Collagen and elastin cross-linking: a mechanism of constrictive remodeling after arterial injury

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Collagen and elastin cross-linking: a mechanism of constrictive remodeling after arterial injury. Am J Physiol Heart Circ Physiol 289: H2228–H2233, 2005. First published June 10, 2005; doi:10.1152/ajpheart.00410.2005—Constrictive remodeling after arterial injury is related to collagen accumulation. Cross-linking has been shown to induce a scar process in cutaneous wound healing and is increased after arterial injury. We therefore evaluated the effect of cross-linking inhibition on qualitative and quantitative changes in collagen, elastin, and arterial remodeling after balloon injury in the atherosclerotic rabbit model. Atherosclerotic-like lesions were induced in femoral arteries of 28 New Zealand White rabbits by a combination of air desiccation and a high-cholesterol diet. After 1 mo, balloon angioplasty was performed in both femoral arteries. Fourteen rabbits were fed 0.25% L-arginine-APN (L-arginine-APN, 100 mg/kg) and compared with 14 untreated animals. The remodeling index, i.e., the ratio of external elastic lamina at the lesion site to external elastic lamina at the reference site, was determined 4 wk after angioplasty for both groups. Pyridinoline was significantly decreased in arteries from L-arginine-APN-treated animals compared with controls, confirming inhibition of collagen cross-linking: 0.30 (SD 0.03) and 0.52 (SD 0.02) mmol/mol hydroxyproline, respectively (P = 0.002). Scanning and transmission electron microscopy showed a profound disorganization of collagen fibers in arteries from beta-APN-treated animals. The remodeling index was significantly higher in beta-APN-treated than in control animals [1.8 (SD 0.3) vs. 0.8 (SD 0.3), P = 0.03], indicating favorable remodeling. Restenosis decreased by 33% in beta-APN-treated animals: 32% (SD 16) vs. 48% (SD 24) (P = 0.02). Neointimal collagen density was significantly lower in beta-APN-treated animals than in controls: 23.0% (SD 3.8) vs. 29.4% (SD 4.0) (P = 0.004). These findings suggest that collagen and elastin cross-linking plays a role in the healing process via constrictive remodeling and restenosis after balloon injury in the atherosclerotic rabbit model. 

METHODS

Animal Model

This investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). This study has been approved by the local Institutional Review Board.

Focal atherosclerosis was induced in femoral arteries of New Zealand White rabbits (n = 28) by a combination of air desiccation and a high-cholesterol diet, as previously described (21, 22). Briefly, rabbits were anesthetized by an intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). Proximal and distal ligatures were used to isolate a 1-cm-long section of femoral artery, which was cannulated. Vascular injury was induced in the isolated segments by air infusion. The animals were then fed a 2% cholesterol-6% peanut oil diet for 4 wk.

At 4 wk after induction of atherosclerosis, balloon angioplasty was performed as previously described (21, 22). Briefly, a baseline iliofemoral angiogram was performed after an intra-arterial injection of molsidomine (250 μg). Angioplasty, consisting of three inflations to 6 atm for 60 s (balloon-to-artery ratio = 1.0–1.2), was performed in both femoral arterial remodeling, particularly after balloon injury. Delayed excessive collagen accumulation has been described after arterial injury and is correlated with the severity of constrictive remodeling (21, 40). Collagen and elastin cross-linking provide structural cohesion of the arterial wall (4, 37). Lysyl oxidase promotes cross-link formation in nascent fibrils of collagen and elastin (23, 31). The difference in wound healing between the fetus and the adult is attributable to differences in the extracellular matrix, i.e., collagen accumulation, scar contraction, and excessive collagen cross-linking (2). Because excessive collagen cross-linking is involved in the skin scar process and lung and kidney fibrosis, we hypothesized that wound contraction by collagen and elastin cross-linking could play an important role in the arterial healing process, leading to constrictive remodeling after injury (3, 8, 17, 32, 33). In contrast, defective connective tissue in nonmechanically injured arteries led to formation of aneurismal-like structures (5, 13, 16, 19). We therefore aimed to investigate the effect of inhibiting lysyl oxidase, a copper-dependent enzyme that controls collagen and elastin cross-linking reactions, on arterial remodeling after balloon injury in the atherosclerotic rabbit model (31, 41). For this purpose, we used the most potent inhibitor of lysyl oxidase, beta-aminopropionitrile (beta-APN).

