Preservation and Structural Adaptation of Endothelium over Experimental Foam Cell Lesions
Quantitative Ultrastructural Study

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To assess the extent to which endothelial cell (EC) structure is modified by hyperlipidemia and by the formation of intimal plaques, we undertook a quantitative ultrastructural study of aortic EC of cynomolgus monkeys after 3 or 6 months on an atherogenic diet. We compared EC in lesion-free areas (LFA) with EC overlying focal discrete foam cell accumulations (FDA) or covering multilayered confluent plaques (MCP). There was a 15% increase in cross-sectional lumen surface profile length over FDA or MCP compared to LFA ($p<0.005$) corresponding to the bulging contours of immediately underlying foam cells. There was, however, no increase in the number of EC per unit of surface area (26.2±4.74 per 10$^4$ mm$^2$ for LFA and 26.0±4.22 for FDA) or, on cross-section, per 100 $\mu$m length of underlying internal elastic lamina (4.79±2.42 for LFA, 8.26±2.01 for MCP). Nor did the number of surrounding cells contacted by each cell over LFA or MCP differ from normolipemic controls (6.56±0.85 for LFA and 5.58±0.86 for MCP). Most ECs were markedly attenuated over lesions, and while the extent and complexity of lateral contact regions between adjacent EC was diminished, the number and complexity of basilar projections was greatly increased. These structures extended among the intimal foam cells to insert on the internal elastic lamina or on intimal matrix fibers, resulting in a 2.7-fold increase in the length of the abluminal portion of the EC profile. The perimeter of the transverse EC profiles was thereby increased from 41.4±2.12 $\mu$m in LFA to 82.2±5.21 $\mu$m over MCP ($p<0.0001$). Polarization of EC in the direction of flow diminished as lesions developed. The ratio of length to width, as well as the standard deviation of the ratio, decreased from 3.51±3.92 in LFA to 2.35±0.25 over MCP, due mainly to increases in the proportion of the cell perimeter exposed to the lumen. Lesion localization bore no relationship to the orientation of EC in corresponding locations in the normolipemic controls or in LFA immediately adjacent to plaques. Organelles of EC in hyperlipidemic animals showed features suggestive of increased metabolic activity in all regions, and stress filaments were increased in the EC attenuated over lesions. There was no evidence of EC degeneration, necrosis, or sloughing regardless of lesion location, size, or complexity. We conclude that EC that cover FDA or MCP undergo alterations in thickness, configuration, extent of intercellular junctional overlap, and in the number and length of their abluminal attachment sites, although with no change in cell number. EC degeneration, disruption, or sloughing are not features of exposure to hyperlipidemia or of the initiation or early progression of diet-induced experimental atherosclerosis.

ultrastructure in the cynomolgus monkey after 3 or 6 months on an atherogenic diet. Plasma cholesterol values in some monkeys in each experimental group exceeded 900 mg/dl, permitting us to also evaluate EC morphology under conditions of extremely high levels of diet-induced hyperlipidemia. We examined EC in lesion-free areas (LFA), over focal discrete lesions with three or fewer layers of foam cells, and over multilayered confluent plaques (MCP) containing both cells and matrix fibers. To minimize artifactual distortions and disruptions, which could be introduced by collapse, redistribution, and handling, the aortas were fixed in situ in anesthetized animals under conditions of controlled pressure perfussion. We avoided agents such as heparin, which are known to alter endothelial structure,6-9 as well as platelet10,11 and leukocyte function.12 Aortas were sampled at standard sites and processed in a manner designed to preserve vessel curvature and to prevent excessive manipulation.1,13,14,15

Methods

Animals and Diets

Twelve cynomolgus monkeys (Macaca fascicularis) were fed an atherogenic diet consisting of 1.75% cholesterol, 21.7% Planter's peanut oil, 61.3% monkey chow, 13.0% orange juice, 1.3% gelatin, and 0.8% of a multivitamin mixture, supplemented periodically with fresh fruit. Six of the animals were killed after 3 months and six, at 6 months. Six additional animals served as controls and were fed a nutritionally complete standard monkey ration 6 months. Six additional animals were killed at 3 months and six, at 6 months. Six additional animals served as controls and were fed a nutritionally complete standard monkey ration which contained 3% fat and virtually no cholesterol. Each animal was offered 300 g of food each morning, and daily consumption was monitored by weighing the uneaten portion. The atherogenic regimen resulted in marked elevations of serum cholesterol levels by 2 months.16,17 Serum lipids were measured initially and monthly. The initial and final blood samples were used for routine hematologic and chemical determinations.

Controlled Pressure Perfusion Fixation

Anesthesia was induced by intravenous sodium thiamylal (25 mg/kg body weight) after sedation with ketamine hydrochloride (10 mg/kg body weight). Each animal was then intubated, and artificial respiration was instituted with a Harvard respirator. The procedure for perfusion fixation to precipitate and accumulate silver granules overcoated by evaporating 12 to 16 cm of gold/palladium

