PROTEOGLYCANS IN PRIMATE ARTERIES

I. Ultrastructural Localization and Distribution in the Intima

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ABSTRACT

Proteoglycans were identified and localized histochemically and ultrastructurally in normal and hyperplastic arterial intimas in nonhuman primates (*Macaca nemestrina*). These regions were consistently more alcianophilic than the adjacent medial layers and this alcianophilia was absent after treatment with glycosaminoglycan-degradative enzymes. Ultrastructurally, the intimal intercellular matrix consisted of numerous, irregularly shaped, 200–500-Å diameter granules possessing 30–60-Å diameter filamentous projections, and these granules were dispersed between collagen and elastic fibers. The granules exhibited a marked affinity for ruthenium red and were interconnected via their filamentous projections. The ruthenium red-positive granules were intimately associated with the plasma membrane of intimal smooth muscle cells and attached to collagen fibrils and elastic fibers. The matrix granules were completely removed after testicular hyaluronidase or chondroitinase ABC digestion but only partially removed after leech hyaluronidase treatment. These results suggest that the matrix granules contain some hyaluronic acid and one or more isomers of chondroitin sulfate. In addition to the large ruthenium red-positive matrix granules, a smaller class of ruthenium red-positive granule (100–200-Å diameter) was present within the basement membranes beneath the endothelium and surrounding the smooth muscle cells. Ruthenium red also exhibited an affinity for the surface coat of the smooth muscle cells. The potential importance of proteoglycans in arterial intimal hyperplasia is discussed.

Although the fine structural localization and distribution of proteoglycans[1] has been examined in several connective tissues (1, 4, 9, 18, 23, 26, 28, 31, 33, 38, 41, 42, 46, 47), relatively little is known concerning the ultrastructural distribution of these macromolecules in arteries.

[1] Proteoglycans are defined as high molecular weight protein polysaccharides which consist of glycosaminoglycans (carbohydrate polymers composed of repeating dimers of monosaccharides, one of which is an amino sugar) covalently linked to a protein core. Glycosaminoglycans do not exist as free polysaccharides in the intercellular space but always occur bound to protein as a complex.

Recent biochemical studies of mammalian arteries have demonstrated large increases in proteoglycans at sites of intimal hyperplasia induced by experimental injury (15). However, fine-structural studies of these intimal thickenings have not demonstrated the presence of proteoglycans in any of the species examined (36, 44). This inability ultrastructurally to observe proteoglycans in a tissue known to be rich in these macromolecules probably results from the fact that they are extracted during conventional tissue fixation and processing for electron microscopy (45).

In the present report we have used special methods to retain arterial proteoglycans *in situ* in the nonhuman primate (*Macaca nemestrina*) and will present observations on the fine structure of these macromolecules, their susceptibility to glycosaminoglycan-degradative enzymes, and their distribution in normal and hyperplastic intimas. The accompanying report presents evidence demonstrating that the arterial smooth muscle cell is capable of synthesizing and secreting proteoglycans in vitro and identifies the types and relative amounts of the macromolecules produced by these cells.

MATERIALS AND METHODS

Experimental De-endothelialization

The right iliac artery and abdominal aorta of the pigtail monkey *Macaca nemestrina* were subjected to experimental de-endothelialization by intra-arterial balloon catheterization (3, 44). Briefly, this procedure consisted of cannulating one iliac artery via the external femoral with a 4F Fogarty catheter, inflating the balloon to a pressure of approximately 700 mm Hg, and passing the catheter through the artery, withdrawing it, and tying off the external femoral. The nonballooned left iliac artery served as a control. The animals were sacrificed 1½–6 mo after injury and the vessels were removed, cut into equal 1.0-mm rings, and processed for light and electron microscope histochemistry.

Light Microscopy

Specimens were fixed in 10% neutral buffered Formalin at room temperature for 4 hr, embedded in paraffin, and sectioned at 6–8 μm. Paraffin sections were stained with alcian blue 8 G-X at pH 2.7 (29). To confirm the specificity of the alcian blue stain for proteoglycans, parallel sections were incubated in either 0.1% testicular hyaluronidase (NaCl-acetate buffer, pH 5.4) or 0.1 U chondroitinase ABC (enriched Tris buffer), pH 8.0, for 4 h at 37°C before staining. Controls were incubated with buffer without enzyme for the same period of time.

Electron Microscopy

Two separate procedures were used to localize ultrastructurally proteoglycans in arterial tissue. A. The first procedure was modified from a study by Matukas et al. (28) who used phosphate-buffered fixatives to demonstrate proteoglycans in cartilage. Arterial rings were placed in phosphate-buffered 2.5% glutaraldehyde-2% paraformaldehyde, pH 7.3 (19), for 3 h at room temperature and postfixed in phosphate-buffered 1% OsO_4 for 2 h at 4°C. After postfixation, the rings were stained en bloc with 2% uranyl acetate for 30 min, dehydrated through ethanol, and embedded in Epon 812 (22). Sections were stained with uranyl acetate and lead citrate (34) and examined in an AEI 6B electron microscope. B. In the second procedure, additional arterial rings were fixed and stained with ruthenium red according to the method of Luft (26) with slight modification. Rings were fixed in 0.1 M cacodylate-buffered 2.5% glutaraldehyde-2% paraformaldehyde (pH 7.3) containing 0.2% ruthenium red (Johnson, Matthey and Co., Malvern, Pa.) for 3 h at room temperature (phosphate buffers cannot be used with ruthenium red because phosphate blocks the ruthenium red-OsO_4 reaction in the tissue [25]). After primary fixation, tissues were rinsed overnight in 0.1 M cacodylate buffer containing 7.5% sucrose and 0.1% ruthenium red and postfixed with cacodylate-buffered 1% OsO_4 (pH 7.3) containing 0.05% ruthenium red for 3 h at room temperature. After a brief buffer rinse, the tissues were dehydrated, embedded, and sectioned as described in section A. Both stained (uranyl acetate and lead citrate) and unstained sections were examined.

Enzyme Digestion

To confirm the presence of proteoglycans, representative rings of arterial tissue were incubated with each of the following enzymes before or after fixation: (*a*) testicular hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) 2,000 U/ml in NaCl-acetate buffer, pH 5.4 (37); (*b*) chondroitinase ABC (Miles Laboratories, Inc., Kankakee, Ill.) (0.5 U/ml) in enriched Tris buffer, pH 8.0 (37); and (*c*) leech hyaluronidase (Biotrics, Inc., Boston, Mass.) 0.15 mg/ml, in McIlvaine's buffer, pH 5.6 (13). Testicular hyaluronidase degrades chondroitin sulfates A and C as well as hyaluronic acid (37). Chondroitinase ABC degrades dermatan sulfate in addition to chondroitin sulfates A and C, and hyaluronic acid (37). Leech hyaluronidase is specific for hyaluronic acid (A. Balazs, personal communication). Using the methods described in Section A, the enzymes were added to tissues after primary fixation and incubated for 18 h at 37°C (28). Controls were incubated with the appropriate buffer minus the enzyme for the same time period. After incubation, the tissues were rinsed briefly and processed for electron microscopy as previously described. Using ruthenium red (Section B), the tissues were incubated with each of the enzymes before fixation for 1 h at 37°C, since it was believed that the ruthenium red would inter-

fere with the enzyme-substrate reaction. After enzyme incubation, the tissues were fixed and processed as described in Section B.

