Hypercholesterolemia Superimposed by Experimental Hypertension Induces Differential Distribution of Collagen and Elastin

Chengpei Xu, Christopher K. Zarins, Pia S. Pannaraj, Hisham S. Bassiouny, Seymour Glagov

Abstract—We studied the mural distribution of collagen types I and III and tropoelastin in enhanced experimental atherogenesis induced in rabbits by hyperlipidemia superimposed by hypertension. Animals were fed a high-cholesterol diet for 5 weeks and also subjected to midthoracic aortic coarctation for 4 weeks. Serum cholesterol levels were increased and blood pressure was elevated proximal to the coarctation. Foam cell lesions developed in the aorta proximal to the coarctation. In situ hybridization and immunohistochemistry showed that gene expression of collagen types I and III and tropoelastin was upregulated, with a differential distribution across the arterial wall. New collagen type I was mainly distributed in the intima, the outer media, and the adventitia. New collagen type III was spread more uniformly across the wall, including the adventitia, whereas tropoelastin was mainly localized in intimal foam cell lesions. Morphometric data showed an increase in wall thickness. These results suggest that collagen types I and III play a role in remodeling of the arterial wall in response to hypertension. The remarkable involvement of the adventitia in this response indicates that the adventitia is an important component of the arterial wall. Tropoelastin is closely associated with foam cell lesion formation, suggesting a role for this component in atherogenesis as well. (Arterioscler Thromb Vasc Biol. 2000;20:2566-2572.)

Key Words: collagen ■ elastin ■ aortic coarctation ■ hypertension ■ hyperlipidemia

Hypertension is an important risk factor in the development of cardiovascular disease. It is closely associated with atherogenesis and aneurysm formation. It has been shown that hypertension facilitates the development of atherosclerosis in the presence of hyperlipidemia.1-3 Hypertension has also been implicated in arterial wall remodeling.4 Studies have shown that hypertension is responsible for the increase in arterial wall thickness and changes in the structural composition of the arterial wall.5,6

Collagen and elastin are major extracellular matrix (ECM) components of the arterial wall. They ensure wall resilience and maintain tensile strength.7 Sustained hypertension leads to structural changes of the arterial wall. These alterations include increases in the degradation and synthesis of collagen and the destruction and reconstruction of elastin fibers,8-10 which eventually lead to remodeling of the arterial wall and modifications of its mechanical properties.11 Collagen and elastin are also major extracellular components of the diseased artery wall. Studies have shown that the arterial wall is less distensible in hypertension owing to the increased wall stiffness and decreased vascular compliance.12

Collagen and elastin play different roles in maintaining arterial wall mechanical properties. The distribution of these ECM components closely corresponds to hypertension and the development of atherosclerosis. We have previously demonstrated that gene expression of type I collagen is increased in the intima, outer media, and adventitia after an acute elevation in blood pressure.13 However, the differential distribution of collagen and elastin in the arterial wall during atherogenesis is largely unknown. The present study was designed to assess the response and distribution of collagen types I and III and tropoelastin in the arterial wall in a model of hyperlipidemia superimposed by acute and sustained elevations of blood pressure.

Methods

Animal Model and Experimental Design

Adult, male, New Zealand White rabbits weighing 2.5 to 3.0 kg were used in this study. Five animals underwent midthoracic aortic coarctation for 4 weeks (4WC, n=5). Six animals were fed a high-cholesterol diet for 5 weeks without coarctation (5WD, n=6). Animals in the third group were fed the atherogenic diet for 1 week followed by the midthoracic aortic coarctation. These animals were then kept on the high-cholesterol diet for 4 more weeks and designated group 5WD+4WC (n=6). Sham-operated animals that were not fed the high-cholesterol diet served as controls (n=6). Two additional animals in each group were killed without subsequent perfusion/fixation for frozen sections.

