuello, Filipinas F. Natividad, Dorothy E. Vatner, Stephen F. Vatner, Edward G. Lakatta

Abstract—To seek evidence that the nonhuman primate arterial wall, as it ages in the absence of atherosclerosis, exhibits alterations in pathways that are involved in the pathogenesis of experimental atherosclerosis, we assessed aortic matrix metalloproteinase-2 (MMP-2) and its regulators, ie, membrane type-1 of matrix metalloproteinase (MT1-MMP) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), and the expression of angiotensin II (Ang II), angiotensin-converting enzyme (ACE), and chymase in young (6.4±0.7 years) and old (20.0±1.9 years) male monkeys. With advancing age, (1) the intimal thickness increased 3-fold and contained numerous vascular smooth muscle cells and matrix, but no inflammatory cells; (2) the intimal MMP-2 antibody–staining fraction increased by 80% (P<0.01); (3) in situ zymography showed that MMP-2 activity, mainly confined to the intima, increased 3-fold (P<0.01); (4) the MT1-MMP antibody–staining fraction increased by 150% (P<0.001), but the TIMP-2 antibody–staining fraction did not significantly change; (5) steady levels of the mRNA-staining fraction (via in situ hybridization) for MMP-2 increased 7-fold, for MT1-MMP increased 9-fold, and for TIMP-2 increased 2-fold (all P<0.001); and (6) intimal Ang II and ACE immunofluorescence were increased 5-fold and 5.6-fold, respectively, and colocalized with MMP-2. Thus, age-associated arterial remodeling and the development and progression of experimental atherosclerosis in young animals share common mechanisms, ie, MMP-2 activation and increased Ang II signaling. This might explain, in part, the dramatically exaggerated prevalence and severity of vascular diseases with aging. (Hypertension. 2003;41:1308-1316.)

Key Words: aging ■ vasculature ■ enzymes ■ angiotensin II ■ monkeys

Although advancing age is the major risk factor for vascular diseases, eg, hypertension and atherosclerosis, mechanisms that underlie the “risky” aspects of aging remain obscure. One hypothesis to explain this exaggerated risk is that age-associated changes occurring within the vascular wall render it a more susceptible substrate on which the more-often-studied lifestyle risk factors for atherosclerosis, eg, hypercholesteremia, can flourish. In unselected humans at autopsy and in animal models studied to date, diffuse age-associated intimal thickening and increased arterial stiffness are accompanied by breaks in the internal elastic lamina and impaired endothelial function.1 Many of the aforementioned features of age-associated intimal thickening also characterize the intimal thickening of vein grafts subsequent to (1 to 5 years) the acute-injury phase after arterialization but before the subsequent development of atherosclerosis.2

Studies aiming to elucidate specific links between the aging vascular wall and increased risk of atherosclerotic arterial lesions require an animal model in which mechanisms that underlie both age-associated arterial remodeling and atherosclerosis can be investigated. Nonhuman primates appear to be ideal in this regard. Although atherosclerosis can be experimentally produced and manipulated in these species,3 it does not develop in the absence of the introduction of “human” risk factors, eg, dietary risk factors. However, diffuse, age-associated aortic intimal thickening and other features of matrix remodeling occur in nonhypertensive, nonhuman primates with adult aging, as in other animal species and humans,4 and are accompanied by increased stiffness and endothelial dysfunction,4 which are also salient features of human aging in otherwise healthy persons and in other animal species.1,5-7 This profile of age-associated remodeling might place these vessels at high risk for the subsequent development of atherosclerosis, because equivalent increases in plasma lipids induced by high-cholesterol diets in younger and older nonhuman primates produce markedly more severe atherosclerotic lesions in the latter than in the former.8 Although these observations support the idea that aging of the vascular wall in nonhuman primates confers additional risk to plasma cholesterol with respect to the
development of atherosclerosis, the basis for the augmented age-associated risk of the aging artery remains obscure. We hypothesized that some of the mechanisms involved in mediating the development and progression of experimental atherosclerosis in young animals, ie, matrix metalloproteinase type II (MMP-2); the factors that regulate its activity, ie, membrane type-1 of matrix metalloproteinase (MT1-MMP) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2); and the expression of angiotensin II (Ang II), angiotensin-converting enzyme (ACE), and chymase are also implicated in age-associated arterial remodeling in the nonhuman primate.