RESULTS

Light Microscope Histochemistry

1.5–6 mo after endothelial injury, the intimas of de-endothelialized vessels were extensively thickened as compared to noninjured controls. This intimal thickening was markedly more alcianophilic than the underlying medial layer (Fig. 1). The noninjured vessels did not exhibit this intimal thickening (36, 44), although the narrow intima displayed the same increase in alcianophilia when compared with the underlying media (Fig. 2). Glycosaminoglycan-degradative enzymes (testicular hyaluronidase and chondroitinase ABC) reduced most of the alcianophilia, confirming the specificity of the stain for proteoglycans.

Electron Microscopy

INTERCELLULAR MATRIX AFTER PHOSPHATE-BUFFERED FIXATION WITHOUT RUTHENIUM RED: Examination of normal and experimentally induced hyperplastic intimas prepared with phosphate-buffered fixatives revealed the presence of numerous 200–500-Å diameter polygonal granules throughout the intercellular matrix in addition to collagen and elastic fibers (Fig. 3). Many of the granules possessed filamentous projections giving them a stellate configuration. A smaller class of granule (100–200 Å in diameter) was present on both sides of the partial basement membranes surrounding the smooth muscle cells (Fig. 3).

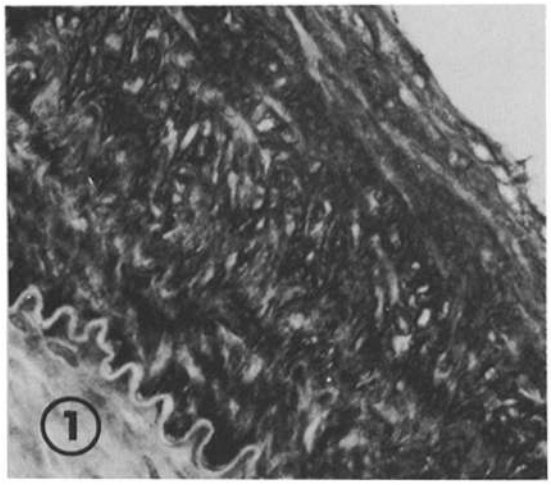


FIGURE 1 Right iliac artery stained with alcian blue 1.5 mo after experimental de-endothelialization. The thickened intima is characteristic of the smooth muscle hyperplasia that follows such injury and demonstrates a marked alcianophilia in comparison with that seen in the underlying media. × 555.

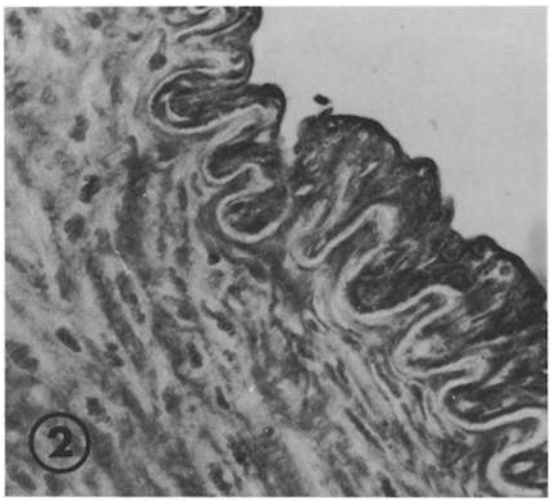


FIGURE 2 This light micrograph illustrates the relatively narrow intima of a noninjured left iliac artery stained with alcian blue. As in Fig. 1, the noninjured intima also appears markedly alcianophilic and stains more intensely than the underlying media. × 555.

INTERCELLULAR MATRIX AFTER FIXATION AND STAINING WITH RUTHENIUM RED: The intimal matrix granules exhibited a marked affinity for ruthenium red and their fine structure resembled that observed in preparations with phosphate-buffered fixatives. Thus it is possible to preserve the matrix granules of the artery wall by the use of phosphate-buffered fixatives or by prefixing in the presence of ruthenium red. In both instances the size and distribution of these granules were similar. The granules were polygonal in shape, measured 200–500 Å in diameter, and possessed weakly stained 30–60-Å thick filamentous projections which appeared to interconnect adjacent granules (Figs. 4, 5, 6).

The granules and their constituent filaments were closely associated with other connective-tissue matrix components. They were frequently observed on the periphery of elastic fibers. In some regions, adjacent elastic fibers were interconnected by the matrix granules and their filaments (Figs. 4, 6). The granules were often deposited on the surfaces of collagen fibrils at their major period

