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Spontaneous Atherosclerosis: An Ultrastructural Study in the White Carneau Pigeon*

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Summary. The atherosclerotic lesions, associated with the celiac intimal smooth muscle cushions, of four and five year old White Carneau pigeons were studied with the light and electron miscroscopes. Light microscopic examination of the spontaneous lesions demonstrated large intimal cushions composed of smooth muscle, abundant collagen, clusters of foam cells and cholesterol crystal clefts.

Ultrastructural examination of the intimal atheroma revealed dilatations between apposing endothelial cells which contained a flocculent material, similar to that seen in the subendothelial space. The subendothelial compartment contained abundant collagen, extracellular lipid, vesiculated material and cell processes which contained a flocculent matrix and tubular-like elements. In addition, fibroblast-like interlaminar cells were often observed. Numerous intimal smooth muscle cells were seen which displayed varied morphology. Abundant foam cells were also present within the intimal atheromas.

The presence of atherosclerotic lesions in preexisting intimal smooth muscle cushions suggests that hemodynamic factors may be important in the progression of these spontaneous lesions. Endothelial cell dilatations may provide an important route of transport for circulating elements which may accumulate within the subendothelial space. Morphologically, it appears that the smooth muscle cells undergo modification and may represent the precursors of foam cells in this species.

Key words: Electron microscopy – Atherosclerosis – Pigeon.

Introduction

The White Carneau pigeon has been extensively utilized for the study of spontaneous atherosclerosis (Armitage et al., 1976; Lofland and Clarkson, 1965; Pri-

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chard, 1965; Prichard et al., 1964; Santerre et al., 1972). Atheroscerlotic lesions frequently occur adjacent to the celiac bifurcation in close proximity to an intimal smooth muscle cushion, which is a normal feature of the aorta at this level (Lauper et al., 1975).

A prominent feature of atherosclerotic lesions is the proliferation and alteration of smooth muscle cells (Ross and Glomset, 1973). In addition, connective tissue elements such as elastin, collagen and glycosaminoglycans are present in great abundance within these lesions. In vivo (Ross and Klebanoff, 1971) and in vitro (Ross, 1971) experiments have confirmed that smooth muscle cells possess the ability to synthesize elastin and collagen, as well as accumulate intracellular lipids (Ross and Glomset, 1973). Endothelial cell injury may also play a prominent role in the genesis of the arterial lesions. A variety of approaches involving the metabolic parameters associated with early initiating events and the ongoing processes of plaque formation are currently under investigation. The spontaneous lesions of the White Carneau pigeon offer an excellent model for the study of metabolic and genetic facets associated with atherosclerosis (Prichard, 1965). The following study was performed to describe in detail the ultrastructural characteristics of the atherosclerotic lesions found at the celiac bifurcation in the White Carneau pigeon.

Materials and Methods

Electron Microscopy. Four and five year old male White Carneau pigeons were obtained from the Palmetto Pigeon Plant (Sumter, South Carolina), and anesthetized by halothane inhalation. The body cavity was opened and the pigeons were perfused through the left ventricle with a fixative solution consisting of 1.5% glutaraldehyde and 1.5% paraformaldehyde buffered with 0.1 M sodium phosphate (pH 7.2), preceded by a saline flush. Following a ten minute period of perfusion of the fixative, the aorta, from the heart to the ischiadic arteries, was removed and the area adjacent to the celiac artery dissected free from the remainder of the aorta. This segment was then sliced transversely into 2 mm portions and totally immersed in fresh fixative solution for an additional 2–4 h. The tissues were washed overnight in 0.1 M phosphate buffer containing 10% sucrose. The tissues were post-fixed in a mixture of 1% osmium tetroxide, 1.5% potassium ferrocyanide for one hour at room temperature (Karnovsky, 1971). After osmium fixation, the tissues were washed in maleate buffer and en bloc stained in uranyl acetate for one hour. The tissues were then dehydrated in a graded series of ethanols, passed through propylene oxide and embedded in Epon-Araldite (Mollenhauer, 1964). Thin sections exhibiting gold interference colors were cut on a Porter-Blum MT-2 ultramicrotome, stained with lead citrate (Venable and Coggeshall, 1965) and viewed in a Siemens 1A electron microscope.

