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SCANNING ELECTRON MICROSCOPY OF EARLY ATHEROSCLEROSIS IN RABBITS USING AORTIC CASTS†

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Abstract

Our research involves measuring the size and location of atherosclerotic lesions on the intimal surface of arteries. To this end we have developed a new method in which scanning electron micrographs of vascular casts with impressions of these lesions on their surface are used. This method is compared with light microscopy and scanning electron microscopy of tissue with lesions. All three methods are found to detect lesions equally well. We also examine the cellular elements in the lesion to determine how the images are formed.

KEY WORDS: Atherosclerosis, Endothelium, Scanning Electron Microscopy, Aorta, Vascular Casting, Macrophage, Rabbit

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Introduction

Atherosclerosis is a patchy disease which occurs at bends and bifurcations in the innermost layer of the artery wall (tunica intima). Two very useful quantities in atherosclerosis research are the percent area covered by lesions and measurement of lesion location. Commonly, these quantities are obtained from arteries that are dissected free, sometimes after aldehyde perfusion fixation. The arteries are cut open longitudinally and spread to examine the intimal surface. The lesions are visible as lighter, white, or yellow regions on the surface. Lipid soluble dyes such as Sudan III can be used to differentiate the fatty lesions from the fibrous lesions which occur in advanced disease.

There are several sources of error in measuring lesion size and location from cut and opened arteries. If the tissue is fresh the artery can contract to as little as 50% of in vivo length and diameter. Straight segments of pressure fixed artery can be opened to give good representation of in vivo lesions, however curved and branching segments are distorted on opening and flattening. Cornhill and Roach (1976) pinned fresh rabbit aortae to in vivo dimensions and used a polar mapping technique to measure lesions associated with the branch. Roach et al. (1978) found that lesions which were actually on the branch could be made to appear as though they were on the aorta. For these reasons, we have recently applied vascular casting to the measurement of lesion size and location.

The outer surface of the vascular cast provides a negative relief of the inner surface of the artery. This surface can now be imaged without cutting, opening, and distorting the artery. The casts of large elastic arteries can be made to have dimensions closely approximating those of arteries in vivo in spite of 20% volume shrinkage of the casting material during setting (Kratky and Roach, 1984; Kratky and Roach, 1987). This is done by injecting the casting material at a high physiologic pressure so the elastic recoil of the artery during setting gives a cast of low-mid physiologic pressure. Our interest in the fluid mechanical aspects of atherosclerosis makes the preservation and measurement of arterial geometries and dimensions important. Conduits for flow modeling can be made using the data from casts. Three different methods of detecting atherosclerotic lesions are compared in this study. 1) Light Microscopy (LM) of aortae pressure fixed in situ and examined wet, both with and without Sudan III staining. 2) The identical tissues, as above, were examined by Scanning Electron Microscopy (SEM) after dehydration and coating with gold. 3) SEM of gold coated aortic casts made by in situ injection of liquid acrylic polymer. Early

atherosclerosis has been induced in the cholesterol fed rabbit animal model by feeding 2% cholesterol feed for one to eight weeks. Over this time course the lesions which develop are of a simple fatty type with no fibrous cap and no ulceration (Roach and Fletcher, 1979). We feel that the slight changes which occur at one week to the very definite fatty lesions at eight weeks provide a good test for comparing the three methods. It has been previously shown that the lesions increase in size in a consistent and reproducible manner on 2% cholesterol diet over a ten-week time course (Roach et al., 1976).

Two specific questions were asked: 1) Will early plaques be detected at the same time by all three methods? 2) Will the location of the plaque's edge be the same by each of the methods?

Materials and Methods

Four New Zealand White rabbits weighing 2.5-3.0 kg. were fed a 2% cholesterol diet in chow (ICN Pharmaceuticals Inc., Cleveland, Ohio) for periods of 8, 18, 21, and 56 days. At the conclusion of the selected time period the animals were anesthetized intramuscularly with 35 mg Ketamine and 5 mg Xylazine per kg body weight. Anesthesia was maintained with intravenous doses of 10% of this amount. One carotid was cannulated to record aortic pressure and the other cannulated for perfusion. This was important since the pressure head required to maintain pressure in the aorta ranged from 10-80% above aortic pressure.