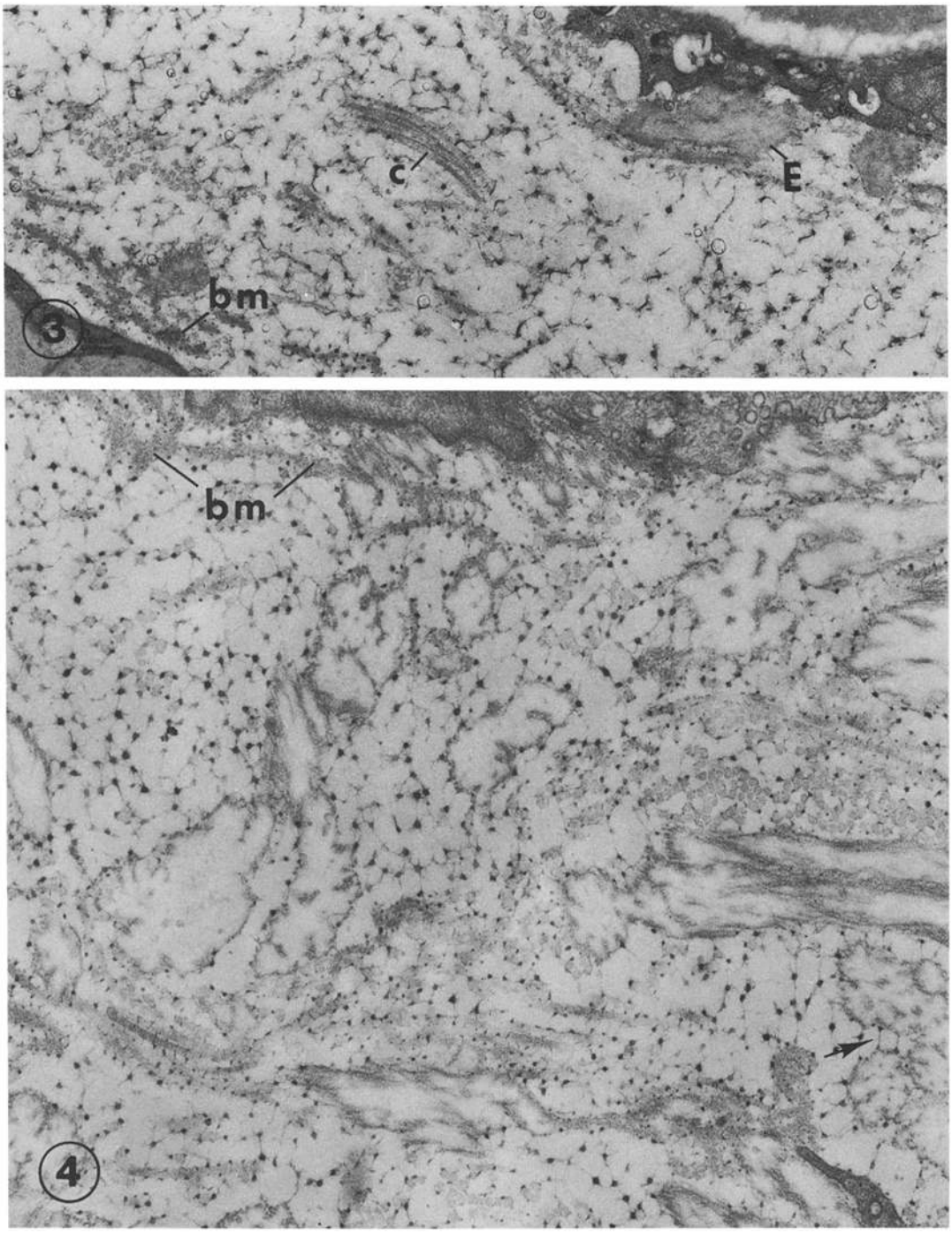


FIGURE 3 This electron micrograph illustrates the ability to preserve the proteoglycan granules of the matrix in the absence of ruthenium red of an arterial intima (3 mo after endothelial removal) by using phosphate-buffered glutaraldehyde and osmium tetroxide. Numerous (200–500-Å diameter) polygonal granules with associated filamentous projections occupy the intercellular space in addition to collagen (*C*) and elastic fibers (*E*). Smaller (100–200-Å diameter) granules are present on both sides of the fragmented basement membrane (*bm*) of the smooth muscle cells. × 22,000.

FIGURE 4 Part of a hyperplastic intima prepared with ruthenium red. The numerous (200–500-Å diameter) matrix granules are distinct and possess interconnecting filamentous projections. The granules are associated with the periphery of elastic fibers and collagen fibrils. Note how the granules appear to connect adjacent elastic fibers (arrow). Smaller granules (100–200-Å diameter) are associated with the basement membrane (*bm*) of the smooth muscle cell. × 24,000.

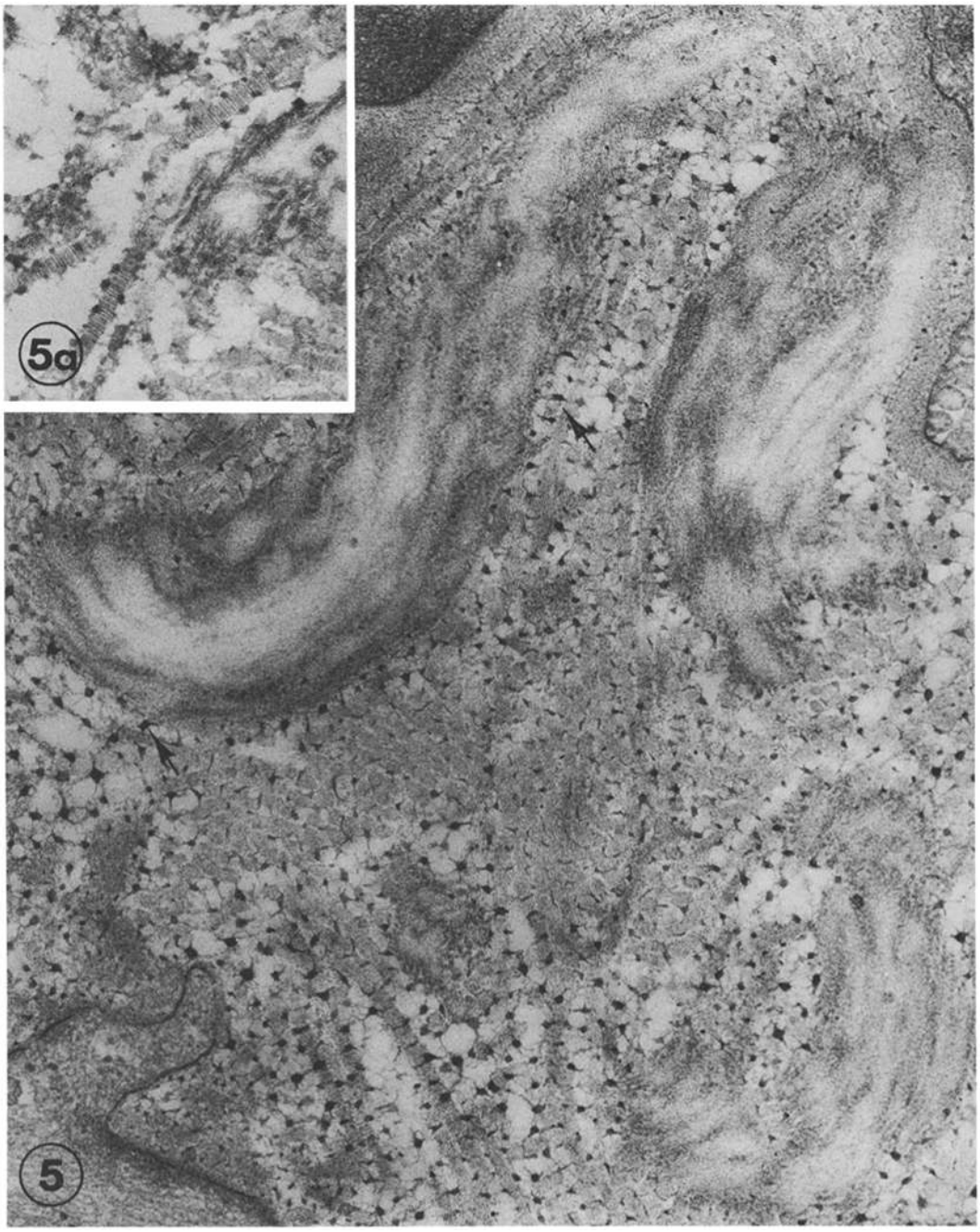


FIGURE 5 High magnification of the intercellular matrix from the same intima shown in Fig. 4, containing abundant collagen and elastic fibers. The matrix granules are closely associated with both collagen and elastic fibers and in some instances appear to serve as a link between these two types of fibers (arrows) × 55,000 (*a*) The granules are tangentially located on the surface of the collagen fibrils at major period band intervals. × 49,000.

bands (Figs. 4, 5, 5 *a*). Occasionally, a thin band of ruthenium red material extended across the collagen fibril at the major period band (Fig. 5). In some regions, adjacent collagen fibrils appeared to be linked to one another at these bands by ruthenium red-positive granules (Figs. 5, 12). Matrix granule connections between collagen and elastic fibers were also observed (Figs. 4, 5). These relationships are particularly well demonstrated in regions containing cross sections of collagen fibrils as seen in Fig. 5.

The size and spatial distribution of matrix granules in both the normal and the hyperplastic intimas appeared to be qualitatively similar. The matrix granules were closely associated with the plasma membranes of the smooth muscle cells. They appeared to be attached to the cell surface via their 30–60-Å thick filamentous projections in regions where the cell surface was devoid of basement membrane (Figs. 6, 6 *a*, and 8). Apparent connections between membrane-associated granules and granules attached to adjacent elastic fibers were observed (Fig. 8).

Similar ruthenium red-positive granules were present within the deeper medial layer of the arteries, although they were less numerous than those observed in normal and hyperplastic intimas due mainly to the fact that the medial cells were more closely approximated and the intercellular space contained abundant closely packed collagen and elastic fibers (Fig. 7).

Susceptibility of Matrix Granules to Enzyme Digestion

Digestion of vessels, after phosphate-buffered fixation or before ruthenium red fixation and staining, with testicular hyaluronidase or chondroitinase ABC eliminated the majority of the matrix granules, leaving the other intercellular matrix components intact (Figs. 10, 11). On the other hand, leech hyaluronidase reduced the size and number of granules but did not eliminate them. The ruthenium red-positive granules associated with collagen fibrils were the most resistant to leech hyaluronidase (Fig. 12).

Ruthenium Red Staining of the Smooth Muscle Cell Surface and Basement Membrane

In sections not stained with uranyl acetate and lead citrate, ruthenium red-staining material could be observed either in the form of intermittent discrete granules or as a thin coat lying along the outer leaflet of the smooth muscle cell plasma membrane (Figs. 8, 9). This membrane-associated staining was not affected by testicular hyaluronidase, chondroitinase ABC, or leech hyaluronidase (Fig. 9).

The basement membrane surrounding smooth muscle cells also exhibited affinity for ruthenium red. In unstained sections, the basement membrane exhibited uniform density of an intensity similar to the matrix granules (Fig. 9). In stained sections, small granules (100–200-Å diameter) were present within the interstices of the basement membrane and slightly larger granules were located on both sides of the basement membrane (Figs. 4, 5, 6). The larger granules associated with the edges of the basement membrane were removed by the enzyme treatments but the smaller granules within the interstices appeared to resist enzyme digestion.

Ruthenium Red Staining of the Endothelial Cell Surface and the Subendothelial Basement Membrane

Ruthenium red was also deposited on the luminal surface of endothelial cells but failed to stain their abluminal surface (Fig. 13). The luminal surface coat was approximately 120 Å thick, adhered closely to the outer leaflet of the plasma membrane, and, in contrast to that of smooth muscle cell surface, staining appeared to be continuous. The coat penetrated into the endothelial junctions but did not extend beyond regions of tight junctions (Fig. 13).

Immediately below the endothelial cells, numerous small (100–200-Å diameter) ruthenium red-positive granules were dispersed throughout the amorphous matrix of the basement membrane (Fig. 13). In thick regions or in appropriately sectioned areas of the basement membrane, the granules appeared to be regularly dispersed. These granules differed from the larger intercellular matrix granules in that they did not possess interconnecting filaments. The ruthenium red staining of the subendothelial basement membranes differed from that surrounding smooth muscle cells in sections that were not counterstained in that the smooth muscle cell basement membrane stained uniformly with ruthenium red, whereas the subendothelial basement membrane