EXTRACELLULAR MATRIX REMODELING plays an important role in arterial development, atherosclerosis, and healing after arterial injury (29). Moreover, it has been demonstrated that unfavorable arterial remodeling (i.e., constrictive remodeling) is the principal mechanism of restenosis in various experimental models and in humans after balloon angioplasty (7, 18, 22, 27). However, little is known about the mechanisms controlling arterial remodeling, particularly after balloon injury. Delayed excessive collagen accumulation has been described after arterial injury and is correlated with the severity of constrictive remodeling (21, 40). Collagen and elastin cross-linking provide structural cohesion of the arterial wall (4, 37). Lysyl oxidase promotes cross-link formation in nascent fibrils of collagen and elastin (23, 31). The difference in wound healing between the fetus and the adult is attributable to differences in the extracellular matrix, i.e., collagen accumulation, scar contraction, and excessive collagen cross-linking (2). Because excessive collagen cross-linking is involved in the skin scar process and lung and kidney fibrosis, we hypothesized that wound contraction by collagen and elastin cross-linking could play an important role in the arterial healing process, leading to constrictive remodeling after injury (3, 8, 17, 32, 33). In contrast, defective connective tissue in nonmechanically injured arteries led to formation of aneurismal-like structures (5, 13, 16, 19). We therefore aimed to investigate the effect of inhibiting lysyl oxidase, a copper-dependent enzyme that controls collagen and elastin cross-linking reactions, on arterial remodeling after balloon injury in the atherosclerotic rabbit model (31, 41). For this purpose, we used the most potent inhibitor of lysyl oxidase, beta-aminopropionitrile (beta-APN).

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arteries. At 10 min after angioplasty, an iliofemoral angiogram was repeated. Thereafter, the animals were allowed to recover, and the high-cholesterol diet was replaced by normal rabbit chow. At 4 wk after angioplasty, an iliofemoral angiogram was performed after intra-arterial injection of molsidomine (250 mg). The animals were killed by an overdose of pentobarbital sodium (120 mg/kg), and femoral arteries were immediately perfused and fixed under pressure with 10% phosphate-buffered formaldehyde for histological analysis (n = 22) or removed and stored at -80°C for biochemical analysis and electron microscopy analysis (n = 6).

Study Design

Two groups of animals were evaluated. Fourteen rabbits (i.e., 28 dilated arteries) were treated with β-APN (Sigma, St. Louis, MO) dissolved in the drinking water at 100 mg·kg⁻¹·day⁻¹ po (25, 35). This dose, chosen to successfully inhibit collagen and elastin cross-linking without significant clinical adverse effects (i.e., bone, tendon, skin, and cartilage toxicity), was defined by preliminary data. The treatment started 1 wk before balloon injury and was continued until the animal’s death. The amount of β-APN effectively taken daily per animal was calculated from the amount of water consumed on a daily basis. Animal weight was periodically controlled to adjust the target dose of β-APN. Fourteen non-β-APN-treated rabbits (i.e., 28 dilated arteries) served as controls. Twenty-two animals were used for histological and scanning and transmission electron microscopy analysis.

Biochemical Analysis

Collagen and elastin cross-links were measured as previously described (1). Briefly, femoral arteries were hydrolyzed in 6 M HCl for 18 h at 110°C, and lysine concentration and desmosine and isodesmosine (elastin cross-links) were measured by ion-exchange HPLC (model 800, Hitachi) in an aliquot of the hydrolysate of the samples (1). Collagen cross-links (pyridinoline and pentosidine) were measured by HPLC (1). For each artery, the concentration of collagen cross-links was expressed as millimoles per mole of hydroxyproline, and the concentration of elastin cross-links was expressed as millimoles per milligram of dry weight.

Electron Microscopy

For scanning electron microscopy, the specimens were fixed using 2% glutaraldehyde in phosphate-buffered saline, pH 7.4, for 1 h at room temperature. Then they were directly dehydrated, critical point dried, covered with a thin layer of gold-palladium in a JEOL JFC 1100 ion sputter, and examined in a JEOL 5400 LV scanning electron microscope at 15 kV.