Immediately following a fatal dose of anaesthetic the circulation was perfused with 50 ml of Tyrode's solution containing 5-10 g of polymethylmethacrylate particles which were 30-80 μm in diameter (Polysciences Inc., Warrington, PA). The particles blocked many of the arterioles and made it easier to maintain physiologic pressures during the remaining perfusions. After this, Tyrode's solution without particles was infused for 2-5 min to remove blood which drained out through a cut jugular vein. Then the arterial tree was fixed by perfusion with 2% glutaraldehyde (by volume) in 0.05 M sodium cacodylate and 0.115 M sodium chloride titrated to pH 7.2-7.3. All solutions were perfused at 37°C and maintained at a pressure of 95-105 mm Hg. The aorta was well fixed after 2 h and was then dissected out, and kept in 2% glutaraldehyde-cacodylate buffer at 4°C until used.

The aortas were then cut into 2-4 cm long segments which included complete aorto-branch junctions. A single longitudinal cut was made and the segment pinned out with its luminal surface exposed. The segments were assessed visually, and some of them were then stained with Sudan III using the method described by Cornhill and Roach (1974), and photographed. Photomicrographs were made with a dissecting microscope using epi-illumination at a shallow 15-20° angle.

The pinned segments were then dehydrated through 100% ethanol, placed in acetone for 5 min and then critical point dried from carbon dioxide. The dried tissue was then glued on SEM stubs with epoxy, coated with ~45 nm of gold in a Polaron cool sputter coater and viewed with a Philips SEM 501 scanning electron microscope.

Another series of six rabbits of the same weight were fed 2% cholesterol in chow for 15 days, while a second group of six rabbits were fed for 8 days. At the conclusion of this period the animals were killed with an overdose of pentobarbital (100 mg/kg) and then the left ventricle quickly cannulated. Casting has been previously described (Kratky and Roach, 1983). Briefly the vessels were perfused with saline, then 0.25% $AgNO_3$ in 10% sucrose to outline the endothelial cells, then saline, then Batson's liquid acrylic at 110 mm Hg. Once set, the cast and surrounding tissue was removed and digested in 15-20% KOH at room temperature for 24 h or occasionally for 48 h. The cast was then washed in distilled water, dried, cut into segments and mounted on a spit. This allowed the cast to be rotated through 360° so that all of the aortic surface could be viewed at roughly 0° tilt.

Results

Figures 1-3 compare the first and second methods, i.e., the light photomicrographs of the wet tissue and the SEM photomicrographs of the same tissue dehydrated. Note that in each of the three pairs, lesions occur in the same locations and have the same extent and shape. The lesions were small and not visible to the naked eye after 8 days, and stained very little with Sudan III. The lesions were visible to the naked eye and stained better with Sudan III for the rabbits fed 18, 21 and 56 days.

Figures 4-9 show these lesions as seen on casts. Note that the surface is more undulating over lesions (figures 6-9), and there seem to be two sizes of depressions (which would be raised spots on the tissue). Larger rounded indentations of 20 μm or more diameter which coalesce in more advanced plaques. Endothelial cell outlines can be seen in these depressions (figure 9) indicating they are caused by subendothelial accumulations. A second group of smaller sharper indentations appears to be caused by blood cells adherent to the endothelium (figures 5,7,8,9). For comparison figure 10 shows an unusual example where red cell impressions can be seen on the surface of a cast.

We often found tissue residue on the casts in the region of lesions (figures 4,7,8,9), but never saw this residue in regions without plaques. This tissue residue is not lipid as it was not removed by xylene. It was removed if the KOH digestion was maintained for 48 h at room temperature (figure 6). We suspect, based on other digestion studies by Song and Roach (1983) that the residue may be elastin as this material is very inert chemically. As shown in figure 8 this residue has a very high contrast image and we found it useful to help us identify the regions with plaque at low magnifications.