Methods

Animals and Aortic Histology
Six young (6.4±0.7 years) and 8 old (20.0±1.9 years) male monkeys (Macaca fascicularis) were maintained and humanely killed according to methods previously described. Segments of the thoracic aorta were harvested, stained, and studied as described previously in rodents.6

Immunohistochemistry and Immunofluorescence
Immunostaining was performed according to the modification of methods previously described.6

In Situ Hybridization
In situ hybridization was performed by the methods previously described.6

Gelatin Zymography and Western Blots
Gelatin zymography and western blotting for MMP-2, MT1-MMP, and TIMP-2 were performed according to the methods as previously described.5,6

In Situ Zymography
In situ zymography was performed by the method previously described.6

Statistical Analysis
All results are expressed as the mean±SEM. Statistical comparisons of both groups were made with Student t tests. A probability value <0.05 was taken as statistically significant. A detailed account of the methods is provided in the appendix and online.

Online Supplement
An expanded Methods section can be found in an online supplement available at http://www.hypertensionaha.org.

Results

Histomorphometry and Cellularity
The intima and media increased in thickness by 3.2-fold and 1.5-fold with age, respectively, from values at 6.4 years old of 29.9±21.8 and 589.1±103.2 μm, respectively (P<0.01 for both). The thickened intima contained both cells and matrix. Nearly all of the cells stained positively for α-smooth muscle actin (SMA; Figure 1). The average intimal α-SMA–staining area increased by 3.4-fold with aging (P<0.01). Staining for anti-CD68 was negative, indicating that cells were not monocytes/macrophages.

Total Aortic MMP-2 and Regulators of MMP Activity
Western blotting (Figure 2) indicated a 2-fold average increase in total aortic protein levels of both MMP-2 and MT1-MMP, a tissue activator of MMP-2, and a smaller (25%), but statistically significant, increase in aortic TIMP-2, an endogenous inhibitor of MMP-2. The ratios of MMP-2 and MT1-MMP protein to TIMP protein increased by 50%. In vitro polyacrylamide gel electrophoresis zymography indicated no significant difference in latent MMP-2 between young and old aortas. However, activated MMP-2 was markedly (3-fold) increased in the old aortas (data not shown).

Localization and Semiquantification of MMP-2, MT1-MMP-2, and TIMP-2 Within the Aortic Wall
The intimal MMP-2– and MT1-MMP–staining fraction increased nearly 2-fold with aging (examples in Figure 3 and average data in Table 1). The staining fraction for TIMP-2 within the intima did not vary with age. The ratios of intimal MMP-2 and MT1-MMP staining to TIMP-2 staining increased with age. MMP-2 staining was particularly noticeable near the internal elastic membrane in the thickened intima of the older animals (Figure 3, upper right). The increased MMP-2 staining within the old intima was localized not only to the matrix but also within cells. Figure 4 indicates that MMP-2, MT1-MMP, and TIMP-2 were colocalized within the intima. Doubling staining indicated that MMP-2 staining colocalized with cell-specific markers for endothelial cells (CD31) and for smooth muscle cells (α-SMA) (Figure 5).

The MMP-2–staining fraction within the aortic media also increased with age (Figure 3) by ~70% (Table 1, right columns). In contrast to the intima, the TIMP-2–staining
fraction within the media also increased with age (Figure 3) by ≈70% (Table 1). The medial MT1-MMP–staining fraction increased with age (Figure 3) by ≈50% (Table 1). Thus, in contrast to the intima, the ratios of medial MMP-2 and MT1-MMP to TIMP-2 staining did not change with age (Table 1).

**In Situ mRNA Hybridization of MMP-2, MT1-MMP, and TIMP-2**

We used in situ hybridization to determine whether the age-associated changes in aortic MMP-2, TIMP-2, and MT1-MMP protein staining could be attributed, in part, to changes in the levels of mRNA coding for these proteins. Figure 6 illustrates representative aortic sections from a young and an old monkey stained for mRNA probes. In situ MMP-2–, MT1-MMP–, and TIMP mRNA–staining fraction were increased in the old aorta. Increases in MMP-2 and MT1-MMP mRNA were particularly marked in the intima (Figure 6, upper and lower left; Table 2). Steady levels of the mRNA-staining fraction for MMP-2 increased 7-fold; for MT1-MMP, it increased 9-fold; and for TIMP-2, it increased 2-fold in the thickened intima of the older monkey (all *P*<0.001) (Table 2). This pattern of altered staining for mRNA in the aged aorta is similar to that of staining of their proteins.

**Figure 2.** A, Western blot for MMP-2, TIMP-2, and MT1-MMP in aortic extracts from monkeys. B, Quantitative analysis of ratios of MMP-2 to TIMP-2 and MMP-2 to MT1-MMP in aortic extracts from monkeys. *P*<0.01 vs young.