For evaluation by transmission electron microscopy, vessel samples were fixed using 2% glutaraldehyde in phosphate-buffered saline, pH 7.4, for 1 h at room temperature. Then they were postfixed with 2% osmium tetroxide, washed in buffer solution, dehydrated in graded alcohols (30, 50, 70, 95, and 100%) for 5 min each, and embedded in Agar 100 resin (Oxford Instruments, Orsay, France). Sections (70-nm thick) were obtained using a Reichert Ultracut-E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a JEOL 1010 transmission electron microscope operated at 80 kV.

Angiographic Analysis

Minimal luminal diameter (MLD), i.e., the most narrowed site, was measured by two blinded physicians using electronic calipers before, immediately after, and 4 wk after angioplasty. The average of the two separate measurements determined the final results.

Histological Analysis

Each femoral artery was cut (5 μm) serially at 1- to 2-mm intervals from the proximal to the distal end and embedded in paraffin. Sections were stained with orceine for morphometric analysis (IPS 38, version 4.32, Alcatel). The solutions were prepared by pouring 55 ml of boiling glacial acetic acid over 1 g of orceine powder. Briefly, the orceine solution (1% solution in 45% acetic acid) was cooled. 45 ml of distilled water were added, and the solution was filtered. After 5 min of incubation in the orceine solution, each artery was evaluated at two sites: the lesion site, defined by the cross section with the smallest luminal area of the proximal reference area, and the uninjured reference site (9, 21, 22). Luminal area, internal elastic lamina, and external elastic lamina were manually identified. Histological restenosis evaluates the percentage of residual stenosis 1 mo after angioplasty. Usually, <50% stenosis indicates the absence of restenosis, and >50% residual stenosis indicates restenosis, which is the difference between the luminal areas of the reference and lesion sites normalized by the luminal area of the reference site. The remodeling index evaluates the chronic variation of the area surrounded by the external elastic lamina compared with a reference normal segment upstream from the lesion site. The remodeling index is defined as the ratio of the area circumscribed by the external elastic lamina of the lesion site to the area circumscribed by the external elastic lamina of the reference site. Neointimal medial growth evaluates the importance of neointimal hyperplasia after balloon angioplasty compared with a reference nondilated segment. This index includes the media, because the internal elastic lamina is frequently disrupted after injury. It is defined as the difference between the area of intima + media of the proximal reference site and the area of intima + media of the lesion site normalized by the area of intima + media of the reference site (Fig. 1). Sections were stained with

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<th>β-APN Group</th>
<th>Control Group</th>
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<tr>
<td>Pyr, mmol/mol Hypro</td>
<td>0.30 (0.03)</td>
<td>0.52 (0.02)</td>
<td>0.0022</td>
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<tr>
<td>Pen, mmol/mol Hypro</td>
<td>15.9 (1.4)</td>
<td>28.0 (2.0)</td>
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<tr>
<td>Lys, mmol/mg dry wt</td>
<td>228.0 (18.4)</td>
<td>191.5 (16.3)</td>
<td>0.01</td>
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<tr>
<td>Des + Isodes, mmol/mg dry wt</td>
<td>7.2 (0.1)</td>
<td>13.0 (2.2)</td>
<td>0.001</td>
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Values are means (SD); n = 4, β-APN, β-aminopropionitriile; Pyr, pyridinoline; Pen, pentosidine; Hypro, hydroxyproline; Lys, lysine; Des, desmosine; Isodes, isodesmosine.
picrosirius red, orceine, and hematoxylin-eosin to evaluate collagen, elastin, and cell density, respectively. Collagen density was quantified in neointima, media, and adventitia in the lesion and reference sites, as previously described (21). The average length of elastin fibers of the internal elastic lamina and the number of segmentations were automatically determined on section areas with dedicated software, which allowed measurement of length when the magnification was specified (IPS 38, version 4.32). Cell density was also automatically determined on section areas with IPS 38 software.

Statistical Analysis

All the variables were analyzed in a blinded fashion. Values are means (SD). Comparisons between β-APN-treated and control groups were analyzed with Student’s nonpaired t-test. Differences were considered significant when \( P < 0.05 \).