Light Microscopy. Portions of the aorta which demonstrated atherosclerotic involvement adjacent to the celiac bifurcation where taken from the perfused animals and immersed in 10% formalin. These samples then were routinely dehydrated in a graded series of ethanols, embedded in paraffin, sectioned, and mounted. These sections were stained with a combination of Verhoff's elastic and Gomori's trichrome stains (Richardson, 1975), and viewed in the light microscope.

Results

Light Microscopy. A transverse section of the aorta, just proximal to the celiac bifurcation, demonstrates a thin elastic lateral wall and features typical of athe-

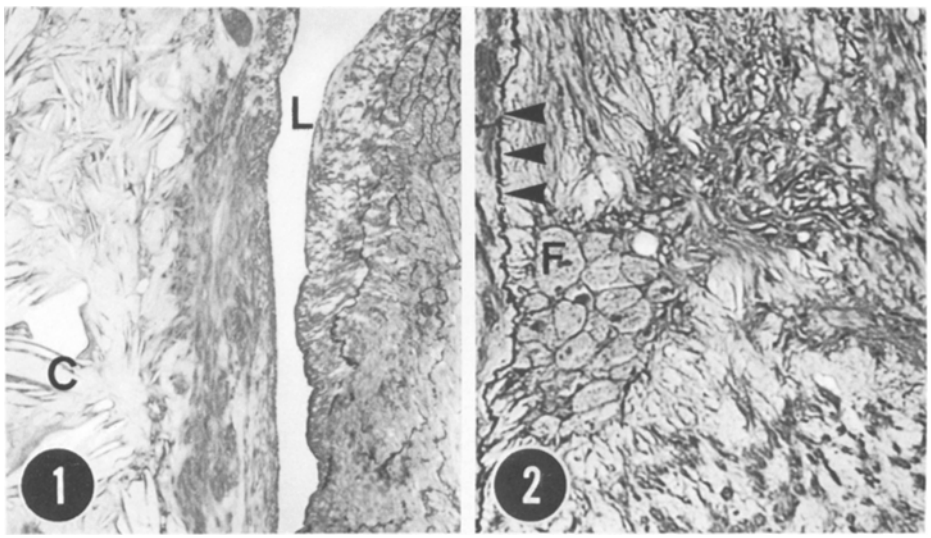

Fig. 1. Figures 1 and 2 are light micrographs stained with Verhoff's elastic and Gomori's trichrome stains. Note the advanced intimal lesion containing cholesterol crystal clefts (*C*). Lumen (*L*). ×65

Fig. 2. Intimal atheroma characterized by foam cells (*F*), an abundance of collagen and smooth muscle cells, and a distinct internal elastic lamina (*arrowheads*). ×190

rosclerotic involvement of the more muscular intimal cushion (Fig. 1). The internal elastic lamina is well preserved and clearly separates the intimal atheroma from the underlying tunica media of the aorta. The smooth muscle cells just beneath the endothelium appear regularly arranged in longitudinal bundles although isolated areas show some discontinuity. Examination of the deeper subendothelial space reveals smooth muscle cells which appear to be more scattered and form discontinuous bundles of aggregates of cells surrounded by abundant collagen fibers (Fig. 2). Collagen fibers are more numerous in the area adjacent to the internal elastic lamina.

Accumulations of extracellular lipid are visisble beneath the endothelium. Occasional structures which appear similar to cholesterol crystal clefts are seen deep within the intimal atheroma (Fig. 1). Lipid can also be observed in larger cells which may be found just beneath the endothelium and/or deeper within the atheroma (Fig. 2). These cells appear in clusters ensheathed in a lattice-work of collagen fibers and probably represent foam cells. Elastic fibers are not observed in the advanced intimal atheroma. Beneath the internal elastic lamina and throughout the tunica media of the aorta, layers of concentric elastic membranes are clearly evident with accompanying collagen strands. Alternating between the elastic lamellae are bands of smooth muscle cells. The adventitia is thin and composed of elastic and collagen fibers.