**Figure 3.** Representative photographs of immunostaining for MMP-2, TIMP-2, and MT1-MMP (all brown) on paraffin sections of aortas from monkeys. L indicates lumen; M, media.
Similarly, the ratios of MMP-2 and MT1-MMP to TIMP mRNA staining, like those of their protein staining ratios, increased with age in the intima but did not change significantly with age within the media (Table 2).

**In Situ Activation of MMP-2**

The colocalization and imbalance of staining for factors that regulate MMP-2 activity within the old intima, coupled to the increased intimal MMP-2 staining, imply that intimal MMP-2 activity increases with age in situ (Figure 4). We used in situ zymography to determine whether MMP-2 was indeed increased in the older aorta in vivo. Figure 7 indicates strong gelatinolytic activity, indicated by a green fluorescence, largely confined to the thickened intima of old aorta. When the experiment was repeated in the presence of an antibody against MMP-2, gelatinolytic activity was absent (Figure 7, right panel).

**Expression and Localization of Ang II, ACE, and Chymase**

Recent evidence indicates that among myriad other actions, Ang II is involved in MMP-2 activation.\(^1\)\(^1\),\(^1\)\(^2\) Figure 8 illustrates that Ang II staining was increased \(\approx 5\)-fold in the thickened intima of older aorta (\(P<0.001\)). Both ACE and chymase can cleave Ang I into Ang II. ACE could not be detected by conventional immunostaining in frozen sections. However, after catalyzed signal amplification, an ACE signal could be detected and was found to be increased \(\approx 5.6\)-fold in old versus young intimas (\(P<0.001\)) (Figures 9A and 9B). Chymase-stained cells were detected only within the older monkey aorta and only within the adventitia (Figure 9C). Figure 10 shows that ACE and Ang II were colocalized in the thickened intima of older monkey, suggesting that intimal Ang II was produced mainly via an ACE-dependent pathway; moreover, Ang II also colocalized with MMP-2 in the thickened intima.

**Discussion**

It has been well established that advancing age is associated with arterial remodeling in diverse species ranging from humans to rodents.\(^1\)\(^3\)–\(^8\) Reduced shear stress due to luminal dilation in the absence of enhanced blood flow velocity and possibly increased wall stress during the cardiac cycle due to arterial stiffening are related in part to reduced endothelial function and in part to changes in the amount and characteristics of the matrix molecules, collagen and elastin, which are potential stimuli involved in age-associated aortic remodeling.\(^1\)\(^4\)–\(^6\) In the nonhuman primate, diffuse, intimal-medial thickening and luminal dilation occur in the context of increased stiffness\(^4\) and endothelial dysfunction.\(^4\) The present results did not detect evidence of lipid or inflammatory components of atherosclerosis in the age-associated aortic remodeling in the nonhuman primate; likewise, there was no evidence of the presence of early, ie, highly proliferative or thrombotic, phases of the arterial response to injury.\(^9\) The present results, however, do indicate that altered activity of growth factors and proteases that characterize the third or

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Intima</th>
<th>Media</th>
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<tr>
<td></td>
<td>Young</td>
<td>Old</td>
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<tr>
<td>MMP-2</td>
<td>16.43±3.72</td>
<td>29.43±4.26*</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>47.25±2.24</td>
<td>48.26±3.96</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>21.95±3.75</td>
<td>53.82±5.03*</td>
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<tr>
<td>MMP-2/TIMP-2</td>
<td>0.36±0.08</td>
<td>0.61±0.06*</td>
</tr>
<tr>
<td>MT1MMP/TIMP-2</td>
<td>0.46±0.07</td>
<td>1.11±0.04*</td>
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Data are expressed as percentages (±SD). *Significant difference old vs young; the ratios of MMP-2 and MT1-MMP to TIMP-2 staining (MMP-2/TIMP-2 and MT1MMP/TIMP, respectively).
chronic stage of arterial injury, perhaps similar to the chronically altered preatherosclerotic stage of vein graft atheromatous occlusion, might be implicated in the chronic process of age-associated arterial remodeling.\(^2\)\(^9\) particularly with respect to intimal thickening. Specifically, aortic MMP-2 activation and Ang II signaling, both of which have been implicated in both arterial injury and atherosclerosis,\(^10\)\(^–\)\(^15\) are increased in aortas of older compared with younger adult nonhuman primates.