RESULTS

Assessment of Collagen and Elastin Cross-Linking

Biochemical analysis of collagen and elastin cross-linking is summarized in Table 1. Pyridinoline and pentosidine were significantly lower in the β-APN-treated than in control animals, confirming inhibition of the collagen cross-linking process. Increased lysine content and decreased desmosine and isodesmosine indicate inhibition of lysyl oxidase (15). Inhibi-

Fig. 2. Collagen fiber organization evaluated 28 days after angioplasty in β-aminopropionitrile (β-APN)-treated and control animals by picrosirius red staining, scanning electron microscopy, and transmission electron microscopy. Picrosirius red staining showed damage in the neointima and the media and lower collagen density in β-APN-treated than in control animals. Scanning and transmission electron microscopy showed indirect features of inhibition of collagen cross-linking: damaged bundles of collagen with loosening of their characteristic striation (C and E) in β-APN-treated compared with control arteries (D and F). Magnification \( \times1,000 \) (C and D), \( \times12,000 \) (E and F), and \( \times20 \) (A and B). *, Noncollagen tissue.
tion of lysyl oxidase was associated with an increase of lysine content in β-APN-treated animals compared with controls. Desmosine and isodesmosine were significantly decreased in β-APN-treated animals compared with controls, indicating inhibition of elastin cross-linking.

**Qualitative and Quantitative Alterations of Extracellular Matrix**

Collagen fiber organization was evaluated 4 wk after angioplasty by picrosirius red staining and electron microscopy. Neointimal and medial collagen fibers were disrupted and fragmented in sections stained with picrosirius red in β-APN-treated animals (Fig. 2A) compared with controls (Fig. 2B). These findings were confirmed by scanning and transmission electron microscopy, which showed severe collagen disorganization and rarefaction, with smaller bundles and less periodic striation in β-APN-treated animals (Fig. 2, C and E) than in controls (Fig. 2, D and F).

Neointimal and medial collagen densities were significantly decreased in β-APN-treated rabbits compared with controls (Table 2). In contrast, adventitial collagen density was not significantly different between β-APN-treated and control animals (Table 2). Collagen content assessed by hydroxyproline concentration inversely correlated to biochemical markers of collagen cross-linking, i.e., pyridinoline and pentosidine: \( r^2 = 0.79 \) (\( P = 0.003 \)) and \( r^2 = 0.82 \) (\( P = 0.002 \)), respectively.

The elastin fibers of the internal lamina were shorter on average and there were more cleavages of elastin in β-APN-treated rabbits than in controls (Table 2).

**Arterial Remodeling and Restenosis**

**Angiography.** MLD was similar before and immediately after angioplasty in treated and control animals. At 1 mo after angioplasty, MLD was significantly greater in β-APN-treated than in control animals, indicating less restenosis (Table 3).

**Histology.** At 4 wk after angioplasty, restenosis significantly decreased in β-APN-treated compared with control animals (Table 4). The remodeling index was significantly higher in β-APN-treated than in control animals (Table 4), indicating less constrictive remodeling. Neointimal medial growth was less in β-APN-treated than in control animals, although the difference was not significant (Table 4).

Cell density assessed in neointimal hyperplasia was similar between the two groups: 6,187 (SD 496) and 8,798 (SD 1,576) cells/mm\(^2\) (\( P = 0.6 \)) in β-APN-treated and control animals, respectively.

**DISCUSSION**

Constrictive remodeling after arterial injury is the principal mechanism of restenosis and is correlated to collagen accumulation (21). In the skin model, it is well established that wound contracture during the healing process is mediated by collagen cross-linking, leading, in most cases, to skin retraction (2, 3). This tissue retraction can be easily translated in the model of a cylinder similar to an artery into constrictive remodeling. An increase in collagen cross-linking has been recently reported after arterial injury (28). We therefore aimed to focus on the role of collagen cross-linking in constrictive remodeling after arterial injury. Interestingly, inhibition of collagen and elastin cross-linking resulted in a decrease in constrictive remodeling associated with collagen fiber disorganization and reduction of collagen content. Consequently, restenosis was decreased.

