Expression of Type VIII Collagen After Cholesterol Diet and Injury in the Rabbit Model of Atherosclerosis

Gabriele Plenz, Anja Dorszewski, G. Breithardt, H. Robenek

Abstract—This study presents an analysis of the expression of type VIII collagen mRNA in response to cholesterol diet and balloon injury in the rabbit iliac artery. The design of the animal experiments was as follows: 28 male New Zealand White rabbits were divided into the 3 different treatment groups. Group 1 received regular chow; group 2 was fed with a 1% cholesterol diet for 6 weeks and normal chow for 5 weeks; and group 3 underwent balloon injury, then 6 weeks of a 1% cholesterol diet, which was followed by 5 weeks of normal chow. The expression pattern of type VIII collagen mRNA was compared with that of the fibrillar collagen types I and III, transforming growth factor-β1, a factor known to exert the most potent stimulatory effect on collagen synthesis in vitro, and matrix metalloproteinase 1, a collagen-degrading enzyme. The cholesterol diet resulted in an upregulation of type VIII collagen, fibrillar collagens, transforming growth factor-β1, and matrix metalloproteinase 1 in the adventitia. Although the number of type VIII collagen mRNA-expressing cells in the media increased, no significant difference in overall expression levels was detectable by northern blot analysis. The ratio of medial smooth muscle cells expressing type VIII collagen mRNA to those expressing type I and type III collagen mRNA (CVIII:CI:CIII) changed from 1:1.88:0.03 in the normal media to 1:0.78:0.29. When cholesterol feeding was preceded by balloon injury, type VIII collagen mRNA expression concomitant with the fibrillar collagens was further upregulated over and above that level reported after cholesterol diet alone. In general, low levels of transforming growth factor-β1 mRNA correlated with high expression of matrix metalloproteinase 1.

Our study indicates that a cholesterol diet resulted in a balanced reorganization of the collagen composition but did not result in marked collagen accumulation. This may provide an extracellular environment that favors migration and proliferation processes during early atherogenesis. It also demonstrates that type VIII collagen is highly expressed and deposited at later stages, and this may be linked to processes such as tissue reorganization during vascular repair and plaque stabilization. (Arterioscler Thromb Vasc Biol. 1999;19:1201-1209.)

Key Words: rabbit ■ balloon injury ■ extracellular matrix remodeling ■ collagen ■ collagenase ■ transforming growth factor-β ■ stenosis

Atherosclerosis involves pathological changes in the normal structure and function of the arterial wall that depend critically on interactions between vessel wall cells and their extracellular environment. The principal vascular cell type responsible for extracellular matrix (ECM) metabolism is the smooth muscle cell (SMC). Medial SMCs, expressing the contractile phenotype, are embedded in an ECM composed of basement membrane molecules, elastin, and collagens. These cells show low proliferative activity and do not migrate. During early atherogenesis, SMCs differentiate from the contractile to the synthetic phenotype. This transition is accompanied by the onset of SMC migration from the medial layer into the intima, increased proliferative activity, and changes in the expression, synthesis, and deposition of ECM components. With consecutive remodeling and accumulation of ECM, the result is intimal thickening and the development of advanced atherosclerotic lesions.

Collagens expressed at low levels are essential constituents of the normal media, but excessive production of collagens is a major feature of advanced atherosclerotic plaque. Ninety percent of the total protein in the plaque consists of collagens, predominantly type I and type III collagen. Fibrillar collagens and other members of the collagen family, types IV and V collagen, appear to play important roles in the processes of SMC phenotype modulation, vascular repair, and plaque stabilization. Although the fibrillar collagens have been investigated extensively in relation to atherosclerosis, our knowledge of the role of type VIII collagen remains limited.

Type VIII collagen is a component of the normal vessel wall, synthesized by endothelial cells (ECs) and...
SMCs. This type of collagen forms 3D networks and is proposed to participate in angiogenesis. Studies on the proliferation and migration of SMCs in the rat balloon injury model provide evidence that type VIII collagen plays a role in promoting migration but not proliferation of SMCs.