The following figures (figures 11, 12) show the tissues studied with SEM. Note that in the tissues elevations correspond with depressions on the casts. Figure 11 shows large undulations which appear to be caused by cells under the surface. Also, cells are found resting on the surface.

Leukocytes, erythrocytes, and platelets were all seen on the intimal surface of the tissue. Red cells and platelets were quite rare while macrophage like leukocytes with ruffled surfaces and sometimes spreading or extending pseudopods were common (figures 11, 12). In figures 11 and 12c leukocytes can be seen in transit through the endothelial layer. The large variation in size within this group of adherent leukocytes is apparent in figure 11.

Discussion

The lesion mapping method of Cornhill and Roach (1974), which involved removing, opening, pinning, staining, and photographing the artery is very rapid and requires little equipment. However, as Roach et al. (1978) found, there is considerable distortion at branch sites, and this is the area we are most interested in. Had they used pressure fixed arteries, as we have, this distortion would have been greatly reduced. Pressure fixed vessels are the most suitable preparation for most purposes. These can be

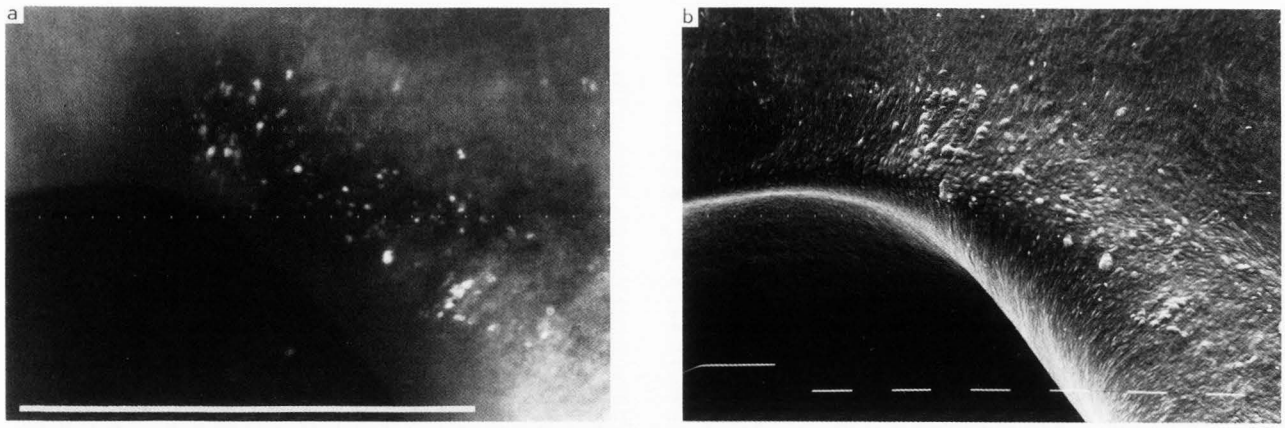


Figure 1. An 8 day plaque on the abdominal aorta at the celiac branch junction. Blood flow is bottom to top in the aorta and into the photo for the celiac. (a) is a light micrograph of wet tissue and (b) is a scanning electron micrograph of the same tissue dehydrated. Bar in 1(a) = 1.0 mm. Bars in 1(b) = 0.10 mm.