**Collagen and Elastin Organization and Remodeling**

Collagen and elastin cross-linking is involved in the stabilization of fibrils via intra- and intermolecular cross-links and the strength of the extracellular matrix (42). Lysyl oxidase promotes cross-link formation in nascent fibrils of collagen and elastin by conversion of lysine and hydroxylysine side-chain residues to aldehydes (23, 31). Lysyl oxidase is a critical enzyme involved in the stability of connective tissue, i.e., the aorta, as well as the maintenance of adult tissues (14, 15). Excessive collagen cross-linking was first described in cutaneous wound healing, leading to a fibrotic scar, i.e., granuloma, after skin damage (38); it has also been observed in the elderly and in patients with hypertension, atherosclerosis, and diabetes. Vessels are characterized by stiffness (i.e., constrictive remodeling), which is reversible after chronic administration of β-APN (6, 36). Recently, an increase of collagen cross-linking has been reported in dilated carotid arteries (28). Because the relation between collagen and elastin cross-linking and remodeling has not been evaluated, we hypothesized that collagen and elastin cross-linking can participate in the healing process, leading to constrictive remodeling. In the present study, inhibition of collagen and elastin cross-linking resulted in disorganization of collagen fibers in β-APN-treated animals and a significant reduction of constrictive remodeling and restenosis. These results suggest that excessive collagen cross-linking may participate in constrictive remodeling after arterial injury. Interestingly, similar results between remodeling and collagen cross-linking have been obtained in a noninjured experimental model. Indeed, lysyl oxidase knockout mice are characterized by excessive enlargement remodeling, leading to aortic aneu-

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<th>Table 2. Collagen and elastin analysis by histomorphometry</th>
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<td><strong>β-APN Group</strong></td>
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<td>Collagen density, %</td>
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<tr>
<td>Neointimal</td>
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<td>Medial</td>
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<td>Adventitial</td>
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<td>Avg length of elastin fibers, μm</td>
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<td>No. of elastin segmentation</td>
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<td>Values are means (SD); ( n = 22 ).</td>
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<th>Table 3. Angiographic analysis: MLD</th>
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<td><strong>β-APN Group</strong></td>
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<tr>
<td>Before angioplasty</td>
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<td>Immediately after angioplasty</td>
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<td>1 mo after angioplasty</td>
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<td>Values are means (SD), in mm; ( n = 22 ). MLD, minimal luminal diameter; NS, nonsignificant.</td>
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<th>Table 4. Histological analysis</th>
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<td><strong>β-APN Group</strong></td>
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<td>Restenosis, %</td>
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<td>Remodeling index</td>
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<td>Neointimal medial growth, %</td>
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<td>Values are means (SD); ( n = 22 ).</td>
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Collagen CROSS-LINKING AND ARTERIAL REMODELING

Collagen Content and Collagen Cross-Linking

Collagen density plays an important role in remodeling after balloon angioplasty and atherosclerosis. An increase in collagen density after angioplasty has been associated with constrictive remodeling and restenosis (21, 40), and a decrease in collagen density has been shown in vulnerable plaques, which are associated in humans with enlargement remodeling (10, 11). In the present study, collagen content was significantly decreased in β-APN-treated animals compared with controls. A similar response was observed with β-APN in bleomycin-induced pulmonary fibrosis and wound healing (24, 34, 43). Lysyl oxidase activity, as assessed by desmosine and isodesmosine analysis (elastin cross-linking), was statistically reduced in β-APN-treated rabbits compared with controls (15). Moreover, biochemical markers of collagen cross-linking (i.e., pyridinoline and pentosidine) inversely correlated to collagen content in the present study. These results emphasize the role of collagen density in the remodeling process after arterial injury. We do not know whether inhibition of collagen cross-linking directly resulted in the decrease of collagen content. It has been shown that non-cross-linked collagen via β-APN treatment is more sensitive to the degradation by matrix metalloproteinases, leading to a reduced collagen content, because collagen cross-linking inhibits matrix metalloproteinase-2 (20).

In conclusion, the results of our study suggest that collagen and elastin cross-linking plays an important role in arterial remodeling and healing after balloon injury. The beneficial effect of β-APN on arterial remodeling in prevention of retrac-tile wound healing may be explained by collagen content reduction and collagen disorganization.

REFERENCES