The high-cholesterol diet consisted of 1% cholesterol and 4% corn oil mixed into standard rabbit chow.14 Blood samples were drawn...
from the ear vein before starting the cholesterol diet and weekly thereafter for measurement of total serum cholesterol level. Surgical procedures and animal care followed the criteria outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23). Surgical procedures were conducted under general anesthesia and sterile conditions. Animals were given 40 mg/kg ketamine and 5 mg/kg xylazine intramuscularly 30 minutes before the operation. After endotracheal intubation, anesthesia was maintained with 1.5% halothane and O₂ administered with a tidal volume of 15 mL (4 to 6 mL/kg body weight) at the rate of 45 breaths/min through a ventilator (Harvard respiration pump). A middescending thoracic aortic coarctation was created through a left thoracotomy. The aortic segment between the orifices of the 5th and 6th intercostal arteries was exposed and incised with a 5-mm-wide Dacron band. Blood pressures from the ear and femoral arteries were monitored throughout the surgery. The degree of coarctation was achieved by adjusting the tightness of the band to obtain 15- to 30-mm Hg pressure gradients between the ear and the femoral arteries. The chest was closed and followed by air suctioning to resume physiological chest pressure. Postoperative analgesia was provided as needed with 0.01 mg/kg IM buprenorphine. Baytril 5 mg/kg IM was given to prevent infection.

Blood pressures were measured through catheters introduced into the central artery of the ear and into the femoral artery. The catheters were connected to a strain gauge with a strip chart recorder (Gould Inc). Mean blood pressures were measured before surgery, during the operation, and at sacrifice. Body weights were documented at the beginning of the experiment and end of the experiment. Heart weights were measured at sacrifice.

**Specimen Preparation**
At sacrifice, the animals were anesthetized; this was followed by injection of an overdose of pentobarbital at 120 mg/kg IV. The animals were immediately perfusion-fixed with 10% buffered formalin through the left ventricle at controlled pressure of 100 mm Hg for 30 minutes in an attempt to preserve normal structural configurations. Two cross-sectional rings, 1 cm proximal and distal to the coarctation site, were immediately taken and further fixed with 10% formalin overnight for paraffin sections. These sections were used for morphometric and histological studies. Fresh tissues were taken from 2 additional non–perfusion-fixed animals for frozen sections at sampling sites corresponding to those for the perfused animals.

**In Situ Hybridization**
In situ hybridization was performed on 8-μm frozen sections from all animals. Both antisense and sense riboprobes were generated from cDNA templates. The original cDNA of pro-α2(I) collagen was a 2.1-kb Hf32 clone from human skin fibroblasts that was carried in plasmid pBR322 (American Type Culture Collection [ATCC], catalog No. 61484). A PvuII- and Xhol-digested 0.25-kb fragment from the Hf-32 clone was subcloned into pGEM-4z (Promega) for in vitro RNA transcription. The original cDNA of pro-α1(III) collagen was a 1.3-kb Hf-934 clone from human fibroblasts that was carried in plasmid pBR322 (ATCC catalog No. 61324). A PvuII- and EcoRI-digested 0.375-kb fragment of this clone was subcloned into pGEM-4z. The cDNA of tropoelastin was a 0.923-kb REL124D clone from human skin fibroblasts that was carried in plasmid pGEM-4z (Promega) for in vitro RNA transcription. The original cDNA of tropoelastin was a 0.923-kb REL124D clone from rat aorta that was carried in plasmid pBI131 (ATCC catalog No. 63179). It was further excised with EcoRI and subcloned into plasmid pBlueScript II KS+ (Stratagene) for RNA transcription.

In vitro RNA transcription for the riboprobes was performed by using the riboprobe Gemini System II (Promega) with the addition of [-α³²P]UTP. The cross-reactivity of the probes with the rabbits was confirmed individually by Northern blot hybridization. The in situ hybridization procedure has been described elsewhere. Sections were incubated in hybridization buffer at 45°C for 2 to 3 hours, followed by hybridization in the buffer with a riboprobe at a concentration of 3×10⁶ counts per minute/mL. Sections applied with the sense probe served as controls. The sections were incubated in a moist box at 55°C overnight. After posthybridization washes, RNase treatment, and dehydration, the slides were emulsified with Kodak NTB-2 autoradiography emulsion and exposed for 10 to 14 days at −80°C before development and fixation. The sections were counterstained with hematoxylin for 60 seconds, dehydrated with graded ethanol, and coverslipped.

