Role of the Extracellular Matrix in Age-Related Modifications of the Rat Aorta
Ultrastructural, Morphometric, and Enzymatic Evaluations
C. Fornieri, D. Quaglino Jr., and G. Mori

Connective tissues such as blood vessels are known to be greatly affected by age because of impaired functional properties and increased susceptibility to diseases. With the aim of providing further information on the role of the extracellular matrix in age-related modifications, we investigated the aorta in the rat model from birth to senescence by means of morphological and morphometric observations and by evaluation of lysyl oxidase activity. Results focused on the dramatic vascular rearrangements due to progressive fibrosis of the extracellular matrix and on prominent elastin modifications. The presence of lysyl oxidase activity, even in the oldest animals, might be at least partly responsible for the increased stiffness of the aging extracellular matrix. The striking age-related remodeling of the aortic architecture and the alterations of the interactions between cellular and extracellular compartments might greatly influence the functional properties of the arterial wall in senescence, at least contributing to the consequences of some apparently age-related vascular disorders. (Arteriosclerosis and Thrombosis 1992;12:1008–1016)

KEY WORDS • age • rats • aorta • collagen • elastin • lysyl oxidase

The extracellular matrix in the arterial wall is an integrated system composed of collagen fibrils, elastic lamellae, proteoglycans, minor collagens, and structural glycoproteins that, together with cells, guarantees the major properties of the vascular compartment, i.e., the diffusion at proper rates of nutrients and other metabolic factors through optimal blood flow.1-4

Apart from calcifications, fibrosis, and atheromatous lesions, which are frequently observed in the vessel wall of several species,5-9 and which are at least partly responsible for the failure of the cardiovascular system with age,7 little is known about the pathogenesis of the age-related modifications of the vessel wall, which may affect vascular properties such as elasticity and resilience and which may be of paramount importance in understanding specific pathological conditions.

The present work was undertaken with the aim of focusing attention on the age-related modifications of the aortic arch in rats from birth to senescence. Qualitative and quantitative morphological data concerning the structure and distribution of the cellular and extracellular components were correlated with the activity of lysyl oxidase, the enzyme that catalyzes the formation of collagen and elastin cross-links and therefore contributes to the stabilization of these two polymers.10

In the light of our results, we suggest that the interactions of the cellular and the extracellular compartments and the ratio between collagen and elastin play key roles in conditioning the morphological-functional properties of the arterial wall and, therefore, explain at least some of the consequences (although not the pathogenesis) of several pathological processes affecting the vascular compartment with increasing age.

Methods

Animals

The thoracic and the abdominal aortas were carefully removed from male Wistar rats, from birth to 2 years of age, all of which were purchased from the same animal breeding company. The rats underwent intraperitoneal anesthesia with 2% pentothal sodium (Nembutal) solution (30 mg/100 g body wt) and were killed by decapitation.

Morphology

Comparable cross sections of the aortic arch that were removed from the external areolar connective tissue were taken immediately below the subclavian artery and placed in a drop of Tyrode's physiological solution. The external and internal diameters of the aortas were measured by means of a reticulated optical magnifier (×10), and the thickness and the cross-sectional area of the vessel walls were calculated from the aforementioned parameters. Samples were then processed for ultrastructural examination, and the remaining tissue was frozen in liquid nitrogen and stored at −80°C for lysyl oxidase activity assay.11

Morphometry

Semithin sections from at least five animals from each age group were analyzed to evaluate modifications in
the thickness of the tunica media and adventitia. From the same specimens, at least 20 electron photomicrographs were taken at ×8,000 through the vessel wall, at the intersections of the grid meshes. Stereological data were obtained with a semiautomatic image analyzer, elaborated by a computerized data processing program, and compared by the two-tailed Student's t-test.

**Lysyl Oxidase Assay**

The tritium-release method, as first described by Pinnell and Martin and later modified by Kagan et al., was used for the ex vivo measurement of lysyl oxidase activity extracted from a pool of age-matched aortic walls. Experiments were done at least two times in triplicate.

### Results

#### Macrostructural Observations

At the time that the animals were killed, examination of the endothelial lining of even the oldest animals did not show any atheromatous degeneration. Optical magnifier observations of aortic arches revealed a striking increase in the lumen diameter during the first month of life of the animals; then, after a further increase during the next month, values became rather homogeneous (Table 1). Wall thickness followed the same behavior, and the increased values appeared to be due mainly to augmentation of the adventitia (Table 1).

#### Morphological Findings

In the rat aortic wall, the tunica intima can be identified by the endothelial lining and by a thin subendothelial space; the tunica media represents the majority of the vessel wall and consists of about 12 concentric elastic lamellae among which smooth muscle cells and small collagen bundles are situated. In the adventitia, cells (mainly fibroblasts) are embedded in an extracellular matrix particularly rich in collagen (Figure 1B). With age, the overall architecture of the aortic wall showed relevant morphological modifications (Figures 1A–1D).

In newborn rats, the endothelial lining was characterized by globular cells showing abundant rough endoplasmic reticulum and numerous cell contacts (Figure 2A). The tunica intima was very thin, with small collagen fibrils and few deposits of amorphous elastin surrounded by several microfibrils (Figure 2B). The media consisted of smooth muscle cells whose volume density was ≈45% of the measured tissue (Table 2); morphologically, cells appeared rounded and showed the synthetic phenotype characteristics, such as abundant rough endoplasmic reticulum with dilated cisternae and only a few contractile cytoplasmic filaments (Figure 3A). The extracellular matrix was only organized; elastic lamellae were discontinuous, extremely thin, and close to the cell layers (Figure 3A), and small collagen fibrils were organized in sparsely scattered fine bundles, even in the adventitia (Figure 4A). Elastin and collagen bundle density represented 8% and 6%, respectively, of the measured tissue (Table 2).

During the first month of life, the aortic wall showed remarkable structural modifications. In 18-day-old rats, in fact, the amount of smooth muscle cells increased, but their volume density was reduced to 22% of the measured tissue because of the consistent extracellular matrix augmentation (Table 2). Elastic lamellae underwent rapid growth, became thicker and continuous, and reached 27% volume density, and although collagen density was higher than at birth (Table 2), the elastin-to-collagen ratio showed its highest value at this age (Figure 5).

In the 60-day-old rats, body growth was still increasing, although at slower rates (Table 1). The endothelium was composed of elongated and differentiated endothelial cells (Figure 2C), and the tunica intima was mainly constituted by the inner elastic lamina (Figure 2C). In the media, smooth muscle cells were still tightly connected to each other but did not show the synthetic phenotype characteristics observed before (Figure 3B); the elastic lamellae were well developed (Figures 1B and 3B), as also observed by morphometric analysis (Table 2). Collagen in the media (Figure 3B) and especially in the adventitia was organized into bigger bundles; the adventitial cells were extremely reduced in number, and elastin fibers were almost absent (Figure 4B). Morphometric analysis revealed that at this age, elastin evaluated as a percentage of volume density started to diminish, whereas total elastic volume per cross-sectional area showed a striking increase (Table 2).

In 8-month-old rats, endothelial cells, which had scarce endoplasmic reticulum, were very elongated and separated from the elastic lamellae by an increasing amount of extracellular matrix (Figure 2D). A remarkable increase in the number and the size of collagen bundles (Figure 3C) was observed in the media, where rarely interconnected smooth muscle cells appeared elongated and exhibited spindle-shaped cytoplasmic

### Table 1. Macrostructural Observations of Aging Rat Aortas

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>1</th>
<th>18</th>
<th>60</th>
<th>240</th>
<th>480</th>
<th>720</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>7.8±1.6</td>
<td>44±3.5</td>
<td>199±7.5</td>
<td>559±69</td>
<td>591±73</td>
<td>666±60</td>
</tr>
<tr>
<td>Lumen diameter (μm)</td>
<td>430±63</td>
<td>1,090±127</td>
<td>1,780±225</td>
<td>1,940±281</td>
<td>2,000±312</td>
<td>1,860±149</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>130±27</td>
<td>166±28</td>
<td>283±28</td>
<td>350±50</td>
<td>350±47</td>
<td>340±79</td>
</tr>
<tr>
<td>Media thickness (μm)</td>
<td>100±9</td>
<td>120±15</td>
<td>141±14</td>
<td>200±16</td>
<td>183±28</td>
<td>175±26</td>
</tr>
</tbody>
</table>

Values are reported as mean±SD and (number of animals analyzed).
protrusions and numerous cytoplasmic filaments. Elastic lamellae showed some areas of disorganization (Figure 1C), and morphometric analysis revealed that the percent elastin volume density was reduced to that of younger ages (2p<0.01). The adventitia was characterized by large amounts of bigger collagen bundles and very few cells (Figure 4C), which caused a dramatic fall in the elastin-to-collagen ratio (Figure 5).