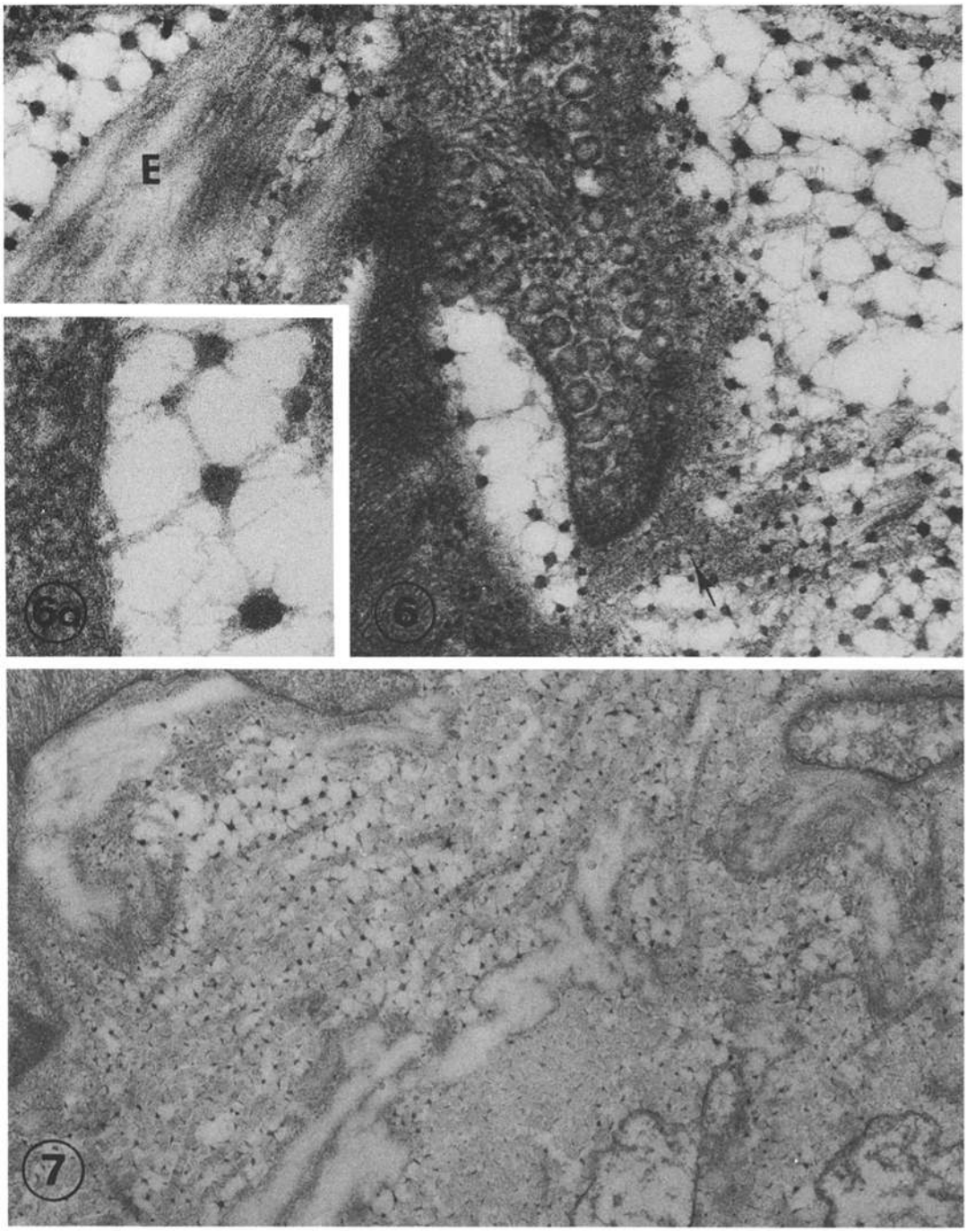
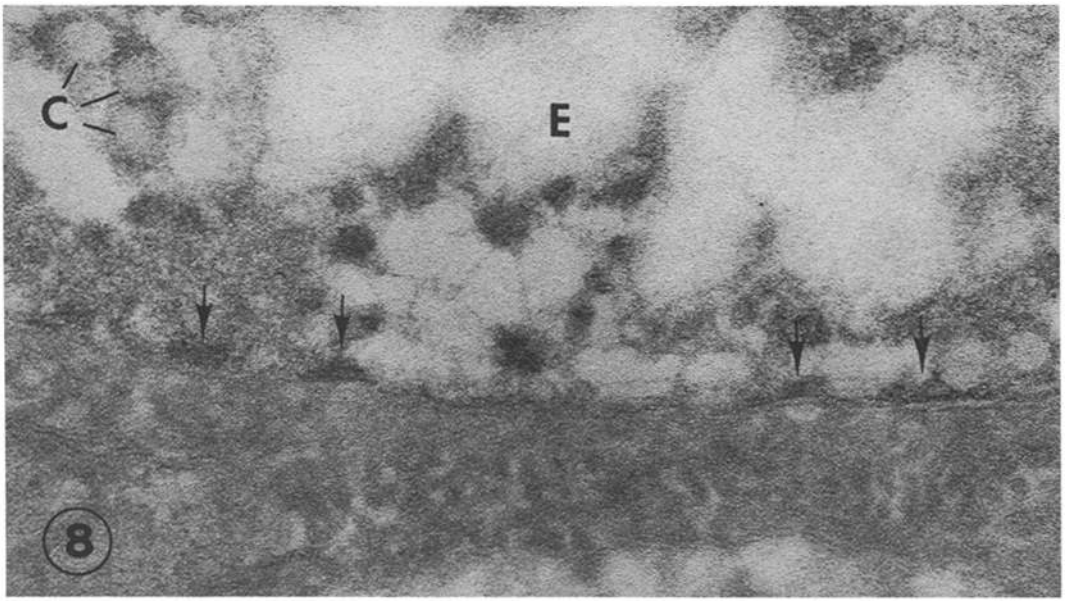


FIGURE 6 A portion of an intimal smooth muscle cell and the adjacent intercellular matrix stained with ruthenium red. Numerous polygonal granules are associated with each other through their 30–60-Å diameter filamentous projections and are also associated with adjacent elastic fibers (*E*). Smaller granules are present within the interstices of the basement membrane of the smooth muscle cell (arrows) × 40,000. (*a*) Frequently the matrix granules are associated with the plasma membrane of the smooth muscle cell through their filamentous projections. × 140,000.

FIGURE 7 Medial layer of an iliac artery stained with ruthenium red. Abundant collagen and elastic fibers occupy the bulk of the matrix and considerably fewer matrix granules are present than were observed in the adjacent normal or hyperplastic intimal layer. × 34,500.