Sampling and Processing

Transverse rings were removed from the thoracic aorta midway between the aortic valve and the first brachiocephalic trunk and from the descending thoracic aorta at one fourth the distance between the origin of the left subclavian artery and that of the superior mesenteric artery. One set of rings was embedded in paraffin, and the sections were prepared for standard light-microscopic study. For demonstration of lipids, frozen sections of some of the fixed samples were treated with oil red O. Immediately adjacent rings were removed for ultrastructural study. These were opened by means of two lateral, longitudinal (axial) incisions to maintain the curvature of the vessel wall and remove distortions attributable to bending or flattening.13,14 Grossly evident lesions, as well as lesion-free areas, were removed from each ring as samples with the full wall thickness and measuring 0.3 x 0.5 cm. Each sample was notched on its proximal transverse edge to indicate the direction of blood flow. Four such blocks were taken for transmission electron microscopy (TEM) and four, for scanning electron microscopy (SEM) at each standard sampling site. The samples for TEM were postfixed in 1% OsO₄ in 1 M Sorensen's buffer for 30 minutes;19 Tissue blocks were dehydrated, were kept overnight in equal proportions of propylene oxide and Epon 812, were transferred to 100% Epon for 2 to 4 hours, and were then vacuum pumped for 2 hours and kept at 10⁻⁵ torr overnight. This procedure assured uniform infiltration and satisfactory sections through the thickness of the block. Specimens for SEM were postfixed in 1% OsO₄ in Millonig's phosphate buffer containing 0.14% CaCl₂ at pH 7.3 for 30 minutes; they were rinsed with buffer, dehydrated, and critical-point dried in a Bomar apparatus after 10 slow flushes with CO₂. These preparations yielded back-scatter electron (BSE) images of lipid vacuoles in intimal foam cells, as well as good secondary electron (SE) images of the lumen surface. For BSE visualization of nuclei, specimens were treated with Gormon's silver methenamine solution at pH 8.8 according to the method of Becker and Sogard.20 The specimens treated with both silver methenamine and OsO₄ provided BSE images of both nuclei and lipid vacuoles in the same specimen. The use of silver binding to disulfide bonds in DNA20,21 for backscatter imaging of nuclei is different from the silver precipitation method commonly used to delineate endothelial cell boundaries.22 The latter method is used before fixation to precipitate and accumulate silver granules beneath overlapping extensions between endothelial cells and to produce preparations with well-demarcated endothelial cell margins. In the present study, exposure to the silver compound followed fixation. No marking of cell borders occurred, and cell contacts were not disrupted. All SEM samples were mounted on aluminum stubs and overcoated by evaporating 12 to 16 cm of gold/palladium
(60:40) wire in an Edwards vacuum evaporator to provide a coating 60 to 80 Å thick.

Selection of Fields

Blocks for TEM were sectioned transversely. Initial semithin sections, 1 μm thick, were stained with methylene blue basic fuchsin azure II23 and were assessed for adequate infiltration, orientation, and vessel distention. Three intimal conditions could be distinguished: 1) LFA, sites with no evidence of intimal foam cell accumulation, 2) focal discrete foam cell accumulations (FDA), areas where large, lipid-vacuolated cells were evident in confined collections one to three cells thick, and 3) MCP, extensive lesions composed of closely packed cells forming four to 10 cell layers. While some MCP were noted in animals killed at 3 months, MCP were abundant at 6 months. Although most MCP obtained at 3 months were almost entirely cellular, many of those obtained at 6 months were stratified and contained prominent zones of connective tissue fibers, amorphous matrix material, or both. Examples of typical lesions are shown in Figures 1 and 2. The blocks were selected to provide fields containing four to six contiguous EC overlying each of the intimal conditions. The same selection of lesion types was made for SEM as for TEM study.

Sectioning and Photography

Serial sets of 30 ultrathin sections were removed from each block face at successive 10 μm steps as shown diagrammatically in Figure 3. The 10 μm interval between each set was traversed by removing 10 semithin sections, the last four of which were retained and mounted. In traversing a block for a distance of 100 to 200 μm, 300 to 360 ultrathin sections and 40 to 48 semithin sections were produced. Semithin sections were studied by light microscopy and photographed to produce prints at a final magnification of ×500. The ribbons of ultrathin sections were mounted on copper grids, the serial order of the sections was indexed by tabs on the grid, and the sections were stained with uranyl acetate and lead citrate. Overlapping TEM photomicrographs were taken of the laterally continuous groups of EC and the underlying intima at a magnification of 4500; selected fields were re-photographed at a magnification of 10 000. Composite mounts were made from prints at final magnifications of 13 500 and 30 000. Each EC of a contiguous group was characterized at two standard levels: on a section passing through the nucleus within the middle 10 μm of its axial length and at one step (10 μm) from one end of the nucleus. A total of 72 blocks were used to study 1776 EC: 540 were at LFA, 314 covered FDA (including 95 over a single layer of intimal foam cells), and 287 covered MCP. For the normolipemic controls, 540 EC were studied. Overlapping SEM photographs of the lumen surface were made at successive magnifications of 500, 1000, and 5000 in both SE and BSE modes. Exposures were made at a constant low tilt angle to reduce the differences in angular distortion. Contact prints were assembled into composites. A total of 319 composites were examined in this manner.

Lumen Surface Profile Length

Modifications of the lumen surface profile were quantified from photomicrograph composites of semithin sections taken at a magnification of ×500. The profile of the endothelial surface representing a 0.5 to 1 mm expanse of lumen was measured by a road-map mileage gauge between reference points marked at each edge of the composite. This distance was then compared with the length of the underlying straightened internal elastic lamina (IEL) between the same reference points (Figure 4). Determinations were made on 50 composites of lesions and on 65 composites of the intima from normolipemic control animals.

Number of Endothelial Cells

Individual EC were identified by their cell margins and were counted on composite photomicrographs of SEM images viewed in the SE mode. The same fields were also viewed in the BSE mode, and the cells were counted by enumerating the silver-stained nuclei (Figure 5). Binucleate cells were scored as a single cell. For the hyperlipidemic animals, 3500 cells were counted over LFA, and 5000 were counted over FDA. For the controls, 2540 cells were counted. The counts were expressed as cells/10^4μm² of lumen area. The number of EC profiles per 100 μm length of internal elastica was also determined.
Figure 2. A. Scanning electron micrograph composite of the intact endothelial lumen surface over a multilayered confluent plaque as seen in monkeys after 6 months on the atherogenic diet. The notch in the specimen (arrow) indicates the proximal (upstream) edge of the specimen. Bar=1 mm. B. Semithin section reveals that such lesions are 5 to 10 foam cells thick (bracket). Crevices between foam cell bulges are one to three foam cells deep. The endothelial lining (arrows) is intact and continuous. Bar=50 μm.

from TEM composites, including 287 endothelial cells over MCP and 540 cells overlying LFA.