In the 24-month-old rats, striking aortic wall modifications were noted (Figure 1D). By light microscopy, elastin fibers were extremely thin and disorganized; smooth muscle cells appeared irregularly shaped, and large amounts of extracellular matrix were present among cells. At the ultrastructural level, the tunica intima was composed of a thin and sometimes discontinuous lining of endothelial cells, whereas extracellular matrix frequently appeared rather swollen and rich in glycosaminoglycans (Figure 2E), as visualized at higher magnification as toluidine blue-stained precipitates (Figure 2F). In the tunica media, numerous collagen bundles were present among multibranched cytoplasmic protrusions of smooth muscle cells (Figure 3D). Cells showed a typical senescent phenotype, with a high nucleus-to-cytoplasm ratio, and maintained contact to more elastic fibers than to adjacent cells. Compared with younger ages, elastin appeared to be organized into thinner laminae, which accounted for the reduced elastin density, for the decreased fiber mean area observed by morphometric analysis (Table 2), and therefore for the very low elastin-to-collagen ratio (Figure 5). Collagen density, representing >35% of tissue, was particularly abundant in the adventitia, where bundles were
FIGURE 2. Transmission electron photomicrographs illustrating the endothelial lining and the tunica intima in the aortic arch of 1- (panels A and B), 60- (panel C), 240- (panel D), and 720- (panels E and F) day-old rats. With increasing age, there is progressive elongation and thinning of endothelial cells (ec), which show reduced contacts among themselves. The tunica intima, moreover, is very thin and poorly organized in newborn animals, whereas it progressively thickens with age. In old animals, the tunica intima appears rather swollen and rich in glycosaminoglycans that can be better visualized at higher magnifications as toluidine blue-stained precipitates (arrows). e, Elastin; *, subendothelial space. Bar=1 μm.
large, numerous, densely packed, and responsible for the increased thickness of this tunica (Figure 4D).

**Lysyl Oxidase Activity**

Lysyl oxidase was sequentially extracted from the aortic tissue in phosphate-buffered saline (soluble, newly synthesized enzyme) and in 6 M urea (insoluble enzyme, tightly bound to the substrate). In both cases, enzyme activity showed the same age-related behavior but at different levels: the highest values were obtained at all ages for the enzyme extracted in urea. Lysyl oxidase activity reached a maximum in the first month of life and remained rather high during the first 2 months; in adult and old animals, values were relatively uniform but lower compared with previous ages (Table 3).

**Discussion**

In this study, by ultrastructural-morphometric analysis and by lysyl oxidase activity assay, we investigated the aortic wall of rats from birth to senescence in an attempt to provide further information on the age-related modifications of the vascular tissue.4-6 Because alterations due to aging are frequently associated with degenerative aspects such as atheromatous lesions, we used the rat model, which has been demonstrated to be quite resistant to developing atherosclerosis.15 The alterations observed, therefore, can be better associated only to age-related effects and may explain how severely aging and/or extracellular matrix modifications might interfere with the pathogenesis of vascular diseases.