**Immunohistochemistry**
Immunohistochemistry was performed on frozen sections. Goat anti-human type I collagen and goat anti-human type III collagen antibodies were purchased from Accurate Chemical & Scientific Corp. Mouse anti-bovine tropoelastin was obtained commercially (Elastin Products Co, Inc). The cross-reactivity of these antibodies with rabbit collagen types I and III and tropoelastin had been confirmed by Western blot analysis before starting the experiment. Corresponding biotinylated secondary antibodies were from Sigma Chemical Co. In addition, antibodies against macrophage and muscle actin (Enzo) were used to determine the origin of the foam cells in the neointima. Sections were rehydrated in PBS, followed by incubation in 3% H₂O₂ in PBS for 10 minutes. After being washed in PBS for 5 minutes, the sections were incubated in 1:100 diluted horse serum for 30 minutes. Sections were incubated with primary antibody at 1:50 dilution in PBS for 1 hour at room temperature. After 3 washes in PBS, the sections were incubated with the corresponding secondary antibody at 1:200 dilution for 1 hour at room temperature. The sections were then incubated with ExtrAvidin-peroxidase (Sigma) for 1 hour at room temperature. After 3 washes in PBS, the sections were incubated for 5 to 10 minutes with 3,3′-diaminobenzidine solution. The reaction product was a brown precipitate. The sections were washed, counterstained with hematoxylin for 60 seconds, dehydrated, and coverslipped.

**Morphometric and Histological Studies**
Cross-trimmed aortic rings were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin as well as with Weigert–Van Gieson’s procedure for matrix fibers. Computer-assisted contour tracing was used to determine the following dimensions: outer media diameter, the diameter derived from the circumference of the outermost elastic lamella; the diameter derived from the circumference of the internal elastic lamella (IEL diameter), luminal diameter, average media thickness, and average wall thickness (total thickness of the media and intima). A semiquantitative method was applied to estimate the differential distribution across the aortic wall for immunohistochemical staining. Three investigators observed the sections without knowing their identification. Plus and minus were assigned to each zone according to the judgment of the observers. The average of the data for the 3 observations was used as the intensity value for each particular zone. Sections were also observed by light microscopy to evaluate the morphological features.

**Data Analysis and Statistics**
All data were entered into a database. Mean and standard deviation were computed for each group. Single-factor ANOVA was performed by using StatView software version 4.5 (SAS Institute Inc) for all groups, and when the ANOVA was significant (P<0.05), Bonferroni’s correction method was performed for multiple comparisons among the groups. The P value was set at <0.002 for statistical significance. In those cases where only 2 groups were involved, Student’s t test for comparing 2 sample means was used. The significance value was set at P<0.05.

**Results**

**Body Weight, Heart Weight, Serum Cholesterol Levels, and Blood Pressure**
Body weights were increased by 14% at sacrifice for all experimental groups (2.98±0.19 kg) compared with the mean body weight at the beginning of the experiment (2.62±0.25 kg; P<0.05). These increases are considered to be normal weight gains over the experimental period. Heart weights were somewhat increased in animals with coarctation (9.26±1.25 versus 7.87±0.61 g in sham-operated controls; P<0.05). These increases can be attributed to the effects of increased resistance due to aortic coarctation. However, the

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ratio of heart weight to body weight was not significantly different from that of controls after 4 or 5 weeks. This result suggests that the effects of aortic coarctation may have not been significant.

The mean total serum cholesterol level for all animals at the beginning of the experiment was 66±16 mg/dL. It increased rapidly after initiation of the high-cholesterol diet. At 1 week it was 15 times (1009±205 mg/dL) higher than normal and peaked by 4 weeks for the 5WD+4WC (2511±444 mg/dL) and by 5 weeks for the 5WD (2715±530 mg/dL) groups (P<0.001 for all compared with normal controls).

Mean blood pressures before surgery were 94±9 mm Hg at the ear artery and 95±10 mm Hg at the femoral artery. For the noncoarcted and sham-operated animals, their blood pressure measurements were not different before versus after the experiment. For the animals with aortic coarctation (5WD+4WC and 4WC groups), blood pressure gradients were established at operation and maintained until the rabbits were euthanized (Table).

Development of Foam Cell Lesions
In the rabbits of the 5WD+4WC group, there were large intimal lesions in the aorta proximal to the coarctation (Figure 1) and very small lesions distal to it. Intimal lesions appeared as characteristic foam cell accumulations. The foam cell lesions in the animals fed the high-cholesterol diet only (5WD) were much smaller than those of 5WD+4WC animals, and no lesions were observed in the distal aortas of the 5WD animals. No foam cell lesions were observed in the aortas of animals with coarctation only (4WC), nor were they seen in the sham-operated controls. Foam cells in the neointima were of both macrophage and smooth muscle cell origin, with the smooth muscle cell origin being dominant, as confirmed by immunohistochemistry (data not shown).