Electron Microscopy. A single layer of endothelial cells forms a sheet over the luminal surface of the intimal atheroma (Fig. 3). The endothelial cells possess

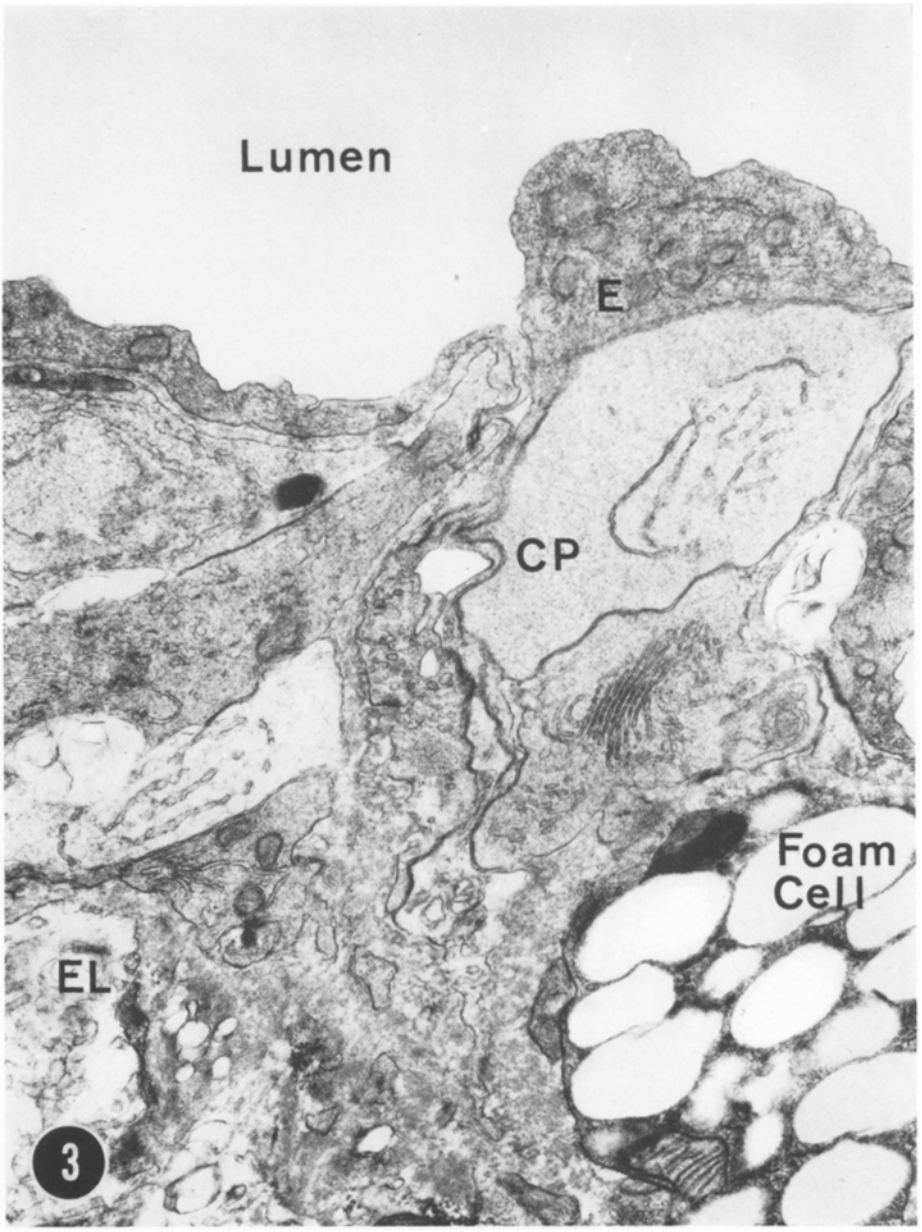

Fig. 3. Figures 3–9 are electron micrographs of tissue stained with uranyl acetate and lead citrate. Endothelial lining (*E*) covering an advanced intimal atheroma. The subendothelial space contains cellular processes (*CP*) which contain a flocculent matrix and tubular-like elements. Aortic lumen (*Lumen*); extracellular lipid (*EL*); portion of a foam cell (*Foam Cell*). ×26,000

an elongated centrally located nucleus which characteristically has an irregular profile. The nucleus is typified by peripheral heterochromatin attached to the inner surface of the nuclear envelope. The endothelial cells are usually narrow, possess attenuated cytoplasmic processes and occasionally short microvilli can be observed projecting into the aortic lumen (Fig. 5). Adjacent endothelial cells are joined by non-specialized endothelial cell junctions or interdigitating portions of apposing endothelial cell membranes (Fig. 4).

Distinct areas of dilatation of the intercellular space are observed between apposing cell membranes (Figs. 6, 9). A flocculent material which has a similar appearance to the ground substance of the subendothelial space can be seen in the intercellular dilatations.

Micropinocytotic vesicles measuring approximately 50 nm in diameter are common along the subendothelial aspect of the endothelial cell plasma membrane (Fig. 4). Juxtanuclear Golgi complexes (Fig. 5), Golgi vesicles and scattered cytoplasmic filaments (Figs. 4, 6) are observed as normal constituents of the endothelial cell cytoplasm. Round and oval mitochondria which display moderately packed cristae are present in the cytoplasm. Membrane-bound endothelial cell granules [perhaps Weibel-Palade bodies (Weibel and Palade, 1965)] exhibiting a dense granular matrix and measuring approximately 80–140 nm are observed within the endothelial cell cytoplasm (Fig. 5). Other cytoplasmic organelles are not abundant with the endothelial cells. An abundance of subendothelial ground substance does not permit conclusive identification of the basal lamina.