Increased extracellular protease activity orchestrated by MMPs is central to multiple aspects of tissue remodeling—cell proliferation, chemotaxis, and invasion, as well as matrix organization. The present results demonstrate that active MMP-2 protein increased within the thickened intima of the old monkey aorta. This might affect the vascular remodeling that accompanies aging in multiple ways. It might be implicated in the disruption of the internal elastic lamina. MMP-2 accumulates in vascular smooth muscle cells (vascular SMCs), in the elastic lamina (vide infra), and in the area surrounding SMCs in the vicinity of breaks in the internal elastic lamina and along elastic laminae throughout the media of the aged aorta.\(^5\) Several members of the MMP family display in vitro elastolytic activity. MMP-2 has a high affinity for components of mature elastic fibers,\(^6\)\(^,\)\(^16\) likely conferred by its repeats that are essential for elastase activity.

Degradation of elastic fibers by activated MMP-2 might facilitate migration of cells from the adventitia/media or from the circulation\(^5\)\(^,\)\(^15\) into the thickened media. The development of a synthetic, migratory phenotype of SMC in vitro with successive passage in culture is accompanied by a high constitutive production of MMP-2; serum withdrawal reduces MMP-2 production and prevents SMC invasion of basement membranes.\(^14\) Thus, increased MMP-2 activity in situ might mediate an age-associated shift in some cells within the aortic media from the contractile to the synthetic-migratory phenotype. Evidence for in situ dedifferentiation of some aortic SMCs (or induction of some precursor cells to differentiate into actin-staining intimal cells) with aging includes the observation that, unlike aortic medial SMCs from young rats, which in early passage in culture fail to migrate in response to a platelet-derived growth factor gradient, early-passaged aortic medial cells from old rats readily migrate in response to this stimulus.\(^5\)\(^,\)\(^17\)

Figure 5. Colocalization of MMP-2 staining with staining for endothelial and SMC markers on paraffin sections of representative aortas from old monkey. Double immunostaining was applied to continuous sections: MMP-2 was detected with AEC (red), and antibodies against α-smooth muscle actin (SMA) or CD31 were detected with NBT/BCIP (dark purple). L indicates lumen; IEL, internal elastic lamina.

Figure 6. Representative mRNA in situ hybridization on paraffin sections of aortas from monkeys. Sections were stained with digoxigenin-labeled cRNA probes for rat MMP-2, TIMP-2, and MT1-MMP (dark blue) with fast-red counterstain. L indicates lumen; M, media.
The age-associated increase in intimal MMP-2 likely results from secretion from endothelial cells and intimal SMCs, because macrophage cells could not be detected. The increased in situ intimal MMP-2 activity of the old monkey is consistent with the prior in vitro and in vivo findings that MMP-2 activity is determined by the balance of TIMP-2 and its activators. Specifically, the ratios of MMP-2 and MT1-MMP mRNA to TIMP mRNA fractional staining, like those of their protein staining fraction ratios, significantly increase with age in the intima, a pattern similar to that observed with aging in FXBN rats.

TIMP-2 has multiple, potential roles in vascular remodeling. It plays dual roles in the in vitro cellular activation of MMP-2. TIMP-2 is bound to MT1-MMP as part of the MMP-2 "receptor" that forms triad complexes, which are required for activation of latent MMP-2; in contrast, free TIMP-2 binding to MMP-2 results in its inactivation. In the present study, although the TIMP-2 protein–staining fraction did not vary with age within the intima, the intimal TIMP-2 in situ hybridization was increased in the old aortas. This apparent discrepancy between TIMP mRNA and protein is explicable, in part at least, on the basis of a 3.2-fold increase in the intimal thickness with age; ie, total TIMP 2 staining within the intima substantially increases, consistent with an increase in TIMP-2 in situ hybridization within this vascular compartment. In addition to regulating the activity of MMP-2, TIMP-2 might have other roles in age-associated vascular remodeling aspects of cellular function. TIMP-2 stimulates the proliferation of fibrosarcoma cells and normal dermal fibroblasts via coupling Ras to the cAMP/protein kinase A signaling pathway and thus might also be related to age-associated intimal cell proliferation, medial vascular SMC hypertrophy, and vascular matrix production.