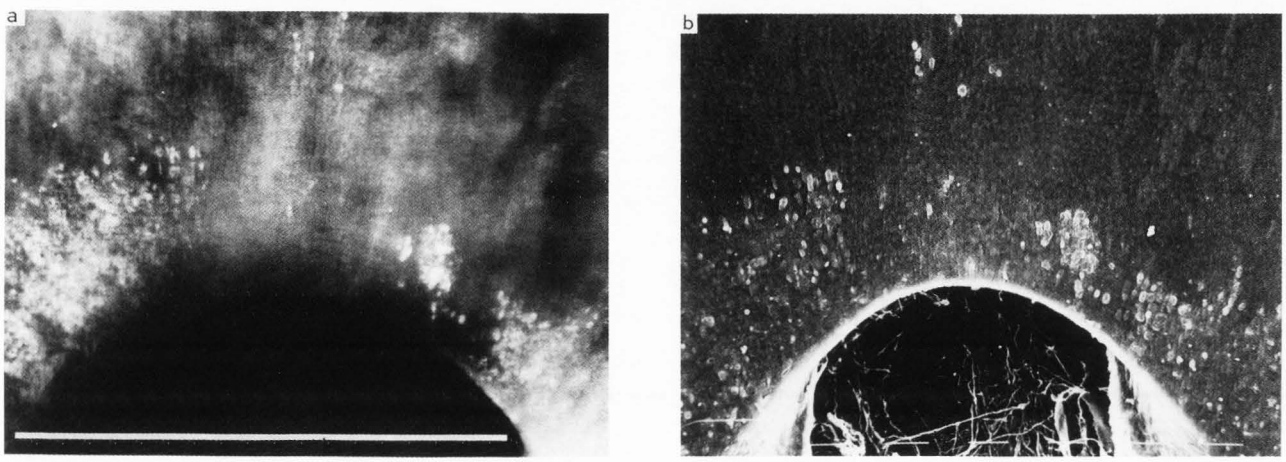


Figure 2. A 21 day plaque on the abdominal aorta at a lumbar branch junction. Explanation as for figure (1). Bar in 2(a) = 1.0 mm. Bars in 2(b) = 0.10 mm.

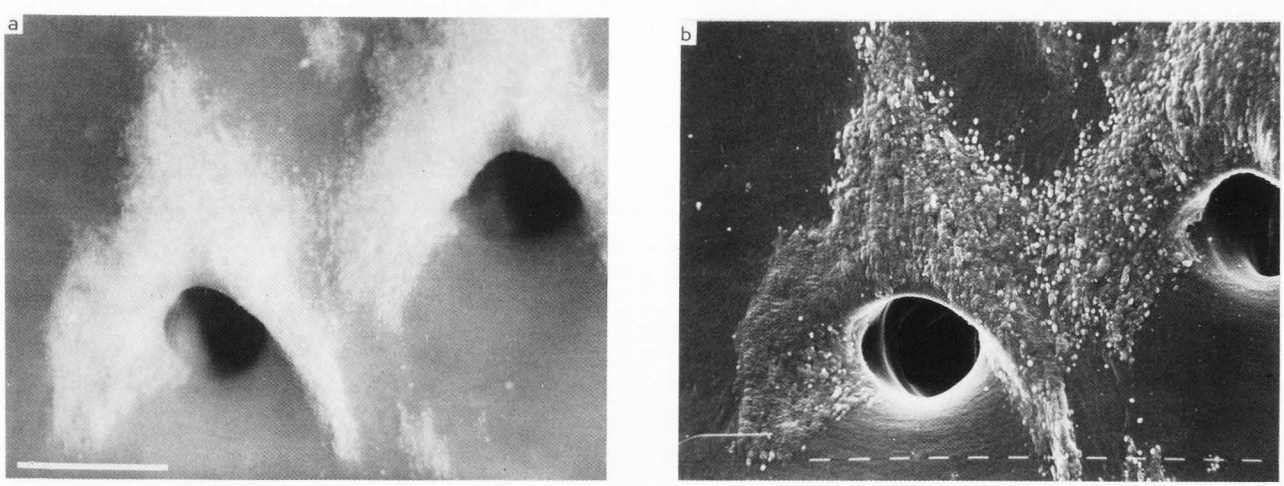
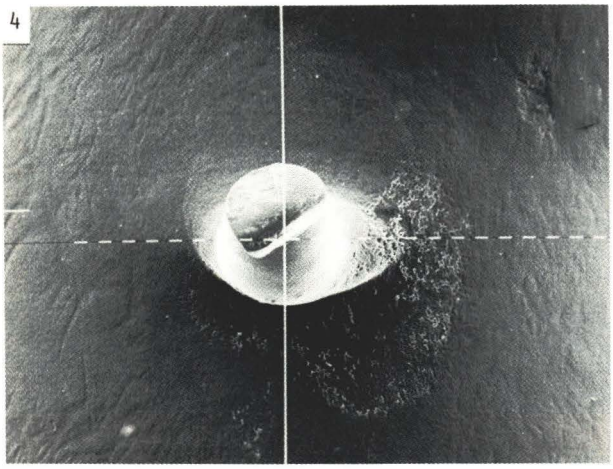
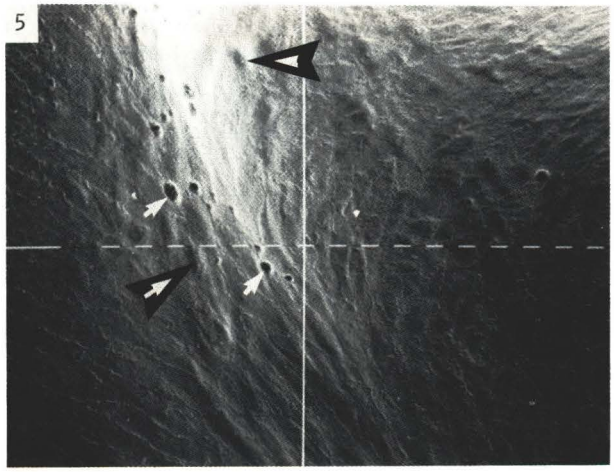