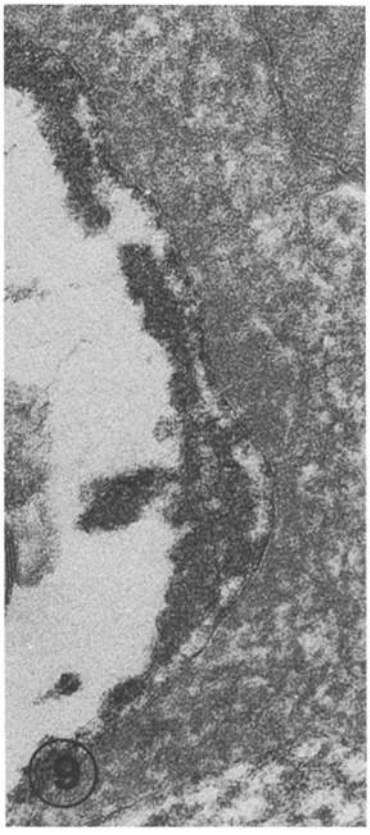
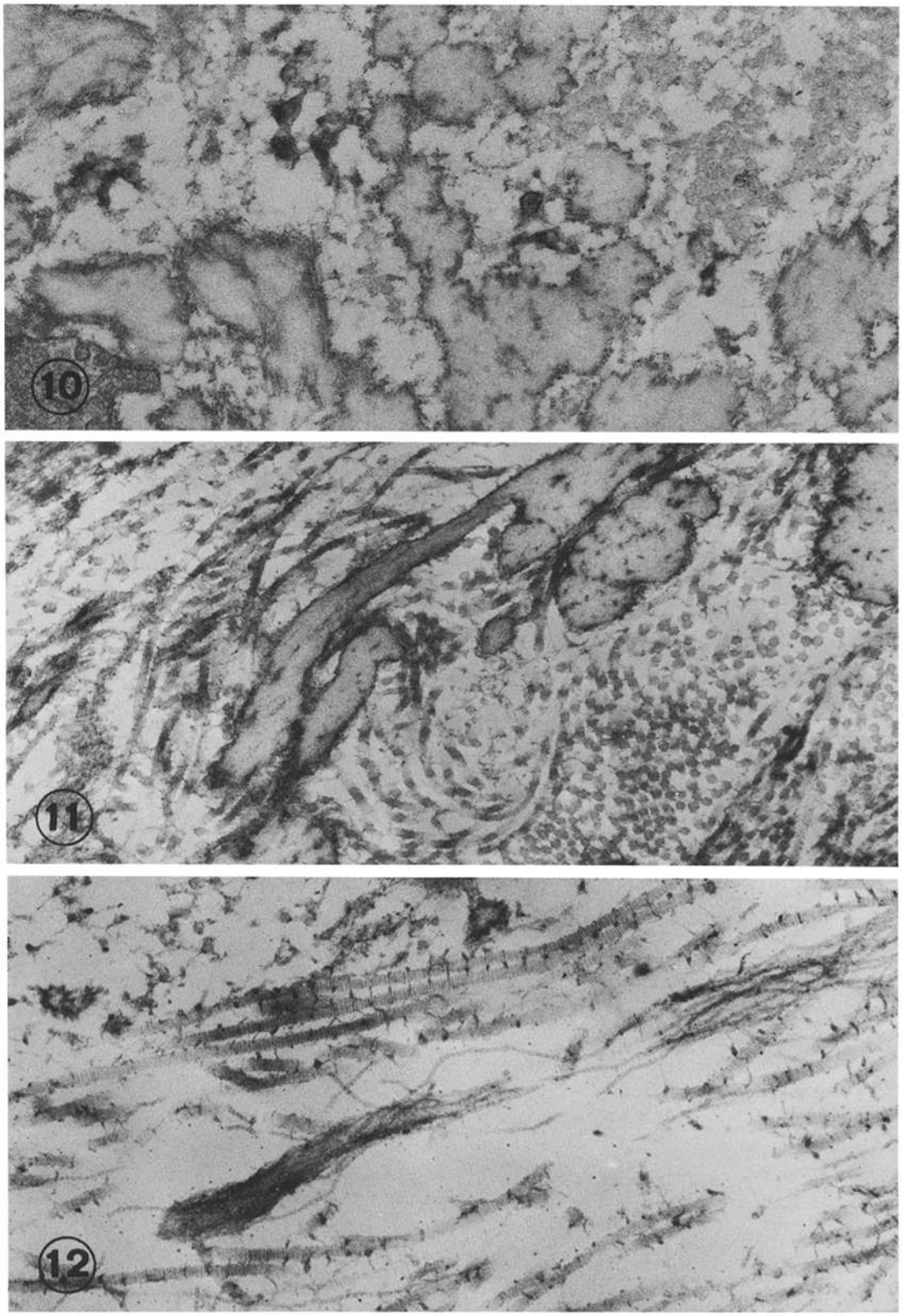


FIGURE 8 Portion of an intimal smooth muscle cell surface and the surrounding connective tissue. Ruthenium red deposits occur intermittently along the outer leaflet of the plasma membrane (arrows), while matrix granules are closely associated with the membrane through filamentous projections. Note the apparent filamentous connection between the membrane-associated granule and the granule lying on the periphery of the elastic fiber (*E*). Ruthenium red deposits surround adjacent collagen fibrils (*C*) which have been transversly sectioned. This section was not stained with uranyl acetate or lead citrate. × 228,000.

FIGURE 9 Periphery of an intimal smooth muscle cell from tissue which has been treated with testicular hyaluronidase before ruthenium red exposure. This section was not stained with uranyl acetate or lead citrate. The ruthenium red staining of the outer leaflet of the plasma membrane and the basement membrane persists although the intercellular matrix granules have disappeared. Similar results were obtained after digestion with chondroitinase ABC and leech hyaluronidase. × 132,000.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contained ruthenium red-positive granules enmeshed in a ruthenium red-negative matrix.

Attempts to elucidate the chemical nature of the endothelial cell coat and subendothelial basement membrane granules with glycosaminoglycan-degradative enzymes have not been successful, since incubation of the vessels with these enzymes causes disruption of the endothelial cell layer and loss of basement membrane material.

DISCUSSION

Ultrastructural Localization of Arterial Proteoglycans

With the use of phosphate-buffered fixatives (28) or ruthenium red (24, 26) we have demonstrated numerous 200–500-Å polygonal granules throughout the intercellular matrix of normal and hyperplastic intimas in the nonhuman primate *M. nemestrina*. Phosphate buffers are known to extract less protein than other agents used to buffer fixatives for electron microscopy (49) and, therefore, appear to facilitate visualization of the protein-containing glycosaminoglycan granules by decreasing their extraction during tissue processing. Ruthenium red, a hexavalent cationic dye, has been shown to bind to proteoglycans, among other substances (24, 26) and, if added at the time of fixation, appears to facilitate the preservation of these macromolecules by precipitating them and rendering them insoluble.

The removal of the matrix granules with glycosaminoglycan-degradative enzymes before tissue processing confirms the glycosaminoglycan nature of the granules and indicates that they contain one or more forms of chondroitin sulfate (A or C), dermatan sulfate, and some hyaluronic acid. It has not been possible to identify precisely the particular "species" of glycosaminoglycan present in the proteoglycan granules, since it is likely that the granules contain a mixture of species and partial digestion of any one of these could affect the solubility of the others during subsequent tissue processing. Although protease digestion was not carried out on the matrix granules, it is likely that these granules contain protein, since glycosaminoglycans have been reported to exist in connective tissue as a protein complex (1, 9, 28, 46). Similar removal of proteoglycan granules by glycosaminoglycan-degradative enzymes has been observed in studies of cartilaginous matrix (1, 9, 28, 46).

Recent observations have demonstrated that arterial smooth muscle cells cultured from the aorta of this same species (*M. nemestrina*) synthesize significant quantities of dermatan sulfate, chondroitin sulfate, and small amounts of hyaluronic acid (48). These results confirm this cell's ability to produce these macromolecules and suggest that this cell is the source of intercellular proteoglycan matrix granules.

The distribution of ruthenium red-positive matrix granules in normal and hyperplastic intimas is remarkably similar to the distribution of similarly stained granules in cartilage matrix (9, 24, 26, 46). They are approximately the same size as cartilage matrix granules and possess similar 30–60-Å thick filamentous projections which appear to connect adjacent granules. Furthermore, they are deposited on the major period band of collagen fibrils in a pattern similar to that observed in cartilage (1, 9, 26, 31, 39, 41, 46). These results indicate that arterial collagen possesses similar proteoglycan binding sites at their major period bands to those suggested for cartilage collagen (39, 41).

The Proteoglycan Matrix Granule as a Link Between Cellular and Acellular Components

The matrix granules are closely associated with all components of normal and hyperplastic in-

FIGURE 10 Intercellular matrix of a hyperplastic intima which has been prepared with phosphate-buffered fixatives and subsequently digested with testicular hyaluronidase. Note the absence of matrix granules (compare with Fig. 3). × 45,000.

FIGURE 11 Appearance of a rutheium red-stained intima after it has been digested with testicular hyaluronidase. Note the paucity of matrix granules and the large number of collagen fibrils and elastic fibers remaining. Identical results were obtained with chondroitinase ABC treatment. × 41,000.

FIGURE 12 Portion of intimal intercellular matrix which has been treated with leech hyaluronidase before ruthenium red exposure. The number of matrix granules is reduced but not toally eliminated as observed in Figs. 10 and 11. The collagen-associated granules were particularly resistant to this enzyme. The matrix granules appear to connect adjacent collagen fibrils at their major period bands and may possibly hold them in register. × 69,000.

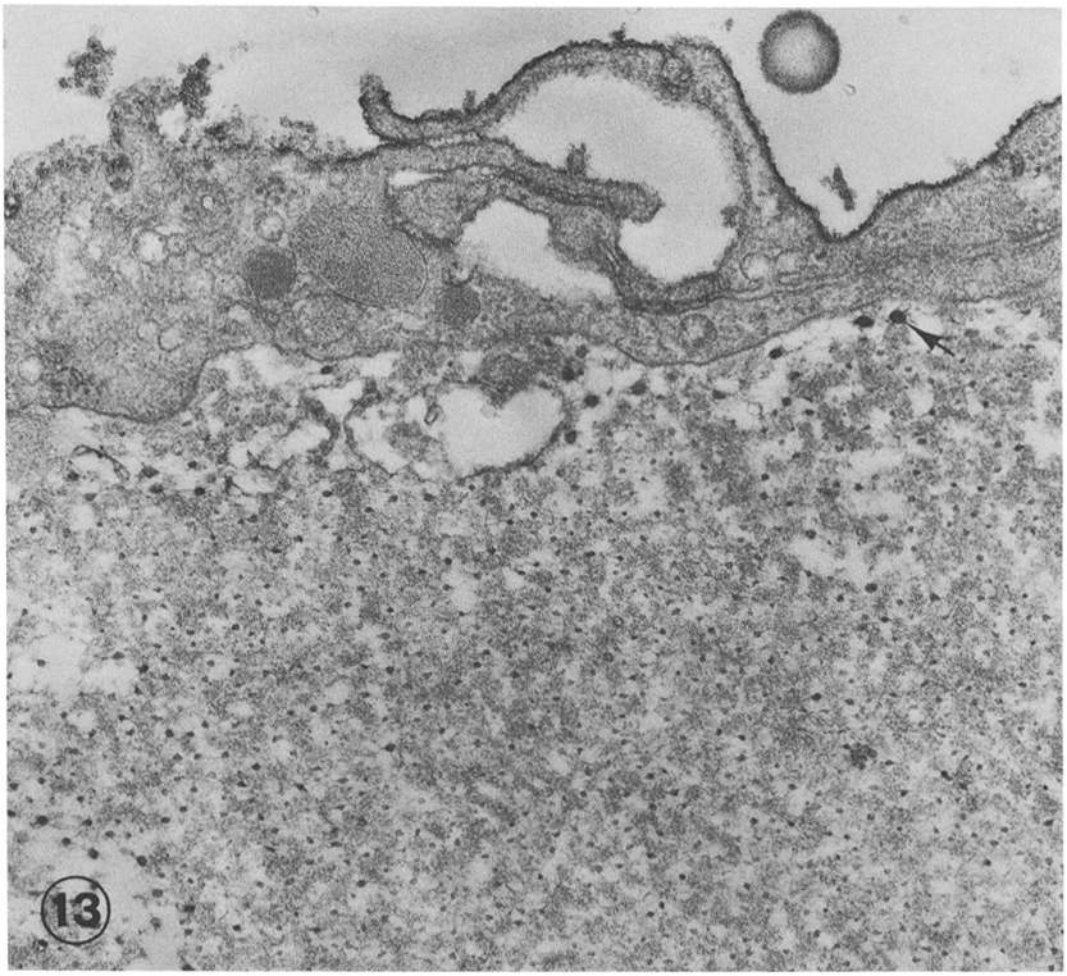

FIGURE 13 This electron micrograph demonstrates the appearance of endothelium and subendothelium prepared in the presence of ruthenium red 3 mo after experimental de-endothelialization. A ruthenium red coat is visible on the luminal endothelial surface but not on the abluminal surface of these same cells. This coating does not penetrate beyond areas of tight junctions between endothelial cells. Numerous round (100–200-Å diameter) ruthenium red-positive granules are dispersed in a regular fashion throughout the matrix of the basement membrane. Larger ruthenium red matrix granules are present on both sides of the basement membrane (arrow). × 56,000.

timas. Their affinity for the periphery of elastic fibers resembles similar findings by Kadar et al. (18) who demonstrated numerous granules associated with newly formed elastic fibers in chick embryo aortas. The frequent observation of adjacent small elastic fibers joined by proteoglycan granules and their constituent filaments in this study suggests that one function of arterial proteoglycan is to hold elastic fibers together. In addition to joining elastic fibers, proteoglycans appear to hold adjacent collagen fibrils in register by forming "bridges" at the major period bands. Similar bridges have been observed associated with collagen in other types of connective tissues (26, 42, 46). Furthermore, the matrix granules appear to interconnect elastic fibers with collagen fibrils, suggesting that the major intercellular matrix components are held together in a type of meshwork by proteoglycan granules and their constituent filaments. These linkages might also serve to keep the major connective tissue components separated and thus could help to maintain turgor in the artery wall. In this capacity, the granules might function as a type of plastic interstitial sub-

stance, important in absorbing and/or dissipating stress imposed on the artery under various physiological conditions (2, 38).

The apparent connections of the granules associated with the plasma membrane of intimal smooth muscle cells to the granules associated with adjacent matrix components further illustrates that the granules may also serve as a link between cells and intercellular matrix components.

These observations demonstrate the pervasiveness and interconnecting nature of proteoglycans in the intercellular matrix of primate arteries and illustrate the importance of these macromolecules in the distribution and function of the various components of the artery wall. Their contribution to the architecture of the artery wall has not been fully appreciated in the past because of the inability to identify them at the ultrastructural level.