Ang II, a main effector of Ras, a vasoconstrictor, and a potent mitogen, is also a potent activator of MMP-2, an action that is mediated by activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and by reactive oxygen species. The local concentration of vascular Ang II is 1000-fold of circulating Ang II, and local Ang II plays an important role in the development of early atherosclerosis and the stability of plaques in the nonhuman primate. Rodents exhibit an age-associated increase in arterial Ang II receptor type 1. The notion that increased vascular Ang II with aging is indeed a factor in age-associated remodeling is strongly suggested by previous studies in which long-term ACE inhibition prevented or delayed age-associated vascular structural changes and endothelial dysfunction. Additionally, ACE inhibition or Ang II receptor antagonist reduce the intimal-medial thickness and improve arterial function in

### Table 2. Intimal and Medial mRNA In Situ Staining Fraction

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<th>Probe</th>
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<th>Medi a</th>
<th>Intima</th>
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<tbody>
<tr>
<td>MMP-2</td>
<td>4.30±1.76</td>
<td>30.65±4.75*</td>
<td>1.60±0.42</td>
<td>3.13±0.57*</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>1.43±0.39</td>
<td>3.83±0.56*</td>
<td>1.41±0.33</td>
<td>3.40±0.45*</td>
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<tr>
<td>MT1-MMP</td>
<td>1.76±0.50</td>
<td>7.50±2.87*</td>
<td>2.24±0.87</td>
<td>2.87±0.42</td>
</tr>
<tr>
<td>MMP-2/TIMP-2</td>
<td>3.38±1.91</td>
<td>8.11±1.43*</td>
<td>1.23±0.55</td>
<td>0.94±0.24</td>
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<tr>
<td>MT1MMP/TIMP-2</td>
<td>1.40±0.88</td>
<td>4.66±1.00*</td>
<td>1.67±0.77</td>
<td>0.86±0.17</td>
</tr>
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</table>

Data are expressed as percentages (±SD). *Significant difference old vs young; the ratios of MMP-2 and MT1-MMP to TIMP-2 staining (MMP-2/TIMP-2 and MT1MMP/TIMP, respectively).

Figure 7. In situ gelatin zymographs of monkey aortas. Controls were incubated in the absence of gelatin (left). Protease activity (green) is localized mainly in aortic intima of the old monkey (middle). Specific antibody to MMP-2 blocked digestion of the substrate (right). L indicates lumen; M, media.
hypertension with or without a reduction in blood pressure. In monkeys, local Ang II is mainly generated via ACE- and chymase-dependent pathways. In the present study, intimal ACE and Ang II staining were both increased with age, suggesting the presence of an age-associated increase in Ang II production through an ACE-dependent pathway and signaling. Chymase-positive cells, which contain predominant, chymaselike, Ang II-forming activities were also detected in the adventitia of the older monkey, suggesting the presence of an age-associated increase in Ang II production through chymase-dependent signaling pathways. Chymase also directly converts pro-MMP-1 to activated MMP-1, cleaves TIMP-1 to inactive fragments, and catalyzes complexes of MMP-1–TIMP-1, which have no MMP-1 activity, to form active MMP-1. A link between cardiac matrix remodeling, mast cells that are the major resource for chymase, and MMP-2 has already been established. Thus, increased local Ang II signaling via both ACE and chymase must also be considered as a factors that are involved in the
age-associated increase in intimal MMP-2 activation and arterial remodeling.  
Ang II signaling is involved in additional signaling pathways that modulate arterial remodeling. Downstream signaling events of Ang II via Ang II type 1 receptor signaling include the activation of early growth response factor-1 and activation of transforming growth factor-β1, which modulate cell proliferation, migration, and fibronectin production. Both early growth response factor-1 and transforming growth factor-β1 are increased with aging. Perspective  
The present study provides evidence that age-associated, large-artery remodeling in nonhuman primates occurs in the context of enhanced levels or activity of factors such as MMP-2 and its regulators and local Ang II, ie, molecules that have been implicated in experimental atherosclerosis. The activation of these nonlipid components of experimental atherosclerosis during arterial remodeling with aging supports the notion that these factors might, in part, account for the marked age-associated increase in the risk and severity of vascular disease. Thus, when the lipid risk factor is introduced into rabbits or nonhuman primates, the resulting atherosclerosis is more diffuse and severe. It is tempting to speculate that a similar scenario plays out in humans, in whom lipid and other risk components of atherosclerosis are not experimental but are linked to lifestyle.

References
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Figure 10. Colocalization of Ang II, ACE, and MMP-2 in aortic intima of old monkey. A, Ang II was detected with red color fluorescence; ACE was detected with green fluorescence. Overlap of ACE and Ang II staining produced yellow (rightmost panel). B, MMP-2 was detected with green fluorescence. Staining for Ang II was as in upper panels. Overlap of MMP-2 and Ang II staining produced yellow in the merged images. Nonimmune serum substituted for primary antibody as control (Con). L indicates lumen.


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