Cell Orientation and Transverse Width

EC polarization or orientation was assessed from the SEM composites by establishing a ratio of cell length to cell width (L/W). L was defined as the maximum linear dimension of the cell viewed en face. W was defined as the average of three measurements taken perpendicular to the length line, one at the midpoint of the nucleus, and the two others at one third the distance from the nuclear midpoint to the end of the cell in each direction (Figures 6 and 7). Five hundred EC over LFA, and 151 over MCP were measured.

Endothelial Cell Thickness

An index of cell thickness was established by averaging thicknesses measured at three positions on the ×30 000 TEM composites at the level of the standard transnuclear section. One measurement was made at the lateral edge of the nucleus and the others half-way between this position and the lateral cell edge at each side of the nucleus. Two degrees of reduced EC thickness of the cell body could be distinguished. We have termed these thinning and attenuation. Thinning is narrowing to between 100 and 200 nm; organelles such as endoplasmic reticulum, ribosomes, Weibel-Palade bodies, microfilaments, and vesicles were scant, but present. Attenuation is narrowing to less than 100 nm. In such regions, organelles were almost entirely absent. The cell profiles were scored for the presence of thinning, attenuation, or both, and the length of the transverse cell profile that was thinned or attenuated was measured. Sixty-three cells were studied in LFA and 72 were evaluated over MCP.

Cell Contacts, Extensions, and Attachments

The number of surrounding EC immediately bordering each EC was determined from the SEM photomicrographs (Figures 6 and 7), and the nature and extent of the cell projections extending between adjacent cells was assessed at the three standard step intervals on the TEM photomicrographs. The distribution of abluminal (basal) cell attachments was determined from TEM stepwise sections of 63 cells in LFA of hyperlipidemic animals, of 72 cells over MCP, and of 84 cells from control animals. Basal and interendothelial cell contacts and junctions were characterized from sections taken at the nuclear transection level as shown in Figure 8.

Cell Perimeter

The length of lumenal, ablumenal (basal), and intercellular cell membrane profiles of each EC was determined from the TEM composites at a magnification of 30 000 (Figure 8). Each cell was measured at the level of the central nuclear section and at one of the proximal or distal levels. Calculations for the two positions were averaged to obtain a single value. For hyperlipemic animals, 72 cells overlying lesions and 63 over LFA were studied; 84 cells were studied in the samples from control animals.

Cell Morphology

The abundance and state of EC organelles were approximated by semiquantitative grading from the TEM photo-
Figure 3. Diagram of the sampling levels used to study contiguous endothelial cell groups by transmission electron microscopy. Block faces were four to six cells wide. Serial ultrathin sections were taken at successive 10 μm intervals (Roman numerals). Cells are numbered in the sequence in which they were identified and studied in a typical block.

graphs. For thinned and attenuated cells, only the perinuclear regions were used. The following structures were evaluated: nuclei, free ribosomes, rough endoplasmic reticulum, mitochondria, Golgi complexes, lipid vacuoles, microfilaments, lysosomes, Weibel-Palade bodies, caveolae, vesicle rosettes, and basement membranes. Grading was made on a scale of 0 to 3+, taking into account both qualitative and quantitative changes. The criteria for grading are provided in the legend for Table 2.

Treatment of Data

We compared the mean values of the measurements taken and found that the discontinuities did not invalidate requirements for two-tailed t test statistics. Distributions such as the bi-modal ones seen in cell shape were sufficiently discontinuous to permit graphing values and to compare mean differences by using t tests. We also used mean values to reconstruct “average” cell shapes and used this as a basis for evaluating the degree of cellular plasticity. Our findings are expressed as means±standard deviations. Differences were considered to be significant if p<0.05.

Results

The mean serum cholesterol level for the 18 animals before beginning the atherogenic diet was 134±30 mg/dl. For the 12 which were fed the diet, cholesterol levels rose rapidly, reaching maximum values at 3 months. These levels ranged from 430 to 1200 mg/dl with a mean of 893±192 mg/dl for the 12 diet-fed monkeys. Eight of these had serum cholesterol values above 700 mg/dl within 2 months of starting the diet. For the six animals continued on the diet for 6 months, total serum cholesterol fell slightly during the fifth and sixth months. At termination, levels ranged from 340 to 1005 mg/dl with a mean of 743±314 mg/dl. At 3 and 6 months on the atherogenic diet, cholesteryl esters were 645±81 and 666.1±122.2 mg/dl, respectively, (normal diet, 94.2±19 mg/dl); triglyceride levels were 11.6±5.2 and 34.6±15.6 mg/dl (normal diet, 37.3±14.5 mg/dl); and phospholipid levels were 365.5±56 and 271.1±32 mg/dl (normal diet, 215.5±32 mg/dl). Hematologic and serum electrolyte values remained within normal limits throughout the experimental period for all animals. Body weights remained nearly constant for both the cholesterol-fed and normal-diet control groups. Values were 5.25±1.55 kg for cholesterol-fed animals at 3 months and 5.05±1.23 kg at 6 months. The control animals weighed 5.46±1.31 kg.
Figure 5. Secondary electron (A) and backscatter electron (B) images of the same field showing a typical lesion-free area. Silver staining renders endothelial cell (EC) nuclei clearly visible. Ovoid shape and vesicular chromatin pattern readily distinguishes EC from leukocyte, smooth muscle cell, or foam cell nuclei. Bar=20 \( \mu \text{m} \).