Differential Distribution of Gene Expression for Collagen and Tropoelastin
In situ hybridization and immunohistochemistry demonstrated a distinct distribution pattern for collagen and tropoelastin in the aortas proximal to the coarctation in the animals with both hypercholesterolemia and hypertension. In situ hybridization showed that the mRNA of collagen type I was distributed mainly in the intima, outer media, and adventitia (Figure 2). In contrast, collagen type III mRNA was rather uniformly distributed across the entire wall, ie, the intima, media, and adventitia (Figure 3). The strength of the detected signals, however, was much weaker than that of collagen type I. Immunohistochemistry showed a similar distribution pattern for both collagen types (see online Figures I and II, at http://atvb.ahajournals.org).

Tropoelastin gene expression, on the other hand, had a unique distribution, mainly in the intimal foam cell lesions, as
shown by in situ hybridization (Figure 4) and by immuno-
histochemistry (Figure III at http://atvb.ahajournals.org). This
particular distribution was observed not only in the proximal
aorta but also in the foam cell lesions of the distal aorta to the
coastriction of the 5WD\textsuperscript{14}WC animals (data not shown). It is
apparent that tropoelastin gene expression is more closely
associated with the development of foam cell lesion than with
the elevation of blood pressure.

Table I (please see http://atvb.ahajournals.org) illus-
trates the distribution patterns of these ECM components
in the different zones of the aorta according to their
abundance. It is apparent that elevated blood pressure had
a major effect on the distribution of collagen type I in the
intima, outer media, and adventitia. Hypertension had a
less remarkable effect on the distribution of collagen type
III in the media and adventitia. Tropoelastin localization

Figure 2. In situ hybridization and
immunohistochemistry for collagen type
I gene expression. Frozen sections from
the proximal aortas of 5WD\textsuperscript{+}4WC ani-
mals were hybridized with an antisense
riboprobe (A) and a sense riboprobe (B)
for pro-\(\alpha2(I)\) mRNA. Dark grains are
mainly in the adventitia, outer media,
and intimal foam cell lesions (A). The
signals are so strong that many are
clustered to form larger dots instead of
fine grains. There are fewer grains in
sections hybridized with the sense ribo-
probe (B).

Figure 3. In situ hybridization and immu-
nohistochemistry for collagen type III
gene expression. Frozen sections from
the proximal aortas of 5WD\textsuperscript{+}4WC ani-
mals were hybridized with pro-\(\alpha1(III)\)
anti-
sense (A) or sense (B) probes. The grains
are distributed more uniformly (A).
Strength of the signal is less intense than
that of pro-\(\alpha2(I)\), although the exposure
time was the same (2 weeks). The sense
probe did not hybridize with pro-\(\alpha1(III)\)
mRNA (B).
appeared to be mainly associated with the intima where foam cell lesions had developed.

**Aortic Dimensions and Morphology**

Aortic size at the proximal portion of the aorta, represented by the diameters of the outer media, the internal elastic lamella, and the artery lumen, was significantly increased for animals with coarctation. For example, internal elastic lamellar diameter was $4.38 \pm 0.39$ mm for rabbits in the 5WD+4WC group and $4.67 \pm 0.41$ mm for rabbits in the 4WC group compared with controls ($3.42 \pm 0.25$ mm; $P < 0.001$). The intimal cross-sectional area, representing lesion size, was larger in animals fed the high-cholesterol diet than in controls and in rabbits of the 4WC group ($2.27 \pm 0.40$ mm$^2$ for 5WD+4WC and $0.17 \pm 0.05$ mm$^2$ for 5WD versus $0.04 \pm 0.06$ mm$^2$ for 4WC groups; $P < 0.0001$). The medial thickness and wall thickness were also increased for animals with coarctation. Medial wall thickness, for example, was $0.26 \pm 0.02$ mm for 5WD+4WC animals and $0.27 \pm 0.04$ mm for 4WC animals, versus $0.18 \pm 0.03$ for both the 5WD group and controls ($P < 0.001$).

In the aorta distal to the coarctation, morphometry study showed significant poststenotic dilation of 2 cm distal to the coarctation at 4 weeks for coarcted animals (5WD+4WC and 4WC). However, the media area and thickness were not decreased (data not shown).

