Increased Expression of Matrix Metalloproteinase-2 in the Thickened Intima of Aged Rats

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Abstract—To characterize remodeling of elastic arteries with aging and to investigate its potential mechanisms, matrix metalloproteinase-2 (MMP-2), intracellular adhesive molecule-1 (ICAM-1), transforming growth factor-β (TGF-β), and fibronectin protein levels were measured in the aortas of young adult (6 months) and aged (30 months) Fischer 344XBN rats. At 30 versus 6 months, the thickness of the intima was 5-fold greater and contained marked increases in TGF-β and ICAM-1, and fibronectin expression was enhanced throughout the aortic wall. Total MMP-2 protein (Western blot) of 30-month-old rats was increased 8-fold over that of 6-month-old rats (0.166±0.032 versus 0.020±0.006; P<0.01), and staining and activity were regionally localized to the intima, often near breaks in the internal elastic membrane and lamellae. Early passage, explanted smooth muscle cells (SMC) from aged aorta secreted more MMP-2 than those from young aorta; while basal MMP-2 production did not differ with age, after stimulation with cytokines (interleukin-1, tumor necrosis factor-α, or TGF-β, 10 ng/mL each for 24 hours), MMP-2 production in SMC from 30-month-old rats increased to levels greater than those in 6-month-old rats. Thus, enhanced expression of TGF-β, MMP-2, and ICAM-1 in the thickened vascular intima of aged rats may in part be produced by exaggerated SMC responses to cytokines and may have potential roles in intimal remodeling with aging. (Hypertension. 1999;33:116-123.)

Key Words: intimal thickening ■ aging ■ matrix metalloproteinase-2 ■ cytokines ■ cells, vascular smooth muscle

Since aging is the major risk factor for the development of vascular diseases, such as hypertension and atherosclerosis, that lead to stroke and heart failure,1 there is considerable interest in understanding the mechanisms that underlie the vascular remodeling that occurs in advanced age. Recent evidence2–4 suggests that matrix metalloproteinase-2 (MMP-2), a 72-kd type IV collagenase that degrades native type IV, V, VII, and X collagen, as well as denatured collagens5 and elastin,6 and growth factors, eg, transforming growth factor-β (TGF-β),7,8 are involved in vascular remodeling associated with vascular diseases and injury to promote both matrix protein degradation and enhanced smooth muscle cell (SMC) migration. Synthesis and activation of MMP-2 may be mediated by the stimulation of local cytokines.9

The specific aims of this study were (1) to determine whether latent and activated forms of MMP-2 in the aortic wall increased with aging in a novel hybrid rat strain, the Fischer 344 crossbred with the Brown Norway (F344XBN), in which an isolated increase in systolic arterial pressure occurs with aging, as in humans; (2) to determine whether SMC production of MMP-2 both under basal conditions and after cytokine stimulation was altered by age; and (3) to determine whether expression of other molecules known to be involved in vascular remodeling associated with atherosclerosis, restenosis, or hypertension, ie, the intracellular adhesive molecule-1 (ICAM-1), TGF-β, and fibronectin, differed in their expression with senescence.

Methods

Animals and Aorta Isolation

Male adult (6 months, n=10) and aged (30 months, n=10) F344XBN rats were killed by an overdose of sodium pentobarbital, and 4% neutralized formalin saline was infused through an abdominal vein for in situ fixation. A segment of descending thoracic aorta was removed without stretching or compression of the tissue. In another subset of rats (5 adult and 5 aged), the thoracic aorta was removed for protein extraction. Systolic blood pressure, measured by the tail-cuff technique, was increased by ~35% in 30-month-old rats (170.52±3.40 versus 127.38±6.24 mm Hg in 6-month-old rats; P<0.001), while the diastolic blood pressure did not differ with age.

Histology and Morphometry

Morphometric evaluation was performed with the use of a computerized imaging analysis system with IBAS 2.0 software. Medial thickness was calculated from the medial area and length. The thickness of the intima, corresponding to the layer between the lumen of surface and internal elastic lamina, was measured from images taken at ×400 magnification. To identify cell types within
the intima, the slides were immunostained with monoclonal antibodies against α-smooth muscle actin (Sigma), rat monocyte/macrophage surface antigens (Chemicon), and a polyclonal antibody that recognizes rat macrophages (Biosource International). The number of medial SMC was evaluated by averaging the number of nuclei in $5 \times 5$ random fields, which covered $60\%$ of a cross section.

**Immunohistochemistry and Immunofluorescence**

The slides were incubated with primary antibodies, including a rabbit polyclonal antibody against MMP-2 (a generous gift from Dr. William G. Stetler-Stevenson, National Cancer Institute, National Institutes of Health) and rabbit polyclonal pan-TGF-β antibody (R&D System) diluted in PBS containing 1% bovine serum albumin; nonspecific antigen was blocked with 10% nonimmune goat serum. The incubation and detection were performed as described previously.8 For immunostaining of ICAM-1, slides were incubated overnight with an antibody against ICAM-1 (Chemicon). A slide in which the incubation with the primary antibody was omitted and replaced with nonimmune goat serum served as a negative control.