Changes in ECM environment influence the behavior of SMCs and, thus, the cross talk between SMC and ECM. ECM components might play an important role in the regulation of the availability of cell mediators and the responsiveness of SMCs to mediators such as transforming growth factor-β (TGF-β). TGF-β1 has been implicated in the formation of an ECM during intimal hyperplasia. It is known to stimulate collagen gene expression, fibrillar collagens as well as type VIII collagen, and to influence the balance of synthesis and degradation of the ECM.

Pericellular proteolysis cascades are required for vascular remodeling and activation of growth factor release during atherogenesis. Matrix metalloproteinases (MMPs), a family of potent proteinases, have been implicated in these processes by way of ECM degradation. Structure and stability of the lesion depend on the balance between synthesis and degradation of the collagenous matrix. Several animal models have been used to study the processes of lesion development. Balloon endothelial denudation and medial layer damage with or without subsequent cholesterol diet constitutes a single-injury model in which there is de novo intimal growth in a previously normal artery. This single-step approach has been evaluated in rat, porcine, and rabbit arteries. In rabbits, single balloon injury combined with moderate cholesterol feeding resulted in the development of lesions resembling those of humans, consisting primarily of SMCs. The lesions contain a fibromuscular cap covering a core composed of extracellular lipid and cell debris and show marked depositions of collagenous matrix.

In the present study we focused on the expression of network-forming collagen type VIII mRNA in comparison with fibrillar collagens (types I and III collagen), and in relation to TGF-β1 and MMP-I in rabbit iliac arteries after a 1% cholesterol diet with and without previous balloon injury. The mRNA expression was followed by in situ hybridization, for type VIII collagen also by northern blot analysis, and with immunohistochemistry to examine the corresponding protein. We demonstrate stimulation of type VIII collagen mRNA expression, codistribution of type VIII collagen with TGF-β1 and MMP-I, and changes in the composition of collagens after cholesterol diet and balloon injury in rabbits.

Balloon Denudation
The rabbits underwent arteriotomy of the left and right femoral arteries. A 3 Fr Fogarty embolectomy catheter was advanced retrogradely into the iliac artery by 10 cm and inflated until contact was made with the vessel wall. The lower aorta and both iliac arteries were denuded by gentle advancement and withdrawal of the catheter 3 times as previously described.

Tissue Preparation
Before exsanguination, each animal received an injection of etomidate (2 mg/kg of body weight; Hypnomidate, Janssen) intravenously. The iliac arteries and abdominal aorta were exposed and cannulated for runoff of the perfusion medium. After median sternotomy, the cannula connected to a perfusion apparatus was inserted into the left ventricular apex. Perfusion was performed with a perfusion pressure of about 100 mm Hg for 7 minutes at 22°C. The blood was flushed with 0.9% sodium chloride solution followed by 150 to 200 mL of glutaraldehyde/saline. After perfusion fixation, the iliac arteries and the abdominal aorta were carefully removed and processed for morphological studies (group 1, n=4; group 2, n=5; and group 3, n=5).

For in situ hybridization and immunohistochemistry, fresh material was used (group 1, n=4; group 2, n=5; and group 3, n=6). The arteries were placed in cryoprotective medium on cork disks and snap-frozen in liquid nitrogen. For northern blot analyses, fresh tissue from the same animals was frozen directly in liquid nitrogen.

Histological and Morphometric Evaluation
The lumen, the tunica media, and the intimal layer were measured in perfusion-fixed cryostat cross-sections of 10-μm thickness (MTC-Microtome, SLEE) stained by the Goldner technique. Morphometric analysis of the cross sections was performed by using a video-morphometric system (VIDAS, Kontron Electronics). The length and position of a lesion was calculated in a series of 10-μm-thick sections of injured arteries. In each artery, 10 sections were measured, representing the middle third of the lesions. The areas and the values were summarized as means of the cross-sectional areas of the lumen, intima, and tunica media and as means of the mean and standard deviation values of each group. Significant differences between the experimental groups were calculated with the 2-sided Mann–Whitney U test at a significance level of P<0.05.

Changes in the composition of the arteries were followed by standard histological techniques. General histology and lipid distribution were evaluated by using the hemalum–eosin– and fat-red-staining techniques. ECM was stained by using trichrome-staining techniques.

Probes and Labeling Procedure
For in situ and in vitro RNA analysis, the following recombinant cDNA clones were used: pHf677, containing an insert complementary to the human α1(III) procollagen mRNA; pH33, complementary to human α1(III) procollagen mRNA; pBSIIIα1Col8, complementary to the human procollagen α1(VIII) mRNA; phTGF-β1 complementary to human TGF-β1 mRNA; pC3P2 complementary to human MMP-1; and cG3PDH (Clontech), complementary to the human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA. The in vitro transcription was performed according to the manufacturer’s protocol, using digoxigenin-labeled UTP (Boehringer Mannheim).

Northern Blot Analysis
Total RNA (0.155±0.0856 μg/mg of tissue) was isolated according to Chirgwin et al. For the northern blot analysis, 4.0 μg of total RNA was fractionated by electrophoresis under denaturing conditions on a 1.1% agarose/formaldehyde gel. Hybridization was performed with conditions as previously described. Modifications were as follows: hybridization was performed at 72°C, using either α1(VIII) procollagen or G3PDH antisense riboprobe (50 ng/mL). Detection was performed by using an alkaline phosphatase detection protocol (Boehringer Mannheim) and the chemiluminescent substrate CSPD (Toyochem/Serva). To evaluate the relative expression, the luminographs on x-ray film (Kodak X-OMAT AR)
were scanned by using a laser densitometer (Personal Densitometer, Molecular Dynamics). The absorbance units were normalized to G3PDH mRNA.

In Situ Hybridization
In situ hybridization was performed on cryostat sections (5 μm) following methods modified from those previously described with 0.3 μg of digoxigenin-labeled antisense or sense riboprobe/mL of hybridization solution (50% formamide, 2× SSPE, 10 mmol/L DTT, 2 mg/mL herring sperm DNA, 200 mg/mL yeast tRNA, and 1 mg/mL BSA) at 52°C in a humidified chamber. To evaluate the background from the hybridization procedure, slides were incubated with hybridization solution only. For the lowest stringency the washing buffers contained 2× SSC, and for the highest stringency 0.1× SSC. Washing was performed at 50°C.

For detection of the in situ hybridization signal a modified anti-digoxigenin alkaline phosphatase protocol (Boehringer Mannheim) was used. As a control to the detection procedure, the antibody was omitted. The alkaline phosphatase–staining procedure was performed in the dark overnight by using nitroblue tetrazolium salt (67.5 mg/mL; BIOMOL) and 5-bromo-4-chloro-3-indolyl phosphate (35 mg/mL; BIOMOL) as substrates. Sections were counterstained with methylene green and mounted with Kaiser’s glycerin gelatin. Background was not observed in sections hybridized with the sense probe and in hybridization solution only. Neither the antibody nor the staining procedure caused background.

The number of expressing cells was evaluated microscopically by relating the calculated number of mRNA-expressing cells to the total number of cells counted in a microscopic area (×250). Data are expressed as mean±standard deviation (±SD) values.

Immunohistochemistry
Cell types were identified as follows: (1) for SMCs, mouse anti-human α/γ actin (HHF35; Loxo), and (2) for macrophages, mouse anti-rabbit RAM11 (MG33; Dako) were used. Type VIII collagen was localized by using mouse anti-bovine type VIII collagen (C8; Medac).

For detection, a fluorescence-staining protocol was used, with HHF35, RAM11, and C8 as primary antibodies. As a secondary antibody/detection system, we used donkey anti-mouse immunoglobulin conjugated to Cy3 (Chemicon). After detection, slides were mounted with fluoromount mounting medium.

Negative controls included substitution of the primary antibody by mouse immunoglobulins or omission of the primary antibody.

Confocal Laser Scanning Microscopy and Correlative Histology
Fluorescence-labeled sections were examined by confocal laser scanning microscopy by using a Leica TCS 4D equipped with an argon/krypton laser and fitted with the appropriate filter block for detection of Cy3 fluorescence. The images were taken by using simultaneous dual-channel scanning and transformed into projection views by using sets of 5 consecutive single optical sections taken at 1-μm intervals.

Results
Design of the Animal Experiments
Our experimental design, single balloon injury combined with a phase of moderate cholesterol feeding, resulted in the development of lesions resembling those of humans. The lesions contained a fibromuscular cap covering a core composed of extracellular lipids and cell debris and showed marked depositions of collagenous matrix. Without additional feeding of normal chow, the diet resulted in lesions predominantly composed of cells and lipids, whereas depositions of ECM are sparsely found. Therefore, to generate lesions resembling those of humans requires the 5-week normal chow diet.

Arteries After Normal Chow and Cholesterol Diet
Morphometry and Cellular Composition
In group 2 (1% cholesterol diet for 6 weeks followed by normal chow for 5 weeks), some intimal thickening restricted to limited areas (intimal area, 0.233±0.043 mm²) was found, although there was no detectable change in the size of the vessel lumen (Table 1). The media stained homogenously for HHF35 (Figure 1a). Macrophages were detected in the adventitia only (Figure 1b).

Expression Patterns
In normal iliac arteries as well as in iliac arteries of cholesterol-fed rabbits (groups 1 and 2), type VIII collagen mRNA was expressed in a proportion of the ECs, SMCs, and adventitial cells (Figure 2a and 2b). For type VIII collagen, the strongest response to cholesterol was observed in the adventitia (Figure 2b). Types I and III collagen are concomitantly expressed. MMP-I (Figure 2c and 2d) and TGF-β1 (Figure 2d and 2e) were also preferentially upregulated in the adventitial zone. In the media, transcription of MMP-I was upregulated whereas TGF-β1 was markedly downregulated.

Thirty-three percent of the medial SMCs expressed type VIII collagen mRNA (Table 2). In control animals, the percentage of expressing cells was almost identical in the iliac artery, carotid artery, and abdominal aorta, although

<table>
<thead>
<tr>
<th>TABLE 1. Quantitative Analysis of Atherosclerosis in Rabbits Fed Normal Chow, 1% Cholesterol Diet, and 1% Cholesterol Diet With Balloon Injury</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Lumen, mm²</td>
</tr>
<tr>
<td>Intima, mm²</td>
</tr>
<tr>
<td>Media, mm²</td>
</tr>
<tr>
<td>Intima/media ratio, %</td>
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</tbody>
</table>

P<0.05, intimal area and intima/media ratio (%) of normal diet versus 1% cholesterol versus 1% cholesterol and balloon injury; P<0.05, luminal area of normal diet and 1% cholesterol versus 1% cholesterol and balloon injury.
marked differences in signal intensity were found (data not shown). Cholesterol diet resulted in increased numbers of cells expressing type VIII collagen mRNA (Table 2). Sixty percent of SMCs from control arteries expressed type I collagen. Accumulations of expressing cells were observed adjacent to the external elastic lamina and in the adventitia. After cholesterol diet, the average number of expressing cells per area did not change (Table 2). Enhanced numbers of expressing cells were sporadically found in the subendothelial medial and intimal regions, whereas decreased cell numbers were observed in the inner medial areas. In comparison with type VIII and I collagen, type III collagen mRNA was expressed at low levels in control arteries and restricted to a small number of medial SMCs. Cholesterol feeding markedly stimulated the type III collagen mRNA expression (Table 2). The ratio of medial SMCs expressing type VIII collagen mRNA to SMCs expressing the fibrillar collagens (CVIII:CI:CIII) changed from 1:1.88:0.03 to 1:0.78:0.29.

Low levels of MMP-I mRNA and only a restricted number of expressing cells (11%) were identified in the normal iliac artery (Figure 2c). After cholesterol diet, the expression of MMP-I was markedly enhanced in the media and in the adventitia, as reflected by the number of expressing cells and the intensity of the in situ signal (Figure 2d and Table 2).

In the normal arteries, a low to moderate but homogeneous expression of TGF-β1 mRNA occasionally occurred in ECs, in medial SMCs, and in the adventitia (Figure 2e). After cholesterol diet, the number of TGF-β1 mRNA–expressing cells in the media was reduced (Table 2). Enhanced levels were observed in the adventitia only (Figure 2f).

As demonstrated by immunohistochemistry (Figure 3) cholesterol feeding did not markedly effect the distribution and deposition of type VIII collagen. In arteries of chow-fed rabbits, label for type VIII collagen was found in the endothelium, the subendothelial media, and the adventitia (Figure 3a). After cholesterol diet, type VIII collagen was synthesized by ECs but found sparsely in the subendothelial media. Increased immunoreactivity was observed at the media/adventitia border (Figure 3b).

**Changes in Response to Cholesterol Diet and Balloon Injury**

**Morphometry, Histological Evaluation, and Cellular Composition**

Balloon injury combined with cholesterol diet led to the development of fibrolipid lesions, resembling those of humans (Figure 4). Accumulations of ECM (Figure 4a) and lipids (Figure 4b and 4c) were observed. For the purpose of analyzing the expression patterns, 2 different zones of the lesion were defined.

<table>
<thead>
<tr>
<th>TABLE 2. Expression of Type VIII Collagen (CVIII), Fibrillar Collagen Types I (CI) and III (CIII), TGF-β1, and Collagenase (MMP-1) mRNA in the Media of Iliac Arteries of Normal and Cholesterol-Fed Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal Diet</strong></td>
</tr>
<tr>
<td><strong>Mean (n=10)</strong></td>
</tr>
<tr>
<td>Cell no.</td>
</tr>
<tr>
<td>CVIII</td>
</tr>
<tr>
<td>CI</td>
</tr>
<tr>
<td>CIII</td>
</tr>
<tr>
<td>MMP-I</td>
</tr>
<tr>
<td>TGF-β1</td>
</tr>
</tbody>
</table>

Rel. Exp. indicates relative expression.

*P* ≤ 0.05, normal diet versus 1% cholesterol: CVIII, CIII, MMP-I, TGF-β1.

*P* ≤ 0.05, normal diet versus 1% cholesterol: CI.

Total cell number estimated by calculating the number of medial SMCs microscopically (microscopic area: ×250; n=3, 3 serial sections each) was set to 100%. The number of expressing cells (microscopic area: ×250; n=3, 4 serial sections each) was presented as percentage of the total number of cells.
First the eccentric, cell-rich zone with ECM-rich areas and marked intima formation (intimal area, 1.60 $\pm$ 0.038 mm$^2$) located in the area of the shoulder of the lesion. Parts of the vessel showed a normal composition or adaptive thickening. The principal cell type was the SMC. The plaque core contained lipid-rich glue and lipid-laden macrophages.

The second type comprised the concentric zone of the lesions with complicated composition, a high proportion of ECM-rich areas, and pronounced intima formation (intimal area, 2.363 $\pm$ 0.105 mm$^2$; Table 1), which were located in the area of maximum lesion development. Macrophages were found mainly in the plaque core and base.

Expression Patterns in Response to Cholesterol Diet and Balloon Injury

In the eccentric lesion areas, high levels of type VIII collagen mRNA were detected. Those parts of the vessel that showed the composition of normal arteries or only adaptive thickening revealed expression patterns for type VIII collagen mRNA and the other mRNAs similar to those found in arteries after cholesterol diet only. In general, all parts of the adventitia adjacent to SMC-rich areas at the plaque base were strongly activated. In addition, type VIII collagen was strongly expressed in the endothelium and the subendothelial region of the lesion; cellular mRNA levels varied. An example of the typical expression pattern of type VIII collagen mRNA is shown in Figure 5a. Accumulations of cells expressing type I collagen mRNA occurred at the plaque shoulder, plaque cap, and plaque base. In the adventitia adjacent to the plaque, almost all cells expressed high levels of type I collagen mRNA. Cells expressing type III collagen mRNA were located mainly in the plaque base and in the subendothelial region. In other parts of the lesion the distribution appeared to be homogeneous. Strong expression of MMP-I (Figure 5b) and TGF-$\beta$1 (Figure 5c) mRNA took place in the adventitia, plaque base, and core, in the fibrotic cap, and in the endothelium. Both mRNAs codistribute with type VIII collagen mRNA. The sense control was devoid of label (Figure 5d).

The concentration lesion areas demonstrated heterogeneous expression patterns in line with their complex morphology.
In general, strong expression of type VIII collagen mRNA (Figures 6a and 7) occurred in intimal regions composed of SMCs, in the media, and in the adventitia. Elevated levels of the fibrillar collagen mRNAs were observed in parallel. The type VIII collagen mRNA was codistributed with TGF-\(\beta\)1 mRNA but only in some areas with MMP-I mRNA (Figure 6b and 6c).

The percentage of cells expressing type VIII collagen ranged from 49% to 69% in the ECM-rich and cell-poor intimal areas and the plaque core. In the other areas of the intima and media, type VIII collagen mRNA expression ranged from 50% to 90% of the cell population (Table 3). Immunohistochemistry demonstrated occurrence of type VIII collagen in the media and intima. The major type VIII collagen–synthesizing cell type was the SMC (Figure 7b and 7c). Although of less intensity, label for type VIII collagen was also found in regions composed mainly of macrophages (Figure 7a and 7c).

In comparison with the collagens, the expression of MMP-I and TGF-\(\beta\)1 mRNA was low. TGF-\(\beta\)1 mRNA expression was inversely related to the expression of MMP-I mRNA (Table 3).

The percentage of cells expressing type I collagen mRNA ranged from 36% to 69% in the ECM-rich and cell-poor intimal areas and the plaque core. In the other areas of the intima and media, type VIII collagen mRNA expression ranged from 50% to 90% of the cell population (Table 3). Immunohistochemistry demonstrated occurrence of type VIII collagen in the media and intima. The major type VIII collagen–synthesizing cell type was the SMC (Figure 7b and 7c). Although of less intensity, label for type VIII collagen was also found in regions composed mainly of macrophages (Figure 7a and 7c). Analysis of the overall expression of type VIII collagen mRNA by northern blot analysis revealed no significant stimulation after cholesterol feeding but significant stimulation after cholesterol diet preceded by balloon injury (Figure 8A and 8B).

The percentage of cells expressing type I collagen mRNA ranged from 36% to 69% in the ECM-rich and cell-poor intimal areas and the plaque core (Table 3). In all other areas of the intima and media, type VIII collagen mRNA expression was by almost every cell (up to 96%). Type III collagen mRNA was expressed by 29% to 52% of SMCs in the media, ranging from 27% to 79% of cells in the intima, and 0.02% to 79% of cells in the adventitia (Table 3), whereas the signal intensity was low.

### Table 3. mRNA Expression of Type VIII Collagen (CVIII), Fibrillar Collagen Type I (CI) and III (CIII), TGF-\(\beta\)1, and MMP-I in a Typical Concentric Lesion After Cholesterol Feeding and Balloon Injury

<table>
<thead>
<tr>
<th>cell no.</th>
<th>area</th>
<th>mean ±sd (no.</th>
<th>CVIII</th>
<th>CI</th>
<th>CIII</th>
<th>MMP-I</th>
<th>TGF-(\beta)1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-a</td>
<td></td>
<td>21.6 ±3.85 (122)</td>
<td>21.5 ±3.85 (100)</td>
<td>0.4 ±0.56</td>
<td>2.4 ±1.14</td>
<td>17.8 ±2.77</td>
<td>16.8 ±7.6</td>
</tr>
<tr>
<td>1-m</td>
<td></td>
<td>22.4 ±2.19 (64)</td>
<td>21.6 ±3.85 (53)</td>
<td>6.0 ±1.14</td>
<td>1.8 ±0.94</td>
<td>4.0 ±17.1</td>
<td>20.71</td>
</tr>
<tr>
<td>1-l</td>
<td></td>
<td>34.2 ±13.88 (50)</td>
<td>22.8 ±6.16 (67)</td>
<td>27 ±1.03</td>
<td>12.6 ±5.41</td>
<td>4 ±12</td>
<td>5.2</td>
</tr>
<tr>
<td>2-a</td>
<td></td>
<td>32.0 ±3.83 (94)</td>
<td>31.4 ±4.04 (96)</td>
<td>16.4 ±2.58</td>
<td>3.8 ±1.14</td>
<td>18 ±55</td>
<td>1 ±87.7</td>
</tr>
<tr>
<td>2-m</td>
<td></td>
<td>62.4 ±7.62 (99)</td>
<td>66.4 ±8.99 (89)</td>
<td>23.2 ±2.77</td>
<td>5.4 ±1.15</td>
<td>30.4 ±48</td>
<td>30.4 ±48</td>
</tr>
<tr>
<td>2-m</td>
<td></td>
<td>109±14.37 (90)</td>
<td>111.4 ±7.02 (82)</td>
<td>37 ±10.5</td>
<td>4.0 ±2.79</td>
<td>61.6 ±45</td>
<td>61.6 ±45</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>43.2 ±12.52 (69)</td>
<td>30 ±8.96</td>
<td>31.8 ±7.7</td>
<td>10.4 ±3.96</td>
<td>8.4 ±22</td>
<td>8.4 ±22</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>63.2 ±10.08 (49)</td>
<td>55.8 ±8.04 (88)</td>
<td>52.6 ±7.7</td>
<td>10.3</td>
<td>12.8 ±2.8</td>
<td>12.8 ±2.8</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>42.8 ±7.5 (99)</td>
<td>15.2 ±7.9</td>
<td>14.2 ±1.9</td>
<td>0</td>
<td>6.8 ±10</td>
<td>6.8 ±10</td>
</tr>
<tr>
<td>6-a</td>
<td></td>
<td>32.0 ±7.83 (99)</td>
<td>31.4 ±8.73</td>
<td>20.2 ±5.63</td>
<td>12 ±2.85</td>
<td>32.3 ±2.92</td>
<td>32.3 ±2.92</td>
</tr>
<tr>
<td>6-m</td>
<td></td>
<td>69 ±8.98 (99)</td>
<td>61.6 ±7.85</td>
<td>36.2 ±4.70</td>
<td>12 ±2.44</td>
<td>12 ±17</td>
<td>12 ±17</td>
</tr>
<tr>
<td>6-l</td>
<td></td>
<td>79 ±8.98 (81)</td>
<td>72.2 ±8.79</td>
<td>57.8 ±5.41</td>
<td>44.4 ±1.17</td>
<td>12 ±3.7</td>
<td>12 ±3.7</td>
</tr>
</tbody>
</table>

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**Figure 6.** Concentric area of the lesion; in situ expression of type VIII collagen, MMP-I, and TGF-\(\beta\)1 mRNA after balloon injury followed by cholesterol diet. In situ hybridization using type VIII collagen (a), MMP-I (b), and TGF-\(\beta\)1 (c) antisense riboprobes. In comparison with the eccentric areas, in areas of maximal stenosis, mRNA expression was in general lower. a, Type VIII collagen mRNA expression was by almost every cell. b, MMP-I mRNA expression was drastically decreased. c, TGF-\(\beta\)1 mRNA strongly codistributed with type VIII collagen mRNA. (Original magnification, ×40.) Arrowheads indicate the internal and external elastic laminae. A indicates adventitia; I, intima; M, media; and L, lumen.
Discussion

It is becoming increasingly clear that type VIII collagen is a key structural component of the vasculature and its expression in vitro by the constituent cell types of the arterial wall has previously been well documented.\textsuperscript{12,14,15} In the rat model of early atherogenesis, alterations in type VIII collagen have been demonstrated as a component of the SMC response to injury, and are suggested to play a functional role in mediating migration of SMCs.\textsuperscript{19,53} The present study in the rabbit injury/cholesterol diet model extends existing work by providing the first evidence that the presence of type VIII collagen is not only related to the migration of SMCs during early atherogenesis\textsuperscript{19} but that this component is also highly expressed at later stages, implying additional roles in such processes as tissue reorganization during vascular repair and plaque stabilization.

Cholesterol Diet–Induced Changes

Type VIII collagen mRNA expression is slightly stimulated after cholesterol feeding, with the strongest increase being observed in the adventitia. However, increased deposition of collagen was not observed, indicating a balanced synthesis of collagen and degradation by collagen-degrading proteins (ie, MMP-I).\textsuperscript{54,55}

SMCs are major players in early processes of atherogenesis.\textsuperscript{56} They synthesize collagens and other ECM components and their proliferation leads to intimal hypertrophy. Marked intimal thickening has not been observed after cholesterol treatment, as indicated by the morphometric evaluation. Therefore, the demonstrated ECM reorganization in the media is probably the first step in providing the appropriate environment for the migration of SMCs, as previously suggested in the rat injury model.\textsuperscript{19,53} SMCs are probably capable of responding to atherogenic factors, such as cholesterol or growth factors, by remodeling the ECM and thereby enabling the vessel wall for time-restricted accumulation of lipids and facilitating lipid transgression. Fibrillar collagens have been proposed to trap lipids in the intima by slowing down their diffusion\textsuperscript{57,58} and network-forming type VIII collagen would be another potential candidate for this process.

Our studies demonstrated the strongest transcriptional activation in the adventitia after cholesterol feeding. However, intimal thickening was not observed. It has been thought that the adventitia may play a crucial role in the maintenance of the integrity of the inner layers of the arterial wall. The development of SMC-rich lesions in normal chow–fed rabbits after removal of the adventitia indicates that the adventitia might influence the migratory and proliferative activity of medial SMCs. Components synthesized by adventitial cells such as ECM components, matrix-degrading molecules, and growth factors may, within limits, inhibit intima formation and affect cellular composition.\textsuperscript{59–61}

Changes in Response to Cholesterol Diet and Balloon Injury

To maintain normal remodeling after injury, a balance between ECM synthesis and degradation is necessary.\textsuperscript{39,62} The imbalance between the synthesis of ECM molecules and matrix-degrading enzymes in wound-healing processes in general is reflected in marked accumulation of ECM.\textsuperscript{63,64} In our model this process is characterized on the molecular level by downregulation of MMP-I and upregulation of collagens and TGF-\(\beta\)1 mRNA after mechanical injury of the arteries, particularly in areas of maximum stenosis.

During the progression of atherosclerotic lesions, the SMC phenotype changes from the contractile to the synthetic

Figure 7. Cellular composition and occurrence of type VIII collagen after balloon injury combined with cholesterol diet. HHF35-positive SMCs (a) occurred in media and intima. For type VIII collagen (c), label of varying intensities was found in media and intima. RAM11-positive macrophages (b) were located mainly at the plaque base adjacent to the media. Type VIII collagen (c) was synthesized and deposited in areas predominantly composed of SMCs (circle) but also in regions composed of macrophages (star). However, the strongest signals were observed in those zones composed of SMCs. (Original magnification, \(\times 160\).) A indicates adventitia; I, intima; M, media; and L, lumen.
phenotype. Enhanced capacity for the synthesis of collagen in general is related to the so-called synthetic SMC phenotype. Nevertheless, the occurrence of synthetic SMCs cannot fully explain the demonstrated distribution patterns, in particular the codistribution of type VIII collagen with macrophage-rich areas. In advanced lesions, stimulation of collagen expression by SMCs reportedly correlates with the appearance of macrophages. As indicated here, type VIII collagen mRNA not only colocalizes in some areas with the appearance of RAM11-positive macrophages but is synthesized by macrophages themselves. Our observations in the rabbit model agree well with the human system, as previously shown by our laboratory (G.P., unpublished observations, 1998). Macrophages are thought to be responsible for MMP-mediated destabilization of the plaque cap and thus for plaque rupture as well as for matrix remodeling via TGF-β-dependent mechanisms. TGF-β1 is known to exert the most potent stimulatory effect on collagen synthesis and to mediate the degradation of type I collagen. One might speculate that network-forming type VIII collagen synthesized by macrophages contributes to the maintenance of lesion integrity by substituting other ECM components, i.e., fibrillar collagens.

Thus, changes in the expression, distribution, and metabolism of type VIII collagen may reflect either balanced ECM reorganization, providing the appropriate environment for the immigration of SMCs into the intima and maintaining lesion integrity, or imbalanced ECM remodeling, causing excessive deposition of collagen or plaque destabilization and rupture.

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References


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