Cell counts over lesion-free areas and over lesions were made on photographs taken in stereo-pairs. Since many EC nuclei were hidden within crevices between foam cell bulges, counts of EC nuclei over confluent lesions on scanning electron micrographs may not be accurate, particularly of multilayered confluent plaques in secondary electron mode (C). The same field viewed in backscatter mode (D) isolates the nuclei from other structures but does not entirely resolve the problem of hidden and overlapping nuclei even when the reverse contrast (E) further enhances identification of nuclei. Bar=50 \( \mu \text{m} \).

**Intimal Cells**

The predominant cell type in the aortic intima of the hypercholesterolemic animals was the foam cell, i.e., a relatively spherical cell with extensively vacuolated cytoplasm (Figures 1 and 2). Lymphocytes and other non-vacuolated monocytoid cells were also noted, but these cell types rarely exceeded 10% of the total lesion cell population. The nature of these cells and their relationships to the endothelium and to other intimal cells will be described elsewhere. Foam cells stained strongly with Oil red O and had the ultrastructural features of macrophages.\(^{24,25}\) Intimal foam cells of FDA were usually arranged in nodular clusters or rows oriented in the direction of blood flow (Figure 1). Where lesions formed MCP, the luminal surface was also nodular (Figure 2), with each surface mound corresponding to an underlying foam cell.\(^1\) Crevices between foam cell contours also tended to be aligned axially and were usually one or two foam cells deep regardless of the total thickness of the underlying lesion (Figure 2). Animals with the highest serum cholesterol values had the most extensive lesions at both the 3-month and the 6-month observations. In general, the extent of lesion formation appeared to be related to the individual response to the diet rather than to the length of time on the diet.

**Lumen Surface Profile Length**

The presence of subendothelial foam cells resulted in a 15% increase in lumen surface profile compared to LFA (Figures 1, 2, and 4). For MCP, the endothelial surface profile length was 19.8%±7.90% (n=50) greater than the underlying IEL length. For LFA, the length of a line along the lumen surface was only 4.31%±0.02% (n=65) greater than the length of the underlying IEL segment, the difference corresponding mainly to the projecting surface contours of endothelial cell nuclei.

**Number of Endothelial Cells**

EC nuclei that fell within lumen surface crevices between foam cell bulges could not be visualized adequately by SEM, particularly over MCP (Figure 5). Counting EC on SEM-BSE images could, therefore, be accomplished with precision only in LFA and over most of the single-layered FDA. EC over both MCP and LFA could be counted accurately on TEM photomicrographs. The number of EC, either per 10^4 \( \mu \text{m}^2 \) of lumen surface area or per 100 \( \mu \text{m} \) length of IEL, was the same over lesions as in LFA. The number of neighboring EC contacted by each EC was also the same for the two regions. EC nuclei per 10^4 \( \mu \text{m}^2 \) of intimal surface viewed by SEM-BSE was 26.1±4.01 (n=2540) for control animals, 26.2±4.47 (n=3500) for hyperlipemic animals, and 26.0±4.22 (n=5000) overlying FDA.
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Figure 6. Tracing made from a scanning electron microscopic photograph of an endothelial lumen surface in a markedly polarized region from the straight portion of the descending thoracic aorta in a lesion-free area. The tracing is enhanced by shading to demonstrate the configuration of the intercellular contacts. Endothelial cells in control animals and in lesion-free areas contact adjacent cells by extensive overlapping extensions including spatulate configurations (insert, upper right) and numerous interdigitating overlapping processes (insert, lower left). Each endothelial cell contacts six to seven surrounding cells regardless of the degree of polarization of the cells. Compare with Figure 7.

Determinations from TEM composites revealed 8.79±2.42 EC profiles per 100 μm of IEL length in LFA (540 cells counted) and 8.26±2.01 cells per 100 μm IEL over MCP (287 cells counted). The number of immediately adjacent EC in contact with each EC, as determined from SEM images of 200 fields, was 6.56±0.85 in LFA and 5.68±0.86 over MCP. None of these differences was significant.

Cell Orientation and Transverse Width

The EC in LFA were mostly distinctly elongated and oriented axially in samples from the straight portion of the descending thoracic aorta (Figure 6) and least polarized in samples from the ascending aorta. The distribution of degrees of cell polarization, as expressed by the ratio L/W, was, however, bimodal in both locations (Figure 9). In LFA, L/W ranged from 1.50 to 3.00 with a mean L/W of 2.03±0.40. With the development of MCP, the degree of polarization of EC decreased in both locations, but no strongly polarized cells were in evidence, and the distribution of degrees of polarization was continuous. The range of L/W was 1.77 to 3.37 with a mean of 2.35±0.25, p<0.01, compared to LFA. The apparent absence of a bimodal distribution of L/W over such lesions when viewed on face by SEM was probably due to the difficulty in identifying the EC boundaries that fell within the crevices. Assessed from TEM micrographs, however, the width of EC overlying confluent plaques extended over a wide range, and the distribution was bimodal (Figure 9). Measured from TEM micrographs, the cell width over LFA was fairly uniform. Values ranged from 11.7 to 23.2 μm with a mean of 18.1±4.17. Over MCP, mean EC width was increased to 23.5±9.1 μm with a range of 10.1 to 51.0 μm (Figure 10). A total of 69% of cells overlying lesions fell between 10.1 and 24.9 μm in width with a mean of 16.7±4.7 μm (n=75), while 31% fell between 25.0 and 51.0 μm with a mean of 35.6±5.5 μm (n=76). The larger range of widths and the apparent bimodal distribution over confluent plaques reflected the occurrence of relatively narrow cells overlying crevices between foam cell contours, while the wider cells spanned the projecting foam cell contours (Figures 10 and 11). These data suggest that determination of cell polarization over lesions from SEM micrographs alone may be misleading.