The subendothelial space is composed of a connective tissue matrix containing abundant collagen fibrils, ground substance, lipids and scattered smooth muscle cells (Fig. 7). In addition, cellular processes are frequently observed in the subendothelial space directly adjacent to the base of the endothelial cells. These processes contain a moderate amount of granular flocculent material and abundant tubular-like structures which resemble dilated elements of the smooth endoplasmic reticulum (Figs. 3, 9). Occasionally, myelin figures and mitochondria are observed within these cellular processes. Although these processes closely approximate both the overlying endothelium and underlying smooth muscle cells, they bear little resemblance to either of these two cells. Throughout the subendothelial space large numbers of smooth muscle cells are observed, many of which display a wide variation in morphology (Fig. 7). These subendothelial smooth muscle cells comprise the major cell type in the pigeon atheroma. Most are elongate or stellate with a centrally placed nucleus which has abundant peripheral heterochromatin (Fig. 7). Some smooth muscle cells show only moderate cytological alterations, but most have apparently undergone some transformation in their morphology. Included among these transformations are randomly arranged bundles of myofibrils, slightly dilated sarcoplasmic reticulum, the appearance of lipid droplets and vacuoles, and a thickened basal lamina, which has collagen fibrils closely associated with it (Fig. 7). Numerous micropinocytotic vesicles measuring approximately 50 nm in diameter are present around the periphery of the smooth muscle sarcolemma. Dense bodies (attachment plaques) are numerous and fusiform densities can be observed scattered throughout the filamentous portion of the sarcoplasm

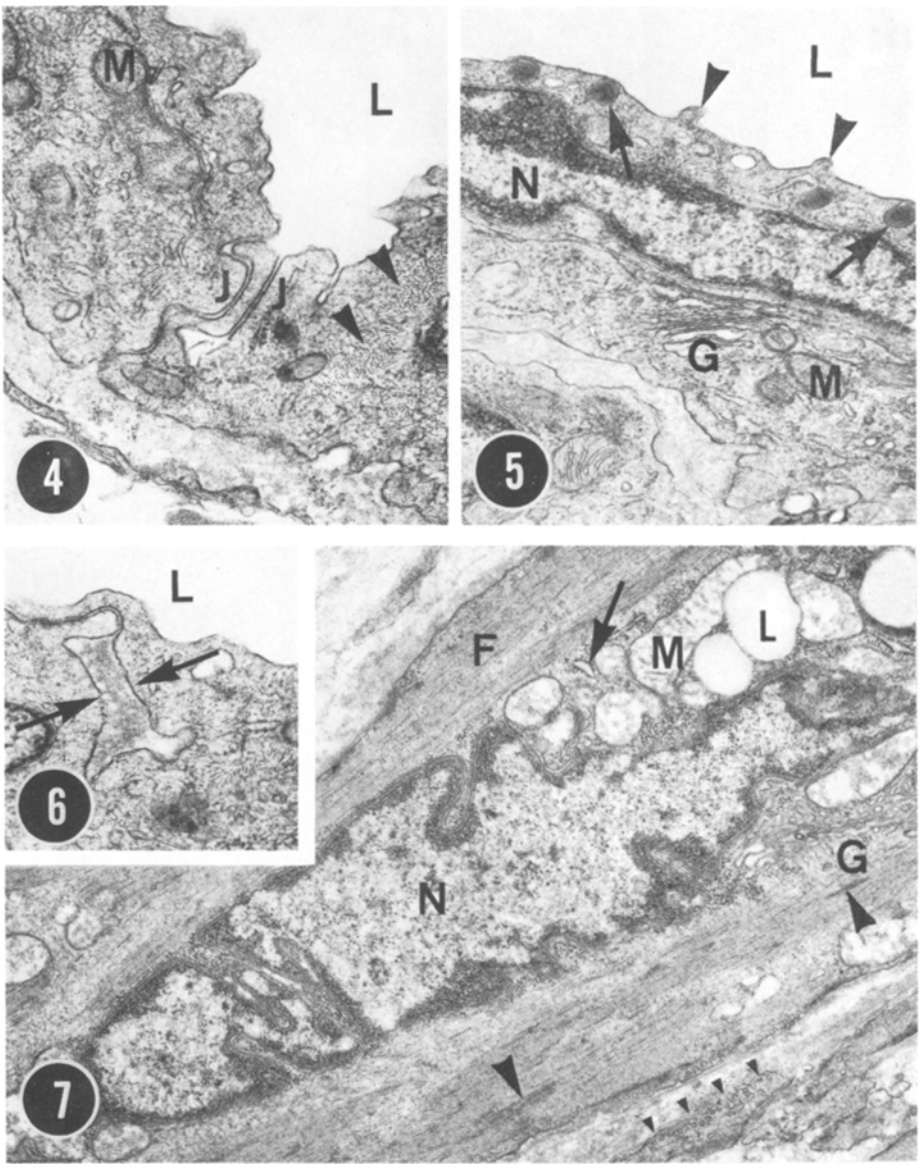

Fig. 4. Two apposing endothelial cells and two non-specialized endothelial cell junctions (*J*). Note cytoplasmic filaments (*arrowheads*). Lumen (*L*); mitochondrion (*M*). ×37,500

Fig. 5. Endothelial cell. Note endothelial cell granules (*arrows*), microvilli (*arrowheads*), nucleus (*N*), Golgi complex (*G*), and mitochondrion (*M*). Lumen (*L*). ×31,200

Fig. 6. Portions of two apposing endothelial cells. Note dilatation (*arrows*) which contains a flocculent material. Lumen (*L*). ×31,200