In newborn and very young rats, the tunica intima is composed of endothelial cells tightly bound together; with time, these cells become progressively more elongated, show reduced contact among themselves, and therefore seem to form a more fragile barrier toward the lumen.16 The importance, for example, of abnormal interactions between collagen and platelets17-19 or between elastin and lipids20 has been suggested in the pathogenesis of several vascular disorders. Endothelial lining integrity is achieved by the presence of tight, adherent, and gap junctions between cells21 and by surface integrins such as α2β1 or α5β1, whereas extracellular matrix proteins such as fibronectin or laminin do not seem to significantly interfere.22 Because it has been demonstrated that integrins are mainly distributed at cell-cell contacts, a reduction in the surface area available for cell–cell interactions might greatly contribute to the fragility of the endothelial lining. In both adult and old animals, moreover, the subintimal space is bigger, sometimes swollen, and generally rich in glycosaminoglycans (visualized as toluidine blue-stained precipitates),11 which might alter the permeability and the behavior of the intima components. Thickening of the tunica intima and increased amounts of proteoglycans were also observed in aging rabbit aortas without any evidence of extracellular lipid deposition,23 suggesting that even in this animal model, which is known to frequently develop vascular diseases, a further stimulus in addition to morphological modification is necessary for atherosclerosis development.

So far as collagen content is concerned, there was a progressive age-related increase in its volume density, although the total amount per cross-sectional area showed a slight but significant (2P<0.01) reduction in aged animals. Moreover, greater amounts of collagen and larger collagen bundles were observed at all ages in the adventitia; this phenomenon could be due to the involvement of more cells in the tunica, to collagen synthesis, or to a greater number of cells susceptible to stimuli for the continuation of collagen deposition, possibly through the selection of a specific cell subclone.24-26 As previously described for in situ hybridization, collagen type I mRNA expression was lower in adult and old animals compared with newborn and young rats, but it was still present and mainly located in the adventitia.26 Therefore, the consistent accumulation of interstitial collagens, morphologically observed in

### Table 3. Morphometric Analysis of Aging Rat Aortas

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>1 (n=6)</th>
<th>18 (n=6)</th>
<th>60 (n=5)</th>
<th>240 (n=5)</th>
<th>480 (n=5)</th>
<th>720 (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount per cross-sectional area (μm²×10⁶)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>14±1.6</td>
<td>96±14</td>
<td>278±24</td>
<td>1,018±67</td>
<td>952±35</td>
<td>867±43</td>
</tr>
<tr>
<td>Elastin</td>
<td>17±2.2</td>
<td>183±18</td>
<td>477±29</td>
<td>443±34</td>
<td>365±37</td>
<td>207±31</td>
</tr>
<tr>
<td>Cells</td>
<td>104±11</td>
<td>162±17</td>
<td>560±32</td>
<td>614±29</td>
<td>541±34</td>
<td>480±46</td>
</tr>
<tr>
<td>Volume density (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>5.9±0.2</td>
<td>14.4±4.4</td>
<td>17.2±4.1</td>
<td>33.9±4.9</td>
<td>31.8±5.4</td>
<td>36.6±1.9</td>
</tr>
<tr>
<td>Elastin</td>
<td>7.7±3.6</td>
<td>27.1±8.1</td>
<td>23.3±4.2</td>
<td>18.2±4.3</td>
<td>15.9±1.7</td>
<td>9.5±3.7</td>
</tr>
<tr>
<td>Cells</td>
<td>44.9±11.5</td>
<td>22.2±4.2</td>
<td>29.3±4.2</td>
<td>24.9±0.9</td>
<td>24.7±4.4</td>
<td>25.4±1.4</td>
</tr>
<tr>
<td>Mean area (μm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>1.7±0.5</td>
<td>2.1±0.8</td>
<td>2.3±1.2</td>
<td>7.3±0.7</td>
<td>7.3±1.2</td>
<td>7.1±1.2</td>
</tr>
<tr>
<td>Elastin</td>
<td>1.1±0.4</td>
<td>3.1±0.9</td>
<td>4.2±1.3</td>
<td>4.9±0.8</td>
<td>3.6±0.1</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>Cells</td>
<td>14.3±4.9</td>
<td>11.3±1.1</td>
<td>12.7±3.5</td>
<td>12.8±0.9</td>
<td>12.4±2.1</td>
<td>10.7±0.4</td>
</tr>
<tr>
<td>Number per unit area (component number/μm²×10³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>3.5±1.1</td>
<td>7.3±1.5</td>
<td>6.6±1.5</td>
<td>5.5±0.2</td>
<td>5.1±1.4</td>
<td>5.2±0.8</td>
</tr>
<tr>
<td>Elastin</td>
<td>6.9±1.7</td>
<td>9.2±3.1</td>
<td>6.2±2.1</td>
<td>3.9±0.3</td>
<td>4.1±0.6</td>
<td>2.6±0.7</td>
</tr>
<tr>
<td>Cells</td>
<td>3.2±0.9</td>
<td>2.2±0.3</td>
<td>2.4±0.9</td>
<td>1.9±0.2</td>
<td>1.7±0.1</td>
<td>1.9±0.3</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. n, Number of animals analyzed.
aging arteries, could be the consequence of altered ratios and/or interactions between connective tissue components or the result of a particularly slow enzymatic extracellular degradation due to chemical modifications of collagen molecules. It is, in fact, largely accepted that, with aging, collagen becomes more insol-
FIGURE 4. Transmission electron photomicrographs of the tunica adventitia in the aortic arch of 1- (panel A), 60- (panel B), 240- (panel C), and 720- (panel D) day-old rats. The progressive and striking thickening of the tunica adventitia is associated with reduction and fragmentation of elastic fibers, whereas collagen (c) is organized in larger and more compact bundles. Bar=1 μm.

