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Scanning Electron Microscope Studies of Rabbit Aortic Endothelium in Areas of Haemodynamic Stress during Induction of Fatty Streaks

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Summary. Young male rabbits were fed a diet containing 0.2% cholesterol for 4, 6, 12 and 20 weeks. At death the aortas of each animal were prepared for scanning electron microscopy (SEM) and the size of the atherosclerotic lesions surrounding the aortic ostia was measured by planimetry. Under SEM the early fatty lesions appeared as small discrete swelling of the endothelial cells. These cells were often larger than normal endothelial cells and their cell boundaries stained poorly with silver salts. Large confluent lesions were observed distal to the aortic ostia both 12 and 20 weeks after commencement of the diet but were still found to be endothelialized. No lesions however were observed immediately proximal to the entrance of an aortic branch. Haemodynamic forces, such as a high shear force, were presumably responsible for the localisation of these lesions.

Key words: Artery – Endothelium – Fatty lesions – Scanning electron microscopy.

Introduction

Injury to arterial endothelium is now considered as an important early step in atherogenesis, since loss of endothelial integrity may lead to platelet aggregation and intimal smooth muscle cell proliferation (Ross et al., 1976, 1977). Endothelial injury may be brought about by haemodynamic forces (Fry, 1969, 1973) and Scanning Electron Microscopy (SEM) has revealed that endothelial cell erosion occurs in the rabbit aorta on and distal to the flow dividers of branches coincident with areas which develop the arrow head fatty lesions induced by hypercholesterolaemia (Reidy and Bowyer, 1977a).

By SEM, early studies suggested that the endothelial surface of developing fatty streak lesions in the rabbit was injured resulting in fissures and cracks to

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the luminal surface as well as loss of endothelial cells (de Bruijn and van Mourik, 1975; Nelson et al., 1976; Weber et al., 1974). We have recently shown, however, that commonly used techniques for preparation of arterial tissue can lead to formation of artefacts. If tissue is not stained to reveal endothelial cell outlines, e.g. with silver salts, or is not fixed for a prolonged period under pressure to flatten elastic lamellae, then endothelial cell detail is obscured (Davies and Bowyer, 1975). Furthermore, when organic solvents are used to dehydrate fatty lesions, lipid may be lost causing collapse of the lesion and giving the erroneous impression that the endothelium is damaged (Davies et al., 1976; Goode et al., 1977). This is avoided by drying in air.

The purpose of this study was to measure the size and location of lesions induced in rabbit aorta by hypercholesterolaemia and to observe the endothelium over developing lesions especially in haemodynamically stressed areas. Artefact formation was minimised by prolonged fixation at physiological pressure, following silver staining, and by drying arteries without the use of organic solvents.

Materials and Methods

Experimental Design

Twenty male New Zealand White rabbits, 2–2.5 kg, 12 weeks of age at the beginning of the experiment were divided into 4 groups and all were fed a semi-synthetic diet containing 20% w/w beef tallow and 0.2% w/w cholesterol for various times as follows:

Group 1 – 4 weeks
Group 2 – 6 weeks
Group 3 – 12 weeks
Group 4 – 20 weeks

Preparation of Arterial Tissue for SEM

The preparation of tissue for SEM has been described in detail elsewhere (Davies et al., 1976; Reidy and Bowyer, 1976; Goode et al., 1977). In summary aortas were cannulated, washed with 4.6% buffered glucose solution and stained with 0.2% silver nitrate solution. The vessels were then fixed at physiological pressure with 2% phosphate buffered formalin for approximately 10 h. After fixation, each aorta was opened along its length and pinned out on a cork board. The aortas were dried in a dessicator over silica gel without prior dehydration with organic solvents. Sections of tissue were then mounted on SEM stubs, coated with gold and viewed in a Stereoscan S600.

Measurement of Lesion Size

The method is essentially as described by Reidy and Bower (1976). Similar sections of each aorