Collagens in Human Atherosclerosis

Immunohistochemical Analysis Using Collagen Type-Specific Antibodies

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This study represents a systematic analysis of the distribution of collagen types in human atherosclerotic lesions. Formalin-fixed, paraffin-embedded aortic tissues of 40 lesions from 16 different individuals ranging in age from 1 month to 84 years were examined immunohistochemically using antibodies to type I, III, IV, V, and VI collagens. Preembedding immunoelectron microscopy was used to simultaneously localize type V and VI collagens within the lesions. Localization of type III collagen was very similar to that of type I, and type VI collagen appeared together with these two types of collagen in the thickened intimas of all stages of the lesion. Type V collagen was not detected in either fatty streaks or the mild intimal thickening of the aortas of children. With advancing age and lesion progression, the immunoreactivity with anti-type V collagen antibody became more intense. Type IV collagen was detected in the basement membrane region of intimal cells. In advanced lesions thick deposits of type IV collagen were found around the elongated smooth muscle cells. Using immunoelectron microscopy, type V collagen was found to be localized to cross-banded collagen fibers, and type VI collagen was found to be localized to beaded filaments present throughout the interstitium of the thickened intima. These findings suggest that collagens preserve the pathophysiological and functional integrity of the vascular wall by providing mechanical support as well as assuring the proper interaction of cells during the formation of atherosclerotic lesions. (Arteriosclerosis and Thrombosis 1992;12:494–502)

Key Words • atherosclerosis • collagen • immunohistochemistry

Atherosclerosis is a disease of large and medium-sized arteries and is characterized by focal thickening of the inner portion of the artery wall in association with fatty deposits.1,2 The common underlying events responsible for lesion formation are intimal smooth muscle cell proliferation, formation of new extracellular matrix by these cells, and the possible accumulation of lipid. Collagens are regarded as important in this disease not only because they represent the major extracellular matrix component in the atherosclerotic plaque and thereby contribute to the occlusive nature of the disease but also because they may play an important role in hemostasis and thrombosis through stimulation of blood platelets.3 In addition, recent studies have indicated that collagens can influence the proliferation and state of differentiation of smooth muscle cells in vitro.4–6 Thus, it seems likely that collagens could play a vital role in the pathogenesis of this disease.

Collagen is now recognized as a family of proteins of at least 13 genetically distinct subtypes, each of which has a unique tissue specificity.7–9 Of these 13 collagen types, six (I, III, IV, V, VI, and VIII) are present in blood vessels.10 All of these collagen types except type VIII collagen are known to be synthesized by smooth muscle cells, and all except type VI collagen, by endothelial cells.10 The distribution of different collagen types in the normal and the atherosclerotic arterial wall has been the subject of extensive investigations.11–15 These observations have documented a predominance of interstitial collagen types I and III in the fibrous stroma of plaques as well as an increase in the amount of type IV and type V collagens relative to the normal vessel wall. However, the method used in these previous studies was immunofluorescence of frozen tissue sections, which do not give the morphological detail afforded by paraffin-embedded tissues. Furthermore, there are no published data available on the distribution of type VI collagen in atherosclerotic lesions.

It is well known that the interstitial collagens, types I and III, are assembled into the cross-banded fibrils that are visible by electron microscopy and that type IV collagen is present in the basement membranes of endothelial cells and smooth muscle cells in the arterial wall. However, little is known about the ultrastructural localization of type V and type VI collagens in the atherosclerotic plaque. The purpose of the present study is to report an investigation of the distribution of collagen types deposited in diffusely thickened intimas and atherosclerotic lesions and to document the changes in this distribution with disease progression, using antibodies against type I, III, IV, V, and VI collagens in deparaffinized, formalin-fixed aortic tissues. In addition, localization of type V and type VI collagens in atherosclerotic lesions has been performed by immunoelectron microscopy.
Methods

Preparation of Collagen

Collagen was extracted from human placentas by pepsin digestion, and then type I, III, IV, V, and VI collagens were isolated by differential salt precipitation procedures.6,7 Type III collagen was further isolated by a differential denaturation/renaturation procedure.8 To remove contaminated noncollagenous proteins, each type of collagen isolated was passed through a DEAE-cellulose column.9 The purity of each type of collagen was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)20 and by SDS-PAGE combined with digestion by pure bacterial collagenase derived from Clostridium histolyticum (form III, Advance Biofactures Co., Lynbrook, N.Y.).21

Preparation of Type-Specific Anti-Collagen Antibodies

Sprague-Dawley rats were immunized with human type I, III, IV, or V collagen and Nippon White rabbits with human type VI collagen. The antibody titers of each antiserum was monitored by enzyme-linked immunosorbent assay (ELISA)22 during immunization. Type-specific anti-collagen antibodies were purified from crude antisera by cross-adsorption of affinity columns consisting of heterogenous collagen types coupled with activated CH-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) diluted 1:2 in PBS, pH 7.4. After incubation the tissues were again rinsed extensively in PBS for 7 hours, transferred to 0.1 M cacodylate buffer, pH 7.4, and postfixed with 1% OsO4 in 0.1 M cacodylate buffer for 1 hour at 4°C. The tissues were then dehydrated with ethanol and embedded in Epon 812. Sections were cut with an LKB ultratome using diamond knives. Thin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-500 electron microscope operating at 75 kV.

Normal Aorta

The intimas of infants within the age of a few months showed normal structures consisting of a single layer of endothelial cells resting on the internal elastic lamina. No collagen fibers were seen in this area with either elastica van Gieson’s or azan staining. At a few months of age, a narrow layer of connective tissue was interposed between the endothelial cells and the internal elastic lamina. The intima developed gradually and increased in thickness over the years. In this study the progressive thickening of the intima with aging was described as either mild intimal thickening in children or diffuse intimal thickening in adults. Aortic media was composed of multiple parallel elastic lamellae with single layers of smooth muscle cells sandwiched between them. The extracellular spaces were filled by collagen and elastic fibers and Alcian blue–positive materials. The adventitia was made up of loosely arranged collagen and elastic fibers, spindle-shaped cells, blood vessels (vasa vasorum), lymphatic vessels, and nerves.

In the normal intimas of infants, type IV collagen was observed as a linear structure in the subendothelium, presumably in the endothelial basement membrane. Type I, III, V, and VI collagen were not detected.

In the media, type I and type III collagens were organized into fibrillar structures localized between smooth muscle cells and elastic lamellae, together with type VI collagen. Type IV collagen was observed as a membranous structure surrounding the smooth muscle cells. Type V collagen was not detected in the media of younger subjects, but the media of older subjects occa-
FIGURE 1. Photomicrographs showing diffuse intimal thickening of the aorta immunostained for type I collagen (panel a) and type III collagen (panel b). Type I and III collagens are diffusely distributed throughout the intima. Bar indicates the boundary between intima and media. ×272.

FIGURE 2. Photomicrograph showing diffuse intimal thickening of the aorta immunostained for type IV collagen. Type IV collagen exists beneath endothelial cells and around intimal cells. ×544.

FIGURE 3. Photomicrographs showing mildly thickened intima of aorta from 3-year-old child (panel a) and diffusely thickened intima of adult aorta (panel b) immunostained for type V collagen. Type V collagen is not present in the immature aorta and only weakly detected in the adult aorta. Bar indicates the boundary between intima and media. ×272.

FIGURE 4. Photomicrograph showing diffuse intimal thickening of the aorta immunostained for type VI collagen. Type VI collagen localizes with a reticular staining pattern in the intima. ×544.

FIGURE 5. Photomicrograph showing fatty streak of the aorta immunostained for type IV collagen. Foam cells show variable immunoreactivity with anti-type IV collagen antibody. Also note the type IV collagen-negative foam cells (arrows). ×544.

FIGURE 6. Photomicrographs showing fibrous plaque without atheroma immunostained for type III collagen (panel a) and type V collagen (panel b). Type III and V collagens are diffusely distributed throughout the thickened intima. ×272.

Mild Intimal Thickening in Children

The lesions with mild intimal thickening were covered by endothelium, and there were a few spindle-shaped or occasionally contained type V collagen. No age-dependent alteration of the distribution of collagen types except type V collagen was detected.

The adventitia was mostly composed of type I and type III collagens. Type VI collagen was also present throughout the adventitia, but type V collagen was not evident. Type IV collagen was localized only in the wall of small blood vessels.
round cells in the abundant extracellular matrix, with predominantly Alcian blue–positive substances intermixed with a few elastic fibers and collagen fibers that were positive for resorcin–fuchsin or azan stain. Using anti-collagen antibodies, we observed that type I and type III collagens were present as sparsely distributed thin fibrillar structures. Type IV collagen was detected in the basement membrane regions of endothelial cells and some subendothelial cells. Type VI collagen was present as a fine fibrillar or reticular pattern. Type V collagen was not detected in the intima of this type of lesion (Figure 3a).

**Diffuse Intimal Thickening in Adults**

In the adult aortas with diffuse thickening, many spindle-shaped cells intermixed with round mononuclear cells were present. There was some Alcian blue–

**FIGURE 7.** Photomicrograph showing fibrous plaque with atheroma immunostained for type III collagen. Type III collagen is distributed diffusely in the fibrous cap (FC) and sparsely in the atheroma (A). x109.

**FIGURE 8.** Photomicrograph showing fibrous plaque with atheroma immunostained for type VI collagen. Type VI collagen is present in the fibrous cap (FC); collagen shows faint staining in the atheroma (A). x109.

**FIGURE 9.** Photomicrograph showing fibrous plaque with atheroma immunostained for type IV collagen. Type IV collagen is seen around cells in the fibrous cap (FC). Note increased deposition of collagen near the atheroma (A). x109. Inset: shows lamellar deposition of type IV collagen around the elongated cell. x544.

**FIGURE 10.** Photomicrograph showing fibrous plaque with atheroma immunostained for type V collagen. Type V collagen is irregularly distributed and concentrated in the fibrous cap (FC) and is not present in the atheroma (A). x109.

**FIGURE 11.** Photomicrographs showing a complicated lesion stained with hematoxylin and eosin (panel a) and a serial section immunostained for type IV collagen (panel b). Thick deposition of type IV collagen is shown in the calcified region. x109.

**FIGURE 12.** Photomicrograph showing a large atheromatous plaque with impending ulceration, immunostained for type III collagen. Collagen is not present in the atheroma (A). Overlying fibrous cap (FC) is thin. x109.
positive material in the intercellular spaces, but there were abundant elastic and collagen fibers. There was a relatively large quantity of periodic acid–Schiff–positive material, especially around the spindle-shaped cells.

Type I and III collagens were diffusely distributed in the thickened intima, and there was much more intense immunostaining than in the comparable lesions of children (Figures 1a and 1b). Type IV collagen existed beneath the endothelial cells and around the intimal cells (Figure 2). Type V collagen was very weakly localized to the intercellular spaces (Figure 3b). Type VI collagen preparations showed a reticular staining pattern in the intima, but the intensity of immunostaining varied from one area to another (Figure 4).

**Fatty Streaks**

Fatty streaks were characterized by the intimal aggregates of foam cells containing numerous lipid droplets. Small but variable numbers of spindle-shaped or round cells were interspersed among the foam cells. Although there were few fibroblastic elements in the area where foam cells aggregated densely, there were various amounts of extracellular accumulation in the areas where cells were sparsely distributed. There were few Alcian blue–positive elements; periodic acid–Schiff–positive elements were observed in the extracellular space, especially around the cells.

Type I and type III collagens were present in the fibrous stroma, where they showed a finely networked distribution around the foam cells. Type IV collagen was seen around the cells, but there were numerous foam cells that were not surrounded by type IV collagen (Figure 5). Along with type I and type III collagens, type VI collagen was present in a fine fibrillar pattern. No positive reaction was noted with the anti-type V collagen antibody.

**Fibrous Plaques Without Atheroma Formation**

These raised intimal lesions were mainly composed of fibrous connective tissue. Cells present were mostly spindle shaped, but occasional cells with vacuoles in the cytoplasm were also observed. Elastic fibers and collagen fibers were present in the intima in large numbers. Periodic acid–Schiff–positive material was present around most of the cells.

In this lesion, the localization of type I, III, IV, and VI collagens was not different from that in diffuse intimal thickenings of adult aortas, although the immunostaining was even more intense (Figure 6a). Type V collagen, however, was present, especially in the deeper portion of the lesions (Figure 6b).

**Fibrous Plaques With Atheroma Formation**

These lesions contained a fibrous cap of dense connective tissue overlying a lipid-filled atheromatous center. This fibrous cap was primarily composed of flattened, spindle-shaped cells and thick collagen fibers. Foam cells and lymphoid cells were present in varying amounts. Atheromatous lesions that existed in the deep intimal layer showed unstructured necrotic debris and needle-shaped cholesterol crystals. Granular elements that were stained dark purple in hematoxylin and eosin preparations (calcium deposition) were rarely observed, and foam cells when present were noted at the periphery of the atheroma. Periodic acid–Schiff–positive material was often seen around the spindle-shaped cells. Elastic fibers were not observed in the atheromata.

Type I, III, and VI collagens were diffusely distributed throughout the fibrous cap, but the immunostaining intensity varied from region to region (Figures 7 and 8). Type IV collagen was clearly detected around the spindle-shaped cells but not around the vast majority of mononuclear cells intermixed with them. Deposition of type IV collagen was observed around the extremely elongated cells in the deep portion of the fibrous cap near the atheroma (Figure 9). Type V collagen was irregularly concentrated between the cells (Figure 10). Type V collagen was occasionally demonstrable in the cytoplasm of some spindle-shaped cells in the lesion.

In the atheroma, sparse deposition of type I, III, V, and VI collagens was observed (Figures 7–10). The intensity of immunostaining decreased from the periphery to the center of the atheroma. Thin filamentous structures that were type IV collagen–positive were also present inside the atheroma. In more advanced lesions, however, no collagen was visible in the center of the atheroma.

**Complicated Lesions**

Conspicuous in these lesions were intimal hemorrhage, ulceration, calcification, and thrombosis. Hyalinization of the fibrous cap was also observed.

Type I, III, V, and VI collagens showed almost the same distribution as in the aforementioned fibrous caps. There was intense immunostaining for type I and III collagens in the hyalinized area. Type IV collagen was found around the spindle-shaped cells. Occasionally, thick deposits of type IV collagen were observed in calcified tissues (Figure 11). Vascularization of these lesions was also noted; small vessels were clearly delineated by type IV collagen expression. Collagen immunostaining was negative in the major portion of the atheroma (Figure 12).

Table 1 summarizes the distribution of collagen types in the normal intima and various lesions.

**Ultrastructural Localization of Type V and VI Collagens**

The cross-banded collagen fibers of 20–40-nm diameter in the intersitial of the thickened intima were labeled with the anti–type V collagen antibody. Gold particles were observed in an irregular distribution on the surfaces of fibers (Figure 13). Microfibrils of 10–15-nm diameter and thin, nonbanded filamentous structures were consistently negative. The basement membrane of smooth muscle cells did not react with antibody specific for type V collagen.

Antibody to type VI collagen was localized along the branching network of filaments present throughout the interstitium of the thickened intima (Figure 14). These filamentous structures encircled interstitial cross-banded collagen fibers but did not appear to interact directly with them. No labeling of the basement membranes was seen. No gold particles were detected on the elastin-associated microfibrils in these studies.

In control experiments using normal IgG, a branching network of “beaded” filaments consisting of a thread measuring 2–3 nm in diameter and a “bead” located on
TABLE 1. Summary of Tissue Localization of Collagen Types I, III, IV, V, and VI in Normal Intima and Various Intimal Lesions of the Aorta

<table>
<thead>
<tr>
<th>Immunoreactivity with type-specific anti-collagen antibody</th>
<th>I</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal intima*</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mild intimal thickening</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Diffuse intimal thickening</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Puffy streak</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fibrous plaque (free of atheroma)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Atheromatous plaque</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Fibrous cap</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Atheroma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Complicated lesion</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>[+</td>
<td>+</td>
</tr>
<tr>
<td>Fibrous cap</td>
<td>-</td>
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<td>Atheroma</td>
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*Intima of infants consisting of a single layer of endothelial cells resting on the internal elastic lamina by light microscopic examination.

- Negative; ±, variably and faintly positive; +, constantly but weakly positive; ++, constantly positive; ++++, constantly and strongly positive.

The filament at intervals of about 100 nm was found. These structures were widespread throughout the extracellular matrix, around or between cross-banded collagen fibers (Figure 15) and elastic fibers, and on the surfaces of basement membranes of smooth muscle cells.

Discussion

The present study clearly demonstrates codistribution of type I and III collagens in the thickened intima of all stages of the atherosclerotic lesion. In this respect our findings corroborate previous immunofluorescence studies.12-15 Mayne10 and Shekhonin et al13 have assumed that type I and III collagens, which are the most important collagens involved in platelet aggregation, play a critical role in the formation and progress of the disease. The increase of the Type I to Type III collagen ratio that has been detected biochemically29-31 is closely associated with intimal sclerosis and lesion progression.

Previous studies of the tissue localization of type VI collagen have revealed that it occurs ubiquitously in most interstitial connective tissues, such as the aortic media,32 skin,32-34 liver,32 cornea,32 tendon,35 cartilage,33,34 vertebral disc,36 and synovia,37 and also in proliferative processes, such as schwannoma,35 fibromatosis,36 and osteosarcoma.39 However, little information is available on its distribution and ultrastructural localization in atherosclerotic lesions. The present immunohistochemical study revealed that type VI collagen appeared together with type I and III collagens in the thickened intima of all stages of lesions. Furthermore, immunoelectron microscopic observations showed that type VI collagen was localized to a branching network of beaded filaments present throughout the interstitium of the thickened intima. Previous immunoelectron microscopic studies have suggested that type VI collagen may be the major structural element of a special class of microfilaments.32-35,37 Our data support this suggestion.

FIGURE 13. Immunoelectron photomicroscopic localization of type V collagen in fibrous plaque of the aorta. Cross-banded collagen fibers are labeled with anti-type V collagen antibody. x36,000.
Type VI collagen is a "short-chain" molecule consisting of a triple helical segment with globular domains at each end. The end-on-end aggregation of tetramers gives rise to filaments that have been observed in cell culture as beaded filaments, with the beads representing a structure formed by the interaction of globular domains from two tetramers. The beaded filaments described in our study appear to be similar to those described by Bruns et al and Keene et al. However, Keene et al have shown that type VI collagen is a component of a ruthenium red-stainable network. Furthermore, Wu et al have suggested the interaction between type VI collagen and a component of the proteoglycan complex in the tissue. Therefore, it seems likely that the beaded filamentous structure of type VI collagen shown in Figure 15 is intimately associated with or entrapped in the proteoglycan complex. The function of type VI collagen is not known, but the ultrastructural studies suggest that it comprises an independent fibrous system, perhaps important to the development and maintenance of the spatial separation of distinct tissue components from large banded fiber bundles as well as providing structural unity to the entire connective tissue. It has also been suggested that type VI collagen has a role in the adhesive mechanism that occurs between cells or between cells and extracellular matrix components.

Our data suggest that type V collagen deposition is a function of age and lesion progression. In fatty streaks as well as in the mild intimal thickening of the aortas of children, type V collagen was not detected in the lesions. However, type V collagen was identified in diffuse intimal thickenings of the adult aortas, and the antibody localization appeared more intense as the lesion progressed, except in atheromatous areas.

Although the immunoreactivity with anti-type V collagen antibody in deparaffinized, formalin-fixed aor-
tic tissues was in general weaker than that previously shown by immunofluorescence studies using frozen sections of unfixed tissues,12-15 our results correlate reason-ably well with the biochemical data that suggest that type V collagen increases in advancing atherosclerotic lesions.29,31,44 The Linsenmayer group has claimed that type V collagen is buried within the collagen fibril, and epitopes for the antibodies to type V collagen are only revealed by dissolution of the fibrils.45,46 Weak staining of type V collagen in our study may be due to the few antigenic domains that were exposed in formalin-fixed tissue sections. Immunoelectron microscopic observations in our study revealed that type V collagen was localized to the cross-banded collagen fibers of 20-40-nm diameter in the intima of the thickened intima. It is well known that type V collagen is copolymerized with type I collagen to form type I-V hybrid collagen.46,47 Birk et al48 have suggested that type V collagen might interact with type I collagen to regulate fibril diameter. Because type V collagen is known to increase not only in atherosclerotic lesions but also in such fibrous lesions as scar tissue and in cirrhous carcinoma,49 this type of collagen appears to be involved in progressive fibrosis and may play a role in the physiological regulation of collagen fibril diameter.

Type IV collagen, a basement membrane collagen,7 was also more intensely localized as the lesions progressed. However, in the fatty streak, the earliest lesion of atherosclerosis, type IV collagen was present around the spindle-shaped cells. These probably represented smooth muscle cells that had migrated from the media, had proliferated within the intima, and had secreted type IV collagen as part of their basement membranes. In advanced lesions greatly increased deposition of type IV collagen was observed, particularly around the extremely elongated cells. This feature was also described in previous immunofluorescence studies.13,15,50 Ross et al51 showed that multilayered basement membranes were formed around the flat smooth muscle cells in the atheromatous plaque, especially in the fibrous cap. Therefore, the present findings on the distribution of type IV collagen seem to suggest a relation between the morphological change of smooth muscle cells and the formation of basement membranes. This finding also seems to be consistent with published biochemical analyses documenting an increase in type IV collagen in advanced atherosclerotic lesions.52 Multilayered basement membrane formation around smooth muscle cells has been suggested to be a reflection of a defense mechanism that protects cells from vascular hemodynamic forces or an indicator of cell senescence.53 Because smooth muscle cells change their phenotype from the synthetic to the contractile state as their proliferative capacity decreases,54 the degree of expression of type IV collagen may be an indicator of the degree of this phenotypic change. In this study thickened basement membranes were also occasionally observed in calcified tissues, suggesting a possible involvement of type IV collagen with the process of calcification. Tanimmura et al55,54 recently reported calcium deposits in association with smooth muscle cell basement membranes in humans and experimental animals.

An additional finding that warrants comment is the presence of cells not surrounded by type IV collagen. It has been shown that a significant number of mono-

cyte-derived macrophages and T lymphocytes appear in the lesions of atherosclerosis along with smooth muscle cells.55-57 It is likely that the vast majority of type IV collagen-negative cells are macrophages and T lymphocytes.

It is interesting to note that collagen of any type were sparsely distributed in the atheroma, and no collagen was detected in the center of the atheroma of more advanced lesions. These findings suggest that there are enzymatic mechanisms for removal of collagens from advanced lesions. Collagenase digests types I, II, III, and X collagens; gelatinase/type IV and V collagens are thought to be involved in the degradation of collagen by digesting gelatin derived from collagen molecules that are cleaved by the action of collagenase59 and by digesting type IV and V collagens.60,61 Previous studies have shown that macrophages secrete collagenase and gelatinase/type IV and V collagens.62-63 Cultured arterial smooth muscle cells can also secrete these enzymes (Y. Okada et al, unpublished observations). Because macrophages and smooth muscle cells are the major cell types found within human atherosclerotic lesions, it is likely that these cells participate in the degradation of collagen by releasing collagenolytic enzymes. The finding that collagenolysis occurs in the atheroma further suggests that these enzymes are secreted and/or activated at specific sites of the lesion. Additional studies are clearly necessary to further characterize collagenolytic activity in atheroma.

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