Thinning and Attenuation of Endothelial Cells

In LFA, EC thickness, expressed as the average of the three standard measurements lateral to the nucleus, ranged from 0.80 μm to 2.17 μm and averaged 1.15±0.19 μm (n=63). These values correspond closely with those previously reported for normal capillary, sinusoidal, and lymphatic endothelium.26,27,28 Over MCP (n=72), there was a 54% increase in the number of cells showing thinning, (narrowing to a thickness of 100 to 200 nm) compared to LFA. The number of cells showing attenuation (narrowing to less than 100 nm) increased markedly. Of the EC in LFA, 61% were found to have regions of thinning, and 19% had regions of attenuation. Overlying MCP, however, 32.8% of the cells showed regions of thinning, while 52.2% showed regions of attenuation. When attenuation was expressed as the percent of the total cell transverse profile at the nuclear level narrowed to 100 nm or less, the extent was ninefold greater over MCP than in LFA. Values were 45.3%±3.1% for MCP compared to 5.13%±0.93% in LFA (p<0.005). Attenuation was seen mainly in EC which were markedly increased in width. Examples of attenuation of EC over foam cell projections are shown in Figures 1, 2, 4, and 11.

Cell Contacts, Extensions and Basilar Attachments

EC projections into the underlying intima were very prominent. In LFA, cell extensions projecting in the lateral (transverse or circumferential) direction tended to remain in continuous contact with similar extensions of laterally adjacent EC. Such extensions, however, often projected further to make contact with cells beyond immediately
Figure 7. Scanning electron micrograph of the endothelial lumen surface overlying a multilayered confluent lesion. Mixed-signal image visualization of endothelial cell (EC) margins and nuclei, as well as clusters of lipid vacuoles in immediately underlying foam cells (arrowheads), are shown. B. Tracing of the EC borders of the field shown in A. The tracing is enhanced by shading to emphasize the simplification of the cell contacts and overlaps. Cell polarization is greatly reduced compared with the lesion-free area shown in Figure 6. These cells are moderately polarized with a length/width ratio of about 2.3. Each EC is surrounded by about six adjacent cells as observed in lesion-free areas. The central prominent cell contains two nuclei. Bar=10 μm.

Figure 8. A. Transmission electron micrograph of an endothelial cell transected at the nuclear level in a lesion-free area. Basal extensions attach to the underlying internal elastic lamina and extend for distances of up to about 3.7 μm from the abluminal cell surface. B. A tracing of the cell perimeter of the cell in A indicates how the perimeter segments were defined for measurement. The luminal aspect is outlined by an interrupted line of dashes, the abluminal aspect, by dots and dashes, and the lateral intercellular contacts, by fine dots. Bar=1 μm.

neighboring cells. Slightly oblique extensions from nearby upstream or downstream cells contributed to the formation of multilayered intercellular junctions. As many as seven overlapping cell extensions could be identified in some junctional zones. Longitudinal or axial extensions projected for distances up to 15 μm but only occasionally contacted other cells. As previously described, basal extensions in LFA projected from the abluminal endothelial surface to insert, by way of focal peripheral dense body attachment sites, into points on the internal elastic
lamina (Figure 8). These extensions were distributed uniformly beneath the cell as short, blunt projections, averaging $3.71 \pm 1.3 \mu m$ in length from adjacent abluminal surface to IEL (72 cells measured). Reconstructed from serial sections, however, these basal extensions proved to be thin, curtain-like structures 100 to 200 nm wide. Overlying MCP, the extent of lateral overlapping between adjacent endothelial cells was much reduced. The EC over confluent plaques overlapped laterally or interdigitated for a length of $2.18 \pm 0.67 \mu m$ (n = 72) compared with $4.81 \mu m \pm 1.30$ (n = 72) for lesion-free regions (p < 0.005).

The length of axial extensions and the number of multilayered interdigitating junctions were also reduced. Spatulate extensions (Figure 6) frequently noted in LFA viewed by SEM were rare over MCP. In contrast to the relative simplification and shortening of lateral contact zones and the absence of axial and lateral extensions over MCP, basal extensions were markedly increased in both number and complexity (Figure 11). The abluminal endothelial processes over FDA projected between intimal foam cells to attach at dense bodies to the underlying IEL, occasionally to islands of elastin, or to amorphous condensations of matrix material within the intima. Over MCP, the basal extensions projected mainly from the more lateral aspects of the EC, curving and sometimes branching as they passed among foam cells to attach to the IEL or to an island of matrix material. An entirely continuous basement membrane was usually absent (Figure 11).

**Cell Perimeter**

There was a twofold increase in overall cell perimeter overlying MCP compared to nonlesion areas (MCP, $82.2 \pm 5.21 \mu m$; LFA, $41.4 \pm 2.12 \mu m$; p < 0.0001) (Table 1). The perimeter profile segment comprising the abluminal (basal) aspect and its projections increased with increasing lesion thickness from $21.4 \pm 2.00 \mu m$ (51.7% ± 4.8% of the total perimeter) in LFA to $34.0 \pm 2.68 \mu m$ (60.2% ± 4.8% of the total perimeter) over FDA (p < 0.001). The cell perimeter segment exposed to the lumen measured $23.5 \pm 5.08 \mu m$ (37.5% ± 6.0% of total) over MCP, compared to $16.2 \pm 1.88 \mu m$ (39.2% ± 4.8% of the total perimeter) in LFA (p < 0.005). The length of intercellular contact regions was reduced from $3.8 \pm 2.36 \mu m$ (9.1% ± 1.2% of total) in LFA to $2.18 \pm 1.33$ (only

Figure 9. Distribution of determinations of endothelial cell polarity as expressed by the ratio of cell length to cell width (L/W) measured on scanning electron micrographs. The histogram of L/W values for cells in normolipemic monkeys was bimodal (upper panel). Of cells in the straight portions of the aorta, 56% were strongly polarized (L/W range = 3.36 to 7.67), while 44% were only moderately polarized (L/W range = 1.50 to 3.00). With the development of confluent foam cell lesions (lower panel), 95% of cells in corresponding standard sampling areas were moderately polarized (L/W range = 1.77 to 3.37), and no cells were strongly polarized.