Fig. 7. Intimal smooth muscle cell. Note invaginated nucleus (*N*) and sarcoplasmic organelles located at the nuclear pole. Mitochondrion (*M*); lipid droplets (*L*); Golgi complex (*G*); longitudinally sectioned myofilaments (*F*): fusiform bodies (*large arrowheads*); dilated sarcoplasmic reticulum (*arrow*); micropinocytotic vesicles (*small arrowheads*). ×20,000

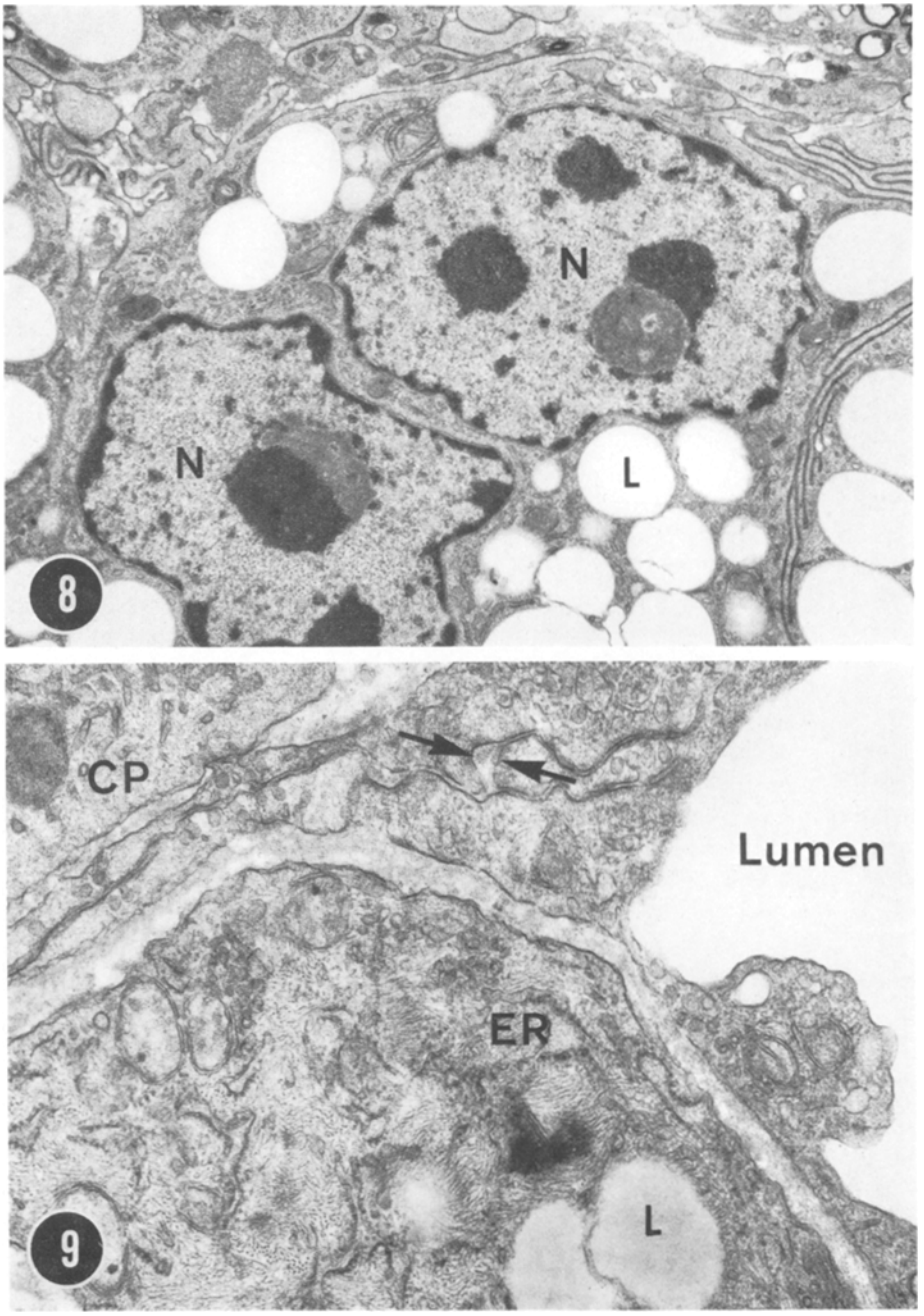

Fig. 8. Binucleate cell. Note both nuclei (*N*) with several prominent nucleoli and cytoplasmic lipid droplets (*L*). The extracellular compartment contains lipid, collagen and vesiculated material. ×15,800