Figure 3. A 56 day plaque on the thoracic aorta at a pair of intercostal branch junctions. Explanation as for figure (1). Note the absence of plaque upstream from the orifices. Bar in 3(a) = 1.0 mm. Bars in 3(b) = 0.10 mm.





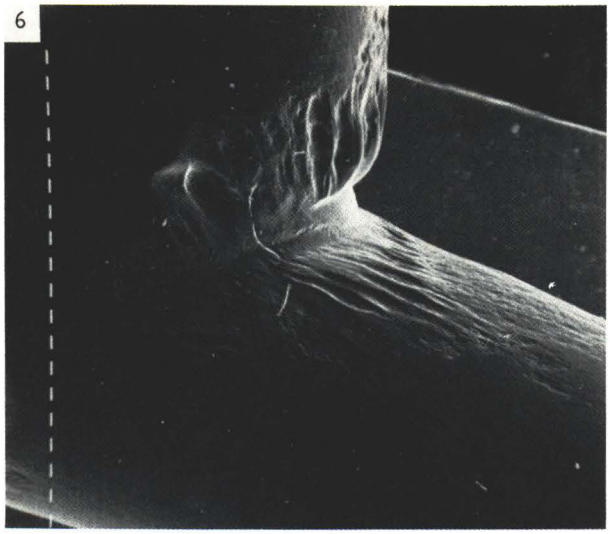


Figure 6. A 63 day cast with a distinct plaque at the distal aorto-right renal junction. Flow is to the right and up into the renal. Bars = 0.1 mm.

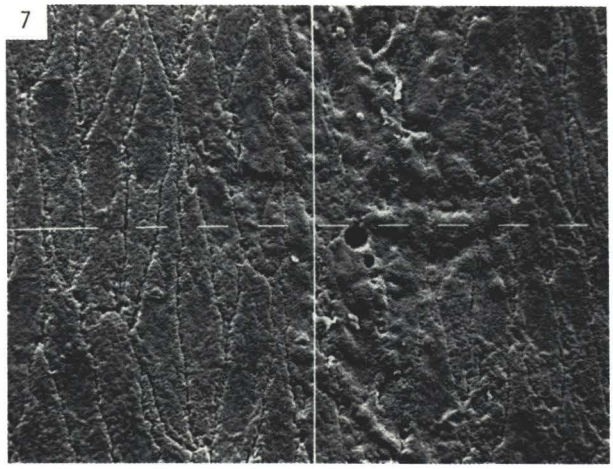


Figure 7. A nonorificial plaque just developing on a 15 day aortic cast. Normal endothelium is on the left side, while plaque is just right of center, and is indicated by the undulating surface, the presence of two adherent cells, and decreased silver staining of cell boundaries. Bars = 10 μm.

Figure 4. A scanning electron micrograph of an aortic cast from a 15 day cholesterol fed rabbit. The stump of a broken off intercostal branch extends up toward the viewer in the center. Note the plaque adjacent to the branch and the small nonorificial plaque in the upper right. Blood flow is top to bottom and up out of the page into the intercostal. Bar = 0.10 mm.

Figure 5. An 8 day aorto-intercostal junction. This is an enlarged view compared to figure 4 and rotated 90° clockwise. Blood flow in the aorta is right to left and upward into the branch. The white arrow heads indicate probable leukocyte impressions and the white and black arrow heads indicate impressions due to subendothelial cells. Bars = 10 μm.

Figure 10. An unusual cast which had erythrocyte impressions on its surface. Bars = 10 μm.

Figure 11. A 21 day plaque on aortic tissue. The white arrows indicate leukocytes and the white and black arrows bulges due to subendothelial cells. Bars = 10 μm.

Figures 12 a, b and c are adherent ruffled cells spreading and extending pseudopodia. The smooth process (S) in (c) was seen for several other such transendothelial cells suggesting it is part of the cell. The (P) indicates a possible activated platelet. The erythrocyte in (d) is for comparison. This aorta was mistakenly perfused at 180-200 mm Hg causing much of the endothelium to slough off, revealing the fenestrated internal elastic lamina with a possible thin fibrous coating of basement membrane on it. Bars = 1.0 μm.

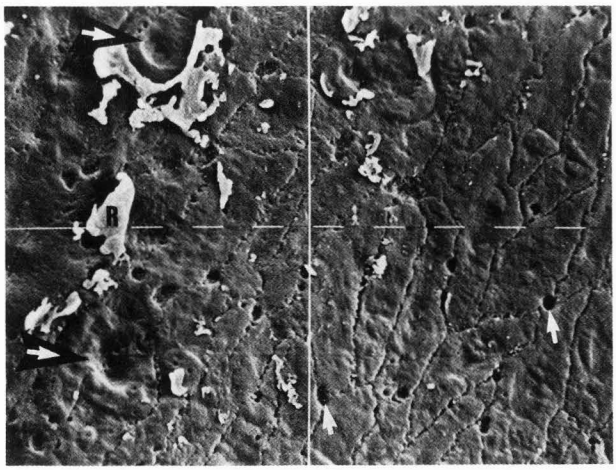


Figure 8. A closer view of a 15 day plaque on an aortic cast. White arrows indicate impressions of the adherent leukocytes and black and white arrows, subendothelial cells. The tissue residue is also shown (R). Bars = 10 μm.

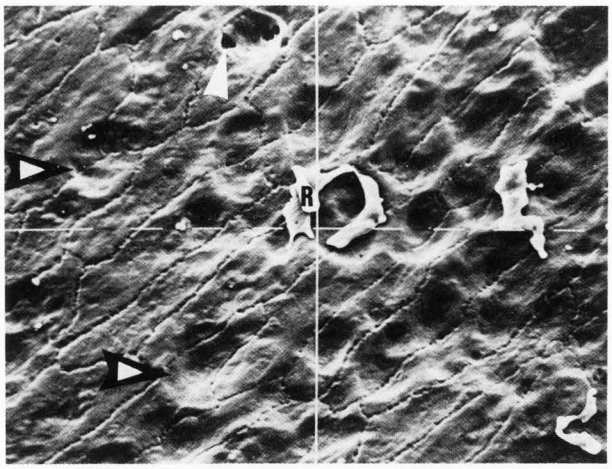
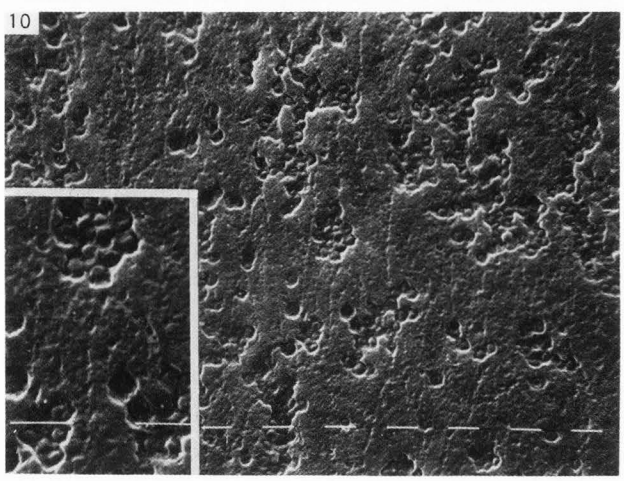
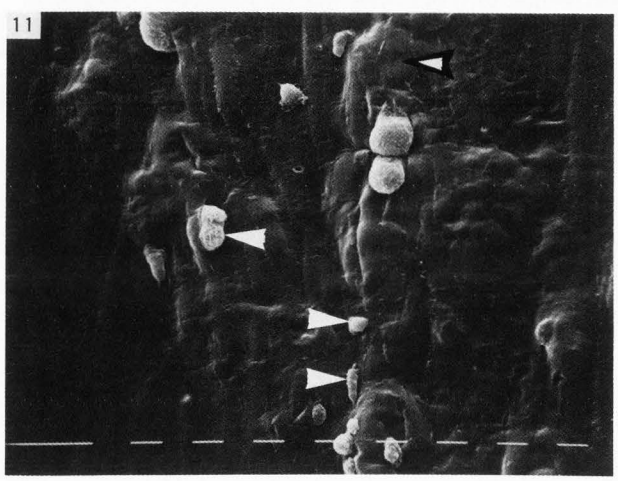
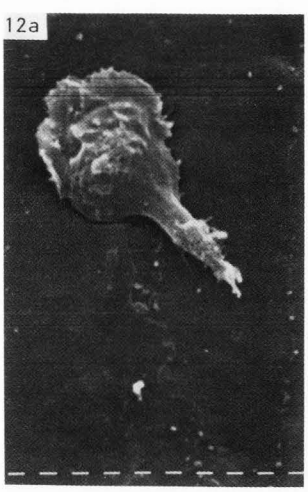
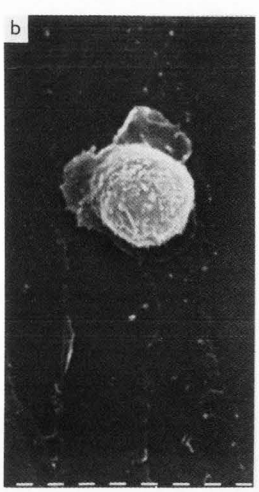


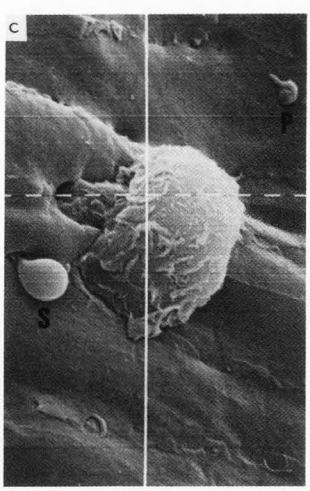
Figure 9. Same as for figure 8. The black and white arrow heads indicate depressions which are traversed by endothelial junctions (see text). Bars = 10 μm.