Figure 10. Distribution of widths of endothelial cells overlying confluent plaques. Widths measured at the transnuclear section level of transmission electron micrographs show a bimodal distribution. Attenuated cells comprise 31% of all cells measured. Widths of these cells ranged from 25.0 to 51.0 μm and averaged twice the width of cells over lesion-free regions. A total of 65% of the cells were narrower than normal, with cell widths ranging from 10.1 to 24.9 μm.
Figure 11. A. Endothelial cells overlying a multilayered confluent plaque. The central cell (C) lies mainly in a crevice. The two lateral cells (L1 and L2) are attenuated over foam cell bulges. Abluminal basal extensions (arrowheads) of the central cell extend to attach to the internal elastic lamina. Basement membranes of the two lateral attenuated cells are not uniformly continuous. Insets. Basement membrane is present but interrupted in the markedly attenuated region on the left (horizontal arrow) but absent from the attenuated region on the right (oblique arrow). B. A tracing of the cell perimeters facilitates identification of the attenuated regions shown in the insets, the intercellular contacts (brackets), and the abluminal extensions (arrowheads). Intercellular contacts are simplified, basal extensions are elongated and complex, and both axial and lateral endothelial cell extensions are reduced in extent compared to lesion-free areas. Bar=1 μm.

2.6%±0.7% of total perimeter) over MCP (p<0.05). Cell contact zone lengths in the LFA of hyperlipidemic animals did not differ significantly from those over FDA.

Subcellular Morphology

In control animals, undistended cisternas were limited to three or four profiles per cell section and showed no alteration in electron lucency from cis- to trans-regions. There was no predominance of either granular or smooth endoplasmic reticulum. Filaments in the 45 to 65 Å range were evident, and microbodies were noted. Profiles of endocytic vesicles averaged 73.1±0.24 nm in diameter and were of the uncoated type, uniformly bounded by a simple unit membrane. Compared to the EC of control animals, those in LFA of hyperlipidemic animals showed a moderate increase of free ribosomes, as well as an increase in rough endoplasmic reticulum, microfilaments, lysosomes, vesicle rosettes, caveolas, and Golgi complexes, including an increase in the number of the Golgi cisterna profiles (Table 2). Intracellular lipid vacuoles were more evident, and basement membranes were less evident than in the EC of controls. Mitochondria were more numerous in LFA but remained elongated and 2 to 3 μm in length. In contrast to those in the EC of controls or over
Table 1. Length of Endothelial Perimeter Segments in Hyperlipidemic Monkeys

<table>
<thead>
<tr>
<th>Segments</th>
<th>Lesion-free areas</th>
<th>Focal discrete lesions</th>
<th>Confluent plaques</th>
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<tbody>
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<td>16.2±1.9</td>
<td>19.2±3.2</td>
<td>23.5±5.1</td>
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<td></td>
<td>(39.2)</td>
<td>(33.9)</td>
<td>(27.5)</td>
</tr>
<tr>
<td>Interstitial</td>
<td>3.8±2.4†</td>
<td>3.3±1.7†</td>
<td>2.2±1.3†</td>
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<td></td>
<td>(9.1)</td>
<td>(5.9)</td>
<td>(2.6)</td>
</tr>
<tr>
<td>Basal</td>
<td>21.4±2.0</td>
<td>34.0±2.7</td>
<td>57.5±4.7</td>
</tr>
<tr>
<td></td>
<td>(51.7)</td>
<td>(60.2)</td>
<td>(69.9)</td>
</tr>
<tr>
<td>Total perimeter</td>
<td>41.4±2.1</td>
<td>56.5±2.5</td>
<td>82.2±5.2</td>
</tr>
</tbody>
</table>

*Values are μm±SD. The percent of total perimeter length is shown in parentheses for each value. N=84 for lesion-free areas, 75 over focal discrete lesions, and 159 over confluent plaques.
†Except for comparison of intercellular segment length between lesion-free areas and focal discrete lesions, comparisons for each of the segment lengths are significant.

LFA in hyperlipidemic animals, mitochondria over MCP were not polarized in the direction of blood flow. There were no cytoplasmic flocculent densities in mitochondria, nor was there any loss of matrix granules. Nuclear chromatin showed a slight decrease, but smooth endoplasmic reticulum and Weibel-Palade bodies showed a slight increase over normal. Microtubules were seen only as components of Weibel-Palade bodies and centrioles. Despite the semiquantitative grading and averaging method used to characterize these features, all of the differences between the controls and the LFA in hyperlipidemic animals were striking. The EC over MCP differed somewhat from the EC in LFA of hyperlipidemic animals. Where EC were markedly thinned and attenuated, organelles were almost entirely confined to the perinuclear region. Nuclei were increasingly euchromatic, and free ribosomes, rough endoplasmic reticulum, and Golgi complexes were markedly more prominent. All of these increases were significant at p<0.05 or less. Microfilament content, mitochondria, lysosomes, Weibel-Palade bodies, intracellular lipid vacuoles, and vescicle rosettes were not further increased over the findings in LFA. Microfilaments were concentrated within the abluminal cytoplasm; mainly at the basal attachments. There was no increase over normal in the number of autophagic vacuoles or lipofuscin granules in endothelial cells over either LFA or MCP. Lipid vacuoles within EC were unusual and occurred mainly in perinuclear regions. Caveolas ringing central vesicles were not observed in EC overlying lesions. Binucleate cells were rarely encountered in the control animals or in the LFA of hyperlipidemic animals; only 1.5% of the cells observed by SEM-BSE over MCP were found to be binucleate (Figure 7). Neither apoptotic nor necrotic changes were noted in any of the samples taken from the aortas of the hyperlipidemic animals.