uble because of increased intermolecular cross-linking. Although we did not measure the types or amounts of collagen cross-links, we found that lysyl oxidase extracted from the aortas of aging animals was always active in vitro, even at low levels in the oldest animals. Because the activity of this enzyme is strongly related to experimental fibrosis and to diet-induced atherosclerosis in the aorta, we hypothesize that the insolubility of aging collagen in the absence of consistent new synthesis might be the consequence of further
lysyl oxidase–catalyzed intermolecular cross-links or the result, as many authors have reported, of oxidative reactions and nonenzymatic glycosylation of amino groups (Maillard reaction), biochemical events that are more frequently detected in aged animals.

Vascular elastin metabolism has been shown to be age related, although there are some differences in the type and/or localization of the cells involved. Elastin synthesis was particularly efficient in the aortas of newborn and very young rats; in fact, elastin, evaluated as the total amount per cross-sectional area or as fiber mean area, was significantly higher only during the first 2 months of life of the animals, whereas the percentage of elastin volume density because of collagen augmentation already started to decline in 60-day-old rats. In old animals, independent of the parameters used, elastin was significantly reduced (2p<0.001) compared with that in younger ages. Incomplete knowledge of the mechanisms involved in the regulation of elastin synthesis hinders the interpretation of these phenomena. The age-related modifications of the elastic component might be related to altered ratios among extracellular matrix proteins, such as collagen or glycosaminoglycans, to intrinsic rearrangements of elastic fiber constituents as glycosaminoglycans, and finally, to the dramatic reduction of elastin mRNA expression. However, in some circumstances, such as after surgically induced intimal injury or in the presence of atherosclerosis or hypertension, at least some smooth muscle cells seem to be able to produce large amounts of elastin together with other matrix proteins, suggesting that factors such as nutrients, cytokines, and growth factors might modulate the phenotypic expression of vascular cells. With increasing age, smooth muscle cells become atrophic and immersed in a progressively more collagenous and stiff extracellular compartment, thereby partly losing their reciprocal contacts; these modifications, moreover, might also negatively interfere with cell–cell signal exchange and also with the mechanical stretching of smooth muscle cells, a stimulus that has been shown in vitro to increase elastin deposition. Moreover, some morphological features of old elastin fibers suggest degradative effects of elastases or other proteases, which are reported to increase in old tissues. It may be hypothesized also that enhanced susceptibility of elastin fibers to elastase activity could be the consequence of intrinsic modifications of the elastic components.

In conclusion, with time, the morphological organization of the rat aorta showed remarkable alterations because of continuous rearrangements between cells and extracellular components, which can explain the functional impairments that are frequently observed in old animals and which can justify the consequences, although not the occurrence per se, of several vascular disorders. These observations might therefore contribute to a better comprehension of the age-related vascular biology of the rat, one of the most commonly used experimental models.

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Role of the extracellular matrix in age-related modifications of the rat aorta. Ultrastructural, morphometric, and enzymatic evaluations.
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doi: 10.1161/01.ATV.12.9.1008

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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