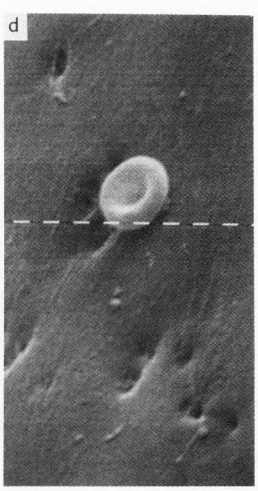












further processed for SEM, or slides can be made for LM or TEM. However, it is difficult to view some regions of the intima in branching or curving vessels without cutting and distorting the tissue, where the entire surface of casts can easily be viewed. The preservation of dimensions is comparable by casts and wet pressure fixed tissue (Kratky and Roach, 1984; Boyde et al., 1977). If the tissue is dehydrated, as in preparation for SEM, it will shrink 10-20% in each dimension (~50% by volume) (Boyde et al., 1977).

Before applying the casting method to the mapping of atherosclerotic lesions we wanted to be certain we were viewing the same lesions. A direct comparison between tissue and casts was attempted, by dissecting the tissue directly off of the cast surface, rather than digesting the tissue away. The intimal surface stuck to or was sheared on the cast surface during removal. Features could not be recognized easily on the damaged surface. Thus we made the three part comparison shown in the results.

The light photomicrographs in figures 1-3, which were produced with oblique epi-illumination, depend on differential scattering and reflection of light by fatty versus normal tissue. The SEM photomicrographic images depend on secondary electron emission from a very thin surface layer, giving images that reflect only surface contours. It is clear that individual cells seen by scattered light produced raised contours on the tissue as seen by SEM. It appears the raised contours on the tissue correspond to the depressions seen on casts. Figure 10 provides convincing evidence that red blood cells produce depressions in the casts surface. Leukocyte impressions cannot be identified with certainty. However, since red cell impressions are formed, and since leukocytes are seen on the tissue it is likely we have correctly identified leukocyte impressions on the cast surface.

The ruffled cells we have called leukocytes have been observed by others in arteries of animals on fatty diets. These cells have been identified as macrophages of monocytic origin in: swine (Gerrity, 1981), primate (Faggioto et al, 1984), pigeon (Jerome and Lewis, 1984), and rabbit (Watanabe et al, 1985). The ruffled cells we have found have the same appearance as the monocyte-macrophages shown by these authors.

Conclusion

Even though plaque was not visible to the naked eye after 8 days on diet, all three methods (LM of tissue, SEM of tissue, SEM of casts) did detect the plaques at this degree of development. The SEM of casts was probably a bit less sensitive at detecting the earliest lesions. After longer times on diet all three methods will detect the plaque equally well. The first two methods will allow one to locate the edge of the plaque at the same position. The SEM of casts should give very similar results, though some very slightly diseased regions are more likely to be excluded. By using SEM of casts in future studies of lesion location, we will be able to study the same lesions as previously with better three dimensional resolution.

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Discussion with Reviewers

M. Richardson: The illustration of the light and correlative scanning microscopy (figures 3a and 3b) show an apparent sparing of a small area immediately distal to the distal lip. Is this a predictable finding? If so would the authors comment on its significance?

Authors: Yes, it is common to observe a small region spared of plaque on the flow divider lip. Fluid dynamically a point of flow division must occur somewhere on this lip. This stagnation point will be surrounded by a small region which is exposed to low fluid shearing stresses, and we feel this coincides with the region spared of plaque. Roach, Hinton and Fletcher (1978) showed that the flow divider was often pulled down onto the aorta when the aorta was opened and pinned at its in vivo dimensions.

R. Laschi: In your experience, did you find any difference (for instance, size and shape changes) between endothelial cells lining the atheromatous lesions and the normal arterial wall?

Authors: We are currently studying this issue. So far we have found no consistent difference in size and shape of endothelial cells over early lesions versus over normal wall in these hyperlipidemic animals.