Ruthenium Red Staining of Intimal Smooth Muscle Cell Surfaces

The reactivity of ruthenium red with the outer leaflet of intimal smooth muscle cell plasma membranes indicates that this cell, like other cells, possesses a cell coat containing anionic components (32). The resistance of this surface staining to glycosaminoglycan-degradative enzymes further indicates that this coat differs in composition from the chondroitin sulfate-hyaluronic acid-containing matrix granules of the intercellular space. These findings are similar to recent studies by Hay and Meier (14), who demonstrated identical enzyme-resistant ruthenium red membrane staining of epithelial cells from cornea, notochord, and neural tube. Zachs et al. (50) demonstrated a neuraminidase-susceptible ruthenium red coat on the plasma membrane of skeletal muscle. We are presently investigating whether the smooth muscle cell surface contains sialic acid residues as demonstrated for skeletal muscle. However, it should be stressed that heparan sulfate, a glycosaminoglycan known to exist in artery walls, is not degraded by enzymes used in this study. Therefore, the possibility exists that the smooth muscle cell surface contains heparan sulfate which has been identified as a cell surface component in some cells (20). It is equally possible that this coat contains anionic proteins since these will also bind ruthenium red (25).

Whatever the chemical nature of this coat, it is evident that the surface of the smooth muscle cell is strongly anionic and may function in the selective uptake of cationic macromolecules. The surfaces of cells known to be involved in uptake of materials have been shown to be particularly reactive toward ruthenium red (26). It is interesting to note that low density lipoproteins, the major cholesterol-carrying lipoproteins which accumulate in arterial smooth muscle cells during atherogenesis, are positively charged (21).

The similar ruthenium red staining of the basement membranes and their resistance to glycosaminoglycan-degradative enzymes indicates some similarity to the cell surface coat. Whether the two parts of the surface layer of intimal smooth muscle cells are chemically identical or different remains to be determined.

Ruthenium Red Staining of Endothelium and the Subendothelial Basement Membrane

The ruthenium red staining of the luminal surface of endothelial cells resembles staining observed in capillaries and small blood vessels (23, 26, 40). Whether this coating represents proteoglycans (26) or absorbed protein from the plasma such as fibrin (7) could not be determined in this study because of the failure adequately to fix endothelium after enzyme digestion. It is interesting that the abluminal surface of the endothelial cells did not stain with ruthenium red. This observation suggests that the two surfaces of endothelial cells may be physically, and perhaps functionally different since the intense staining of the subendothelial matrix granules indicates that a significant amount of the stain penetrated this region. The staining of the endothelial luminal surface indicates that it possesses special anionic properties that may be important in the selective uptake of macromolecules from the plasma (40, 43).

The nature of the small (100–200-Å diameter) spherical ruthenium red positive-granules present in the subendothelial basement membranes is not known. Previous studies demonstrated that the entire basement membrane of capillaries and small blood vessels stained uniformly with ruthenium red and did not demonstrate a separate population of discrete staining granules (23, 26, 40). These results differ from those of the present study which characterize the basement membrane in nonhuman primate large arteries as consisting of spherical ruthenium red-positive granules enmeshed in an amorphous filamentous ruthenium red-negative matrix. Recently, Trelstad et al. (47) demonstrated

a similar fine structure in corneal epithelial basement membranes. In these studies, the basement membrane granules were susceptible to chondroitinase ABC digestion, suggesting that they contained one or more forms of chondroitin sulfate, hyaluronic acid, and/or dermatan sulfate. Since we have not been able to preserve adequately the architecture of the endothelium and subendothelium after enzyme digestion, the chemical nature of the granules in the arterial subendothelial basement membrane remains unknown. It is interesting to note the remarkable similarity in both size and spatial distribution of these granules in basement membranes from arteries and corneal epithelium (47).

Although we have not been able to determine the chemical nature of the basement membrane granules, their affinity for ruthenium red suggests that they may contain proteoglycans or acidic glycoproteins (25). Whatever their nature, their presence within the arterial basement membrane is consistent with at least one of several proposed functions—that of acting as a type of anion exchange filter for macromolecules entering from the plasma (27).

Importance of Proteoglycans in Arterial Intimal Hyperplasia and Atherosclerosis

Arterial intimal fibromuscular hyperplasia appears to occur as a general response to injury to the artery wall (10). Recent studies illustrate that regions of arteries exposed to increased shearing stress and turbulence undergo intimal hyperplasia, which may be initiated by a brief episode of endothelial cell erosion (11). Experiments in our laboratory have demonstrated that similar proliferative lesions can be created by experimental denudation of the endothelium, supporting the hypothesis that injury to the endothelium may be an initiating event in the development of such proliferative lesions (44). In the present study, we have demonstrated that these fibromuscular intimal thickenings are rich in proteoglycans which form an interconnecting matrix between cells and their surrounding connective tissue matrix.

Since no differences could be detected in the size and distribution of the matrix granules between normal and hyperplastic intimas, the induced lesions appear to contain more proteoglycan granules. Examination of the intensity of alcian blue staining of these intimal thickenings and in similar lesions in rabbits (15) further suggests that hyperplastic intimas contain an increased amount of proteoglycans when compared with the noninjured intima. Biochemical studies of experimentally induced arterial lesions in rabbits (15) and developing lesions in man (6) have confirmed the increase in proteoglycans in proliferative lesions. Furthermore, the finding that proteoglycans appear to be more concentrated in both the normal and the hyperplastic intima, as compared to the underlying media, agrees with other biochemical studies which have demonstrated that normal and thickened intimas contain more proteoglycans than their corresponding underlying medial layers (6, 30). These results suggest that proteoglycan accumulation occurs principally within the intima and may be the result of the response of the intimal smooth muscle cells to endothelial injury and/or increased mechanical stress.

The increase in intimal proteoglycans in proliferative lesions probably represents a nonspecific process of repair (15) since injury to various connective tissues elicits increased production of connective tissue matrix components (35). Although an increase in proteoglycans may be advantageous in the repair process, it may also provide an environment conducive to the development of an atherosclerotic lesion. Recent studies indicate that proteoglycans are capable of trapping and interacting with low density lipoproteins (the major class of cholesterol-carrying lipoprotein), causing their precipitation through steric exclusion and ionic bonding (5, 12, 16, 17). An increased amount of proteoglycans coupled with an increased entry of plasma low density lipoprotein into the artery wall (a condition known to exist in blood vessels exposed to shearing stresses and/or endothelial injury [11]) could provide one explanation as to why intercellular lipid eventually accumulates in intimal thickenings. Lipid-glycosaminoglycan complexes have been isolated from fatty streaks in human aortas, indicating that such complexes do exist in atherosclerosis (5). Since we observed no evidence of lipid accumulation in the intimal thickenings from animals fed normal diets, we suggest that increases in proteoglycans are part of early intimal change brought on by endothelial injury, and support the hypothesis that these changes precede, and possibly "set the stage" for subsequent lipid deposition and atherosclerotic development (8).

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