Fig. 9. Portion of a subendothelial cell which resembles an interlaminar cell. This cell contains dilated endoplasmic reticulum (*ER*) lipid droplets (*L*), disorganized filaments and cytoplasmic vesicles. Note a dilatation (*arrows*) between two apposing endothelial cells. Subendothelial cellular process (*CP*); aortic lumen (*Lumen*). ×36,800

(Fig. 7). In many instances, the sarcoplasmic organelles and lipid droplets appear aggregated at one or both of the nuclear poles. Attachment points between adjacent smooth muscle cells are seldom observed.

Accumulations of extracellular lipids are scattered throughout the subendothelial space, along with collagen, myelin figures and vesiculated elements (Figs. 3, 8). In addition to the smooth muscle cells, the subendothelial space also contains cells of a questionable derivation. Usually closely associated with the luminal aspect of the subendothelial space, there appear elongated cells with angular nuclear profiles, Golgi complexes, a few mitochondria, dilated elements of the granular endoplasmic reticulum, an occasional lipid droplet and cytoplasmic vesicles (Fig. 9). The elongated cells observed in this study have many features in common with mesenchymal cells but are also characterized by abundant dilated granular endoplasmic reticulum.

Deeper within the subendothelial space, adjacent to the internal elastic lamina, binucleate cells are sometimes observed. These cells possess some morphological characteristics of smooth muscle cells, however the myofibrils are sparse or completely lacking, and a distinct basal lamina is absent (Fig. 8). Several prominent nucleoli are present within the nuclei of these cells, as well as dense peripheral heterochromatin. The cytoplasm contains numerous lipid vacuoles and the plasma membrane displays extensive interdigitations. Frequently, these binucleate cells also have morphological features in common with foam cells.

Foam cells are observed within the atheroma. They are usually seen arranged in groups and are characterized by having large round and oval lipid droplets, few mitochondria, numerous myelin figures, peripherally displaced nuclei, many smaller vesicles and fragmented remnants of other cytoplasmic organelles (Figs. 3, 8). Numerous myelin figures are present within the cytoplasm of these cells, as well as randomly arranged fibrils and many smaller vesicles. The foam cells are surrounded by extracellular lipid and randomly arranged bundles of collagen. In more advanced lesions, foam cells may appear closer to the luminal aspect of the atheroma and may actually come to lie beneath the endothelial cells.

Discussion

The spontaneous atherosclerotic lesions found at the celiac bifurcation of the White Carneau pigeon have been studied in detail at the level of the light microscope (Lofland and Clarkson, 1965; Prichard, 1965; Prichard et al., 1964). Several studies have briefly described the ultrastructure of the smooth muscle cells (Cooke and Smith, 1968) and normal architecture of the pigeon aorta (Lauper et al., 1975). However, additional ultrastructural detail which may be of significance is presented in this study thereby expanding upon previous reports. Special attention will be given to the morphology of the endothelial cells and the cellular components of the immediate subendothelial compartment.

The normal architecture of the celiac bifurcation of the White Carneau pigeon includes an intimal cushion composed of longitudinally arranged smooth muscle (Lauper et al., 1975). These raised cushions are the sites of apparent

smooth muscle cell proliferation and lipid and connective tissue accumulation associated with the atherosclerotic lesions. Hemodynamic changes apparently are involved with these muscular cushions (Santerre et al., 1972) which may precipitate the intimal smooth muscle cell responses leading to atheroma development. Whether the blood flow over these cushions is great enough to provoke a smooth muscle cell response is not directly known. However, McDonald (1974) has demonstrated that lesions have a predilection for vessel segments which are exposed to high turbulence or eddying of the blood flow.