**Western Blotting**

The thoracic aorta, including both the arch and descending aorta, were prepared for Western blotting as previously described.2 Each band on a blot of a given sample was scanned 3 times, and an average intensity was calculated to represent the relative quantity of MMP-2 or fibronectin.

**SDS-PAGE and In Situ Zymography**

Aortic tissue was homogenized in lysis containing 10 mmol/L sodium phosphate, pH 7.2, 1% Triton X-100, 0.1 SDS, 0.5% sodium deoxycholate, and 0.2% sodium azide, as previously described.9 For detection of MMP-2 in cultured SMC conditioned medium, equal amounts of conditioned medium (see below) and 2× sample buffer were mixed and loaded onto the zymography gel. Activated (100 ng) and a combination of activated and latent forms (100 ng total) of human recombinant MMP-2 (a generous gift of Dr. R. Fridman, Wayne State University School of Medicine, Detroit, Mich) were also loaded onto the gel and served as a standard for MMP-2. The gel was renatured by incubation with NOVEX renaturing buffer for 30 minutes at room temperature and incubated in developing buffer at 37°C overnight. Finally, the gel was stained in 0.5% Coomassie blue followed by destaining to visualize the proteolytic lysis bands. The presence of MMP-2 activity within the aorta in situ was detected by an in situ zymographic technique, as described by Galis.10

**SMC Culture and Cytokine Treatment**

SMC cultures were established from the explanted aortas of young and old rats as described previously.11 SMC (1×10⁶ cells per well) were fed with DMEM containing 10% fetal bovine serum for 24 hours to allow attachment and then incubated in serum-free DMEM for 48 hours. Cells incubated with serum-free DMEM but without any cytokine served as controls. The conditioned culture medium was harvested for detection of MMP-2 by the zymographic technique.

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**Figure 1.** Morphometric changes in the aortic wall of old (A) and young (B) rats. m indicates media; a, adventitia. Arrows show internal elastic lamina (Movat pentachrome stain; magnification ×400). C, The tunica media thickness increased by $\sim 38\%$ in old rats (solid bar indicates old rats; open bar, young rats) ($P<0.001$). D, In old rats, a thicker layer of intima developed, which was 5-fold larger than that in young animals (solid bar indicates old rats; open bar, young rats) ($P<0.001$) and was composed of a layer of endothelium and a subendothelial space that consisted mainly of extracellular matrix and cellular components.
noted above. Cells subcultured for 3 passages were used in this study.

Statistical Analysis
Data are presented as mean ± SD and were statistically analyzed with Student’s t test. Statistical significance was considered at P<0.05.

Results
Morphology and Morphometry
Compared with young rats, the aortic luminal diameter and tunica media in old rats were increased 28.3% and 38%, respectively (Figure 1A, 1B, and 1C). Although the medial thickness increased with age, the number of nuclei in a given medial area decreased by 18 ± 4%. The tunica intima was represented only by a single layer of endothelium in young animals (Figure 1B), but in old rats intimal thickening was observed (Figure 1A and 1D), containing both extracellular matrix and cellular components. The thickened intimal matrix of old aortas stained positively for both collagen and proteoglycans (results not shown).

In some regions of the old rat aortic wall, the continuity of the internal elastic lamina appeared disrupted (Figure 2A); medial SMC were often localized in the vicinity of the disrupted edge and seemed to project through these breaks into the intima (Figure 2B). These SMC, as well as those on the luminal side of the internal elastic lamina, were covered by a thin layer of connective tissue that had the same Movat pentachrome staining features as the

elastic lamina (Figure 2C). In some instances, the medial aspect of these newly formed basement membranes around SMC, ie, the medial part or that contiguous with the internal elastic lamina, became fragmented, while the luminal aspect of the SMC basement remained intact, appearing to become incorporated as a new segment into the internal elastic membrane (Figure 2D). Thus, a SMC that had been surrounded by its own elastic membrane and partitioned within the intima subsequently appeared to have become partitioned into the first muscle layer of the media by local degradation of the medial aspect of its basement membrane.

Smooth Muscle α-Actin Staining and Cell Type Identification
Medial SMC in both young and old rat aortas stained positively with an antibody against α-smooth muscle actin (Figure 3A and 3B). In the intima of old aortas, most cells in the subendothelial space also stained positively for α-smooth muscle actin, suggesting that they are or are derived from smooth muscle (Figure 3C). However, none of the intimal or medial cells stained positively with antibodies that recognize either macrophages or monocytes.