**Discussion**

Collagen and elastin are major structural and functional components of the arterial wall. These components actively participate in arterial wall remodeling in response to hemodynamic alterations and during atherogenesis. Little is known about the molecular mechanisms by which hypertension accelerates atherogenesis under condition of hyperlipidemia. In this study, we focused on the distribution of gene expression for collagen types I and III and tropoelastin under conditions of combined hyperlipidemia and hypertension.

We have shown that collagen types I and III and tropoelastin had a distinct differential distribution across the aortic wall in response to the combination of high blood pressure and hypercholesterolemia. Collagen type I gene expression was remarkable in the intima, media, and adventitia. Collagen type III appeared more uniformly localized in the media and adventitia as well as in the intima. Tropoelastin was distributed in the intimal foam cell lesions. These results suggest different functions for these ECM components during arterial wall remodeling and in the atherogenic process.
Quantitative study of collagen gene expression in response to acute hypertension showed that mRNA levels of collagen types I and III were maximal at 3 days but returned to normal by 4 weeks after aortic coarctation. Accumulation of these collagen types and their precursors was increased by 3 days, peaked at 4 weeks, and decreased toward normal by 8 weeks. These results demonstrate an important role for collagen during arterial wall remodeling in response to an elevation of blood pressure. It was also reported that collagen type I gene expression was mainly localized in the adventitia, outer media, and intima and that collagen type III gene expression was uniformly localized across the arterial wall in response to an elevation in blood pressure. However, in the current study in animals with hyperlipidemia only, gene expression of collagen types I and III in response to hyperlipidemia was rarely observed, except for mild expression in the intimal foam cell lesions. Furthermore, tropoelastin gene expression was mainly seen in the intimal foam cell lesions in the cholesterol-fed animals. These foam cells were both of macrophage and smooth muscle cell origin. Thus, it is evident that hypertension is associated with an immediate response of collagen gene expression and that hyperlipidemia may lead to upregulation of tropoelastin.

Collagen gene upregulation in the adventitia and outer medial zones has been reported under many conditions. Although there has been a lack of in vivo information about tensile stress distribution across the arterial wall, it is conceivable that the adventitia and outer medial zones are subjected to the largest tensile stress, because tensile stress is positively correlated with arterial diameter. Hypertension has been considered an important risk for atherosclerosis, and it accelerates atherogenesis under conditions of hyperlipidemia. Hypertension sustains atherosclerosis development even after hyperlipidemia was normalized in experimental studies. It has been demonstrated that chronic abdominal aortic coarctation aggravates atherogenesis by high blood pressure in the Watanabe heritable hyperlipidemic (WHHL) rabbit. The intimal lesions in the hypertensive descending thoracic aorta are 6 times larger than those in normotensive rabbits. In the present study, we also combined 2 major risk factors, hypertension and hyperlipidemia, in the rabbit model. We further investigated the molecular mechanisms of the ECM participating in arterial remodeling and atherogenesis under these conditions.

The mechanism by which hypertension accelerates atherogenesis remains unclear. Tensile stretch on vascular cells may influence cell behavior, such as proliferation, apoptosis, and alterations in gene expression of the ECM, An increased ECM, for example, may alter the normal metabolism of lipids and favor their deposition. The accumulation of lipids in turn induces foam cell lesion formation, and tropoelastin in foam cell lesions, as shown in the present study, may aggravate lipid retention and deposition. Furthermore, angiotensin II, which induces hypertension, has been shown to be related to a heightened fibroproliferative response, intensive monocyte/macrophage infiltration, and elastin gene expression in affected arteries. All of these consequences could contribute to lesion acceleration by hypertension in our animal model.

The results that media thickness and aortic diameters were increased for segments proximal to the coarctation in the 5WD+4WC and 4WC animals and not in the 5WD animals agrees with the concept of the effects of tensile stress on the arterial wall by hypertension. The intimal foam cell lesions were much larger in the 5WD+4WC animals than in the 5WD animals. This further supports the theory that hypertension accelerates atherogenesis.

This study demonstrates that hypertension induces a differential distribution of gene expression of collagen types I and III across the arterial wall. However, it is hyperlipidemia, not hypertension, that is associated with the upregulation of tropoelastin gene expression in intimal foam cell lesions. We speculate that collagen type I gene upregulation may help the vasculature resist the increased tensile stress in the early stages of hypertension. Collagen type III may be associated with adjustments of cell-cell and cell-matrix interaction so as to preserve artery wall integrity and maintain a maximally efficient structural and functional wall. Tropoelastin is mainly associated with lipid deposition and foam cell lesion formation.

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