A major aspect of hemodynamic stress of the vessel wall is the ability of the endothelial cells to maintain their normal permeability. The endothelial cells appear normal in morphology with the exception of occasional intracellular vacuoles and small dilatations between apposing endothelial cells. Similar intercellular dilatations have been observed by Parker and Odland (1966) in rabbit aortic atherosclerosis. A flocculent material is often observed in these dilatations which resembles the extracellular ground substance found immediately beneath the subendothelial space. Whether the flocculent material observed in the intercellular dilatations represents a possible influx of lipid or other material into the subendothelial space cannot be determined from the observation made in the present study. Moss and Benditt (1970) observed normal-appearing endothelial cells overlying both small and large spontaneous plaques in chickens. However, they also observed irregular accumulations of a homogeneous granular material between the endothelial cells and the subjacent cells. Lewis et al. (1976), utilizing scanning electron microscopy, have observed areas of the aorta denuded of endothelium with the underlying subendothelial matrix exposed.

The subendothelial space is greatly enlarged in the area of the intimal atheroma and contains, in addition to smooth muscle cells, several other cell types which are difficult to identify by purely morphological criteria. These subendothelial cells possess morphological characteristics in common with mesenchymal or fibroblast-like cells. Cooke and Smith (1968) described cells of a similar morphology, termed interlaminar cells, in the media of the pigeon aorta. The interlaminar cell may be the precursor to these fibroblast-like cells observed in the intimal atheroma. In addition, cellular processes which contain a flocculent matrix and tubular-like elements are observed in the subendothelial space. Occasional myelin figures and mitochondria are also seen within these processes. The appearance of the flocculent matrix in these processes and the material observed in the dilatations between apposing endothelial cells is similar. Jorgensen, et al. (1972) have described the accumulation of flocculent material in the subendothelial space and suggest that it probably is indicative of edema. These subendothelial cellular processes may represent cells which have sequestered ground substance or a vascular filtrate which has accumulated in the subendothelial compartment.

Recent emphasis in the area of atherosclerosis has been placed upon smooth muscle cell proliferation as a key event in the genesis of the lesion (Ross and Glomset, 1973). Indeed, the advanced lesions observed in these pigeons are all characterized by an abundance of smooth muscle cells. The accumulation of lipid within the smooth muscle cells, as well as the presence of lysosomes suggests that the intimal smooth muscle cells may be actively involved in phago-

cytosis (Garfield et al., 1975). The morphological appearance of smooth muscle cells in increasingly advanced stages of modification throughout the lesions suggests that hey may represent the precursor of foam cells. Although direct evidence for the transformation of smooth muscle cells into foam cells is still lacking, many investigators believe that foam cells represent transformed smooth muscle cells which have become engorged with lipid (Imai et al., 1966; Wissler et al., 1976). The accumulation of the extracellular lipid may be a result of the dissolution of existing foam cells and/or the complexing of lipoproteins and extracellular glycosaminoglycans, as a result of smooth muscle synthesis (Chen, 1973) or plasma lipoprotein deposition (Shimamoto, 1975).

The major difference between spontaneous lesions in the chicken and spontaneous lesions in the White Carneau pigeon is the absence of foam cells in chicken spontaneous lesions (Moss and Benditt, 1970). The lesions observed in this study contain many foam cells and, in this regard, resemble the human fatty streak (Strong and McGill, 1962; Balis et al., 1964; Doerr, 1970). The usefulness of the White Carneau pigeon for the study of atherosclerosis lies in the predictability of the lesions sites, their spontaneous development, their morphological similarity in advanced stages to human lesions (Prichard et al., 1964) and the availability of inbred strains for studies involving genetic suscpetibility to atherosclerosis, independent of dietary considerations.

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