Expression of MMP-2
The overall level of MMP-2 was 3-fold higher in the aortic wall of old rats than that in young rats (Figure 4A). Figure 4B and 4C shows that MMP-2 staining was more intense in the intima of old than young rat aortas and that MMP-2 was sometimes seen in close proximity to intimal SMC.
Accumulation of MMP-2 along the internal elastic lamina and surrounding SMC just beneath the internal elastic lamina was also occasionally observed (Figure 4D). In old rats, both activated and latent forms of MMP-2 (Figure 5A) were more than 2-fold higher than in young rats (Figure 5B), consistent with the changes observed by Western blotting (Figure 4A). There was no age-dependent change in the ratio of activated to latent forms. MMP-2 activity observed by in situ zymography (Figure 6) can be recognized by areas with decreased fluorescence intensity (appearing as dark dots) or by an absence of fluorescence, resulting from the lysis of fluorescence-labeled substrate. In situ MMP-2 activity in young aortas was observed only in the adventitia, which is commonly thought to be nonspecific staining. There was no evidence of MMP-2 activity in the media and intima of young aortas. However, in old aortas in situ localized MMP-2 activity is present in thickened intimal layer and also within elastic lamellae throughout the media (Figure 6).

**MMP-2 Production in Cultured SMC Stimulated by Cytokines**

All cells cultured from the aortas of young and old rats stained positively for α-smooth muscle actin antibody (Figure 7A and 7B, top panel). In the absence of added cytokines, there was no age difference in MMP-2 levels (Figure 7A, bottom panel). Figure 7B and 7C (bottom panels) shows that after interleukin-1, the ratios of MMP-2 produced by SMC of old rats to that of SMC of young rats were 120.1% and 154.2% (latent and activated, respectively; both *P*, 0.01); after TNF-α the same ratios were 117.4% (*P*, 0.05) and 142.5% (*P*, 0.01) for latent and activated forms, respectively. Figure 7D (bottom panel) shows that the activated form of MMP-2 in conditioned media from SMC of old rats was 111.3% (*P*, 0.05) that of their young counterparts.

**TGF-β ICAM-1 and Fibronectin**

Immunohistochemical staining revealed that TGF-β was mainly present in the thickened intima of old aortas (Figure 8), a distribution pattern similar to that seen for MMP-2 (Figure 4). TGF-β was barely detectable in the media of old animals and was not visualized in the wall of young aortas (Figure 8). ICAM-1 staining (Figure 9) was observed only in the thickened intima of old rats. In contrast, there was no regional staining of this adhesive
molecule in the young rat aortic wall. The intensity of this fibronectin band on immunoblots in the aortic wall of old rats was significantly (5-fold) higher than that of young animals (1.36 ± 0.35 in old versus 0.25 ± 0.03 in young rats).

Discussion

The aorta exhibited a 5-fold increase in intimal width compared with that in young rats and was mainly composed of matrix molecules, including collagen and proteoglycan, and cells staining positively for smooth muscle actin, and it contained markedly higher levels of MMP-2, TGF-β, and ICAM-1. In contrast, the aortic intima of young rats consisted of only a single layer of endothelial cells, suggesting that actin-positive cells in the older intima may have migrated in from the media. The observed intimal changes with senescence in vivo in the present study exhibit several features of cell senescence in vitro, which include a loss of regulation of and overexpression of collagenase activity in fibroblasts12–14 and increased expression of the integrin ICAM.15

Intimal growth during aging in the absence of experimental injury in some ways resembles neointimal formation in response to injury.16 Prior studies have shown that neointimal growth in response to endothelial injury is markedly enhanced in old versus young rats and is due to factors intrinsic to the vessel wall11 and that may be attributable in part to enhanced SMC chemotaxis or proliferation in response to growth factors (eg, platelet-derived growth factor [PDGF]) or to resistance to growth-inhibitory effects of molecules such as TGF-β.17 PDGF-receptor mRNA in rat aorta increases with aging up to 40 weeks of age, and this age-associated increase is accelerated in experimental hypertension.18 In this regard, it is noteworthy that early passage (<5) SMC isolated from aortas of old (24 months) rats exhibit an exaggerated chemotactic response to PDGF, while cells from young aortas require several additional passages in culture to generate an equivalent response.19