Discussion

Both the quantitative and qualitative observations reported here emphasize the adaptability and resiliency of the endothelium of cynomolgus monkeys in response to diet-induced hyperlipidemia, to accumulation of foam cells in the intima, and to development of early multilayered complex lesions. The endothelial lining was modified in relation to intimal lesion formation in a number of ways. The usual distinct polarization of EC in the direction of flow tended to be lost, due mainly to an increase in cell width, while cell length tended to be preserved. The extent of overlap between adjacent endothelial cells was markedly decreased, but there was no change in EC number or in the number of surrounding endothelial cells in contact with each EC. These features were associated with an increase of approximately 15% in the lumen surface profile, as the EC conformed to the contours of immediately underlying foam cells. The radial thickness of individual EC was much less uniform than normal. Thinning and attenuation of the cell bodies occurred over the bulging foam cell profiles, while normal or even increased EC thickness was noted in those cells which overlay crevices between adjacent foam cells. Abluminal projections were increased in both number and prominence as they extended and often insinuated between foam cells to maintain contact with the underlying internal elastica or to insert on interstitial intimal matrix condensations. Compared to controls, the prominence of abuminal insertions and the abundance of 45 to 65 Å cytoplasmic filaments suggest structural modifications that would be expected to favor stability and tenacity of attachment to the underlying matrix. EC have been shown to develop increased myofilament content in response to increased shear stress. Unsteady flow conditions at the blood-endothelial interface, as well as altered tensile stress due to stretching and attenuation of the endothelial cells over the surface irregularities, are also likely to result in deformations, which could induce filament production. Our data do not exclude the possibility that endothelial cell turnover rate is increased under conditions of marked hyperlipidemia because our methods were designed to assess quantitative changes in cell configuration rather than cell turnover. Nevertheless, we could find little evidence to support a prominent effect on EC proliferation. We found no mitotic figures in the aortas of either normolipidemic or hyperlipidemic animals, and the number of binucleate endothelial cells associated with lesions was no greater than that noted by others in association with aging under normal dietary conditions. Our observations are, on the contrary, consistent with a persistence of the low endothelial turnover rate characteristic of normal endothelium.

Decreased EC polarity has been noted at sites of predilection for lesion formation in the coronary arteries of the atherosclerosis-prone Carneau pigeon. We found no evidence for such a correlation in the thoracic aorta of the cynomolgus monkey. EC were somewhat less elongated in the ascending aorta than in the proximal descending segment in LFA, but over early lesions, the degree of cell polarization was the same for both locations. Once lesions formed, however, overlying EC were seldom markedly polarized regardless of location and regardless of the orientation of nearby cells in intervening LFA. These observations suggest that loss of polarization occurred after the lesions formed and do not necessarily reflect prelesion conditions or regional susceptibility.

Although the extent of lateral interendothelial cell contact and overlap was generally greatly reduced over MCP, serial sectioning revealed that continuous contact with...
neighboring cells was always maintained. In contrast, some axial endothelial cell projections frequently appeared as profiles without definite contact with nearby cells. Images of these projections were identical to those described in rat aortas as the intimal "ghost bodies," which have been considered to be extensions of medial smooth muscle cells into the intima through fenestrae in the internal elastic lamina.4344 In nearly all instances, these intimal profiles in our material arose as projections of endothelial cells.

Earlier reports have emphasized the likelihood that endothelial injury and disruption in the presence of hypercholesterolemia are factors in the development of early lesions.45 We found changes in organellar frequency and in size and shape in the hyperlipidemic monkeys, but found no pyknotic nuclei, membrane discontinuities, or disrupted organelles. Nor did we find any of the mitochondrial changes that have been shown to precede irreversible cell damage.4647 There was actually an increase in the abundance of mitochondria, suggesting increased energy demand. Although mitochondria may double in size and shape in the hyperlipidemic monkeys, but found no pyknotic nuclei, membrane discontinuities, or disrupted organelles. Nor did we find any of the mitochondrial changes that have been shown to precede irreversible cell damage.4647 There was actually an increase in the abundance of mitochondria, suggesting increased energy demand. Although mitochondria may double in number before cell division, other indicators of imminent cell division, such as dissolution of the nuclear envelope and euchromatic appearance.4849 We found no blebs or membrane whorls associated with cell injury or considered to be artifacts of specimen preparation.114849646465 There were no qualitative changes in the appearance of Weibel-Palade bodies, although a modest increase in number was noted in the hyperlipidemic animals. Since Weibel-Palade bodies appear to be associated with coagulation factors, the minimal response noted in our experiments could be related to our avoidance of heparin administration. Thus, the changes we observed in cells over confluent lesions, although modest compared to the changes associated with hypercholesterolemia,5667 hypertension,58 and experimentally induced edema,596061 probably reflect modified or increased levels of cell metabolism due to exposure to hyperlipidemia and increased lipoprotein uptake.

The SEM images also failed to reveal changes that have been associated with cell injury6263 or considered to be artifacts of specimen preparation.11,13,14,64,65 There were, for example, no lumen surface craters, stomata, or plasma membrane rufflings. On the basis of the present findings, as well as previous studies,11,12,14 we submit that findings, as well as previous studies,11314 we submit that modifications of organelles in endothelial cells.

Table 2. Modification of Organelles in Endothelial Cells

<table>
<thead>
<tr>
<th>Features</th>
<th>Controls</th>
<th>Hyperlipidemic animals</th>
<th>Lesion-free areas</th>
<th>Confluent lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear chromatin*</td>
<td>2.81±0.40</td>
<td>2.37±0.19</td>
<td>1.45±0.50</td>
<td></td>
</tr>
<tr>
<td>Free ribosomes</td>
<td>0.66±0.52</td>
<td>1.01±0.53</td>
<td>2.56±0.50</td>
<td></td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>0.67±0.46</td>
<td>0.92±0.21</td>
<td>1.35±0.74</td>
<td></td>
</tr>
<tr>
<td>Golgi complexes†</td>
<td>0.68±0.47</td>
<td>0.99±0.20</td>
<td>1.14±0.61</td>
<td></td>
</tr>
<tr>
<td>Lysosomes</td>
<td>0.34±0.48</td>
<td>0.60±0.25</td>
<td>0.49±0.50</td>
<td></td>
</tr>
<tr>
<td>Microfilaments</td>
<td>1.23±0.63</td>
<td>1.49±0.63</td>
<td>1.56±0.61</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.98±0.47</td>
<td>1.68±0.41</td>
<td>2.30±4.39</td>
<td></td>
</tr>
<tr>
<td>Weibel-Palade bodies</td>
<td>0.92±0.53</td>
<td>1.22±0.32</td>
<td>1.25±0.38</td>
<td></td>
</tr>
<tr>
<td>Intracellular lipid</td>
<td>0.16±0.39</td>
<td>0.68±0.40</td>
<td>0.71±0.28</td>
<td></td>
</tr>
<tr>
<td>Basement membrane‡</td>
<td>2.20±0.52</td>
<td>1.97±0.39</td>
<td>0.39±0.23</td>
<td></td>
</tr>
<tr>
<td>Caveolae</td>
<td>1.86±0.35</td>
<td>2.82±0.29</td>
<td>2.70±0.19</td>
<td></td>
</tr>
<tr>
<td>Rosettes of vesicles</td>
<td>0.30±0.46</td>
<td>0.81±0.37</td>
<td>0.75±0.20</td>
<td></td>
</tr>
</tbody>
</table>

Except where otherwise indicated, scoring was based on a semiquantitative comparative grading system with 0 assigned if feature was not evident, 1 when occasionally present, 2 when frequently present, and 3 when abundant. The increase in caveolae was taken to imply an increased rate of non-energy-dependent pinocytosis, while the increase in macropinocytotic vesicles indicates increased energy-dependent uptake of materials. An artifactual increase in caveolae has been associated with aldehyde fixation,51 but comparison with controls fixed in the same manner in our own studies indicates that there was an actual increase under hyperlipidemic conditions.

The increase in microtubular bodies in cells over lesions has been associated with elaboration of "procoagulation" factors52 or Factor VIII5354 in a variety of conditions.11494955 There were no qualitative changes in the appearance of Weibel-Palade bodies, although a modest increase in number was noted in the hyperlipidemic animals. Since Weibel-Palade bodies appear to be associated with coagulation factors, the minimal response noted in our experiments could be related to our avoidance of heparin administration. Thus, the changes we observed in cells over confluent lesions, although modest compared to the changes associated with hypercholesterolemia,5667 hypertension,58 and experimentally induced edema,596061 probably reflect modified or increased levels of cell metabolism due to exposure to hyperlipidemia and increased lipoprotein uptake.

Significance compared to controls: a, p<0.0001; b, p<0.002; c, p<0.05; x=NS. Significance compared to lesion-free areas: d, p<0.0001; e, p<0.001; f, p<0.02; y=NS.
estimates of cell size, shape, and configuration must take into account differences due to conditions of fixation and to differences in cell position relative to the foam cell contours.

Early attempts to illuminate the pathogenesis of atherosclerosis included the hypothesis that plaques were formed mainly by accretion and organization of blood coagulation products at the arterial lumen surface. Recent identification of a platelet growth factor that stimulates smooth muscle cell proliferation in culture has led to a more focused extension of the hypothesis linking endothelial injury to the coagulation system and to the formation of atherosclerotic plaques. According to this hypothesis, platelet deposition at a disrupted or denuded endothelial region and release of a platelet-derived growth factor induces proliferation of underlying smooth muscle cells, which then migrate into the intima. In association with other factors that promote or sustain endothelial disruption and lipid incorporation, this proliferative reaction has been thought to promote the establishment of an intimal atherosclerotic lesion. Direct morphologic evidence in arteries that disruption or desquamation of endothelium occurs before or at the initiation of diet-induced experimental plaques has not been forthcoming. On the contrary, after experimental physical disruption of the endothelium in the presence of hyperlipidemia, characteristic foam cell lesions tend to occur preferentially where the endothelial lining is intact or recently restored. Although platelets may play a role in the progression of foam cell lesions to complex plaques, the conditions under which this complication occurs have not been clarified. Even if EC are damaged only as a consequence of plaque formation, such events must be unusual, at least in the early stages of experimental plaque formation. The data presented in the present report indicate that EC tend to adapt to the presence of intimal lesions and to preserve the integrity of the endothelial lining.

Acknowledgments

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References


Index Terms: endothelium • foam cells • atherosclerosis
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Quantitative ultrastructural study.
K E Taylor, S Glagov and C K Zarins

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