SMC production of proteases and elastases is involved in digestion of the basement membrane surrounding SMC and in their migration through and invasion of the complex extracellular matrix of the vessel wall. SMC in culture secrete numerous factors with elastolytic activity, and that elastase-like activity increases with passage number.20 Recent studies of cultured SMC have demonstrated that chemotactic invasion of a reconstituted basement requires MMP-2 activity.21 Additionally, the expression and activity of MT-MMP (MMP-14), MMP-2, and MMP-9 increase during the response to mechanical arterial injury.3 MMP-2 also presents within atherosclerotic lesions. The proteolytic activity may weaken the fibrous cap, resulting in its rupture.2,22 The present study provides evidence of age-associated discontinuities of the internal elastic lamina in the aorta in the absence of externally imposed experimental injury. It has been hypothesized that both an increased elastase activity with aging, as observed in aortas isolated from humans, and an age-associated increase in Ca2+ and cholesterol deposition on elastin, rendering the latter more susceptible to elastase activity, contribute to elastin fragmentation or reduction in its content with aging.23 Both MMP-2 and MMP-9 (another type IV gelatinase) exhibit elastase activity,6 as does a metalloelastase cloned from macrophages.24 A novel finding of the present study is that both latent and activated forms of MMP-2 are greater in the aortas of old than in young rats and that the active levels of MMP-2 seemed to localize to the intima and elastic lamellae, as shown by in situ zymography. The demonstration of MMP-2 accumulation in the area surrounding SMC located just beneath the broken internal elastic lamina and along elastic laminae throughout the media in the present study (Figure 2) suggests that MMP-2 may have a role in fragmentation of the elastic laminae with aging.

Another novel finding of the present study is that SMC are potentially a source of the age-associated increase in MMP-2 in the aortic wall in situ, as early passage SMC from aged aorta secrete more MMP-2 than those from young aorta. The fact that basal SMC MMP-2 production does not differ significantly with aging, but enhanced MMP-2 levels were observed after stimulation of old versus young vascular SMC by cytokines, including interleukin-1, TNF-α, and TGF-β, suggests that enhanced MMP-2 levels in the thickened intima of aortas from aged...
rats may reflect a chronically enhanced level of cytokine stimuli in vivo.

The cytokine TGF-β exhibited an age-associated increase in situ and also localized to the intima as MMP-2. TGF-β, which normally suppresses protease activity and activates tissue inhibitors of metalloproteinase, is a potent factor for the synthesis of extracellular matrix proteins, and its expression can lead to excessive fibrosis. The accumulation of TGF-β in the aortic wall of aged rats between adulthood and senescence may account for the concomitant increase in fibronectin, which itself has a diverse effect on SMC phenotype properties. That fibronectin levels markedly increase with aging suggests that the observed increased TGF-β levels with aging are also accompanied by an increase in TGF-β activation. There is some evidence to indicate that the collagenolytic and antiproliferative actions of TGF-β decrease with aging.

The regional increase in ICAM-1 observed in the intima of aged aortas might be also related to the augmented levels of TGF-β, since the latter is known to induce the synthesis of cell adhesion receptors, which may lead to increased adhesion and interaction of cells with the surrounding extracellular matrix. Prior studies have shown that human endothelial cell senescence in vitro is accompanied by increased ICAM mRNA and protein and an enhanced capacity to bind monocytes. However, in the present study in rat aorta in vivo, there was no evidence of monocyte or macrophage infiltration in the intima. Enhanced ICAM-1 expression with little evidence of monocyte infiltration has also been observed in the endothelial cells of rat vessels following shear stress. Thus, expression of other adhesion molecules

Figure 7. Top panels, Immunofluorescent staining for α-smooth muscle actin in SMC from explant cultures from the aortas of young (top, A) and old (top, B) rats. All cultured cells in both age groups were SMC, as indicated by their α-actin staining (magnification ×400). Bottom panels, Comparison of in vitro MMP-2 production by aortic SMC of young and old rats without cytokine stimulation (A) and after stimulation by interleukin-1α (IL-1α), TNF-α, and TGF-β (B, C, and D). MMP-2 levels were detected by zymography of SMC-conditioned media. The data in the figure depict the level of MMP-2 measured in the media SMC of old rats (solid bars) relative to that measured in young rats (open bars).
in addition to ICAM-1 may also be required for adhesion of monocytes to the endothelial surface.

In summary, chronic morphological and biochemical modifications in the aortic intima of aging rats, ie, fragmentation of the internal elastic membrane and intimal thickening, and localized increases in growth factors and collagenase activity appear as a muted version of those chronic alterations associated with chronic hypertension or with transient changes that occur in response to acute mechanical injury, eg, after balloon angioplasty.3,7,10 Such vascular remodeling during aging in the absence of an externally imposed injury may underlie exaggerated responses to endothelial injury that have previously been reported to occur in older versus younger rats.16 The novel findings that increased MMP-2, TGF-β, and ICAM-1 levels are chronically elevated and localized to the thickened intima of aged rats in the present study not only provide insights into possible mechanisms of age-associated vascular remodeling but also provide new links between senescence markers in vitro and cell senescence in vivo. These molecular changes during vascular senescence in vivo may be targets for novel strategies for the prevention and treatment of age-associated vascular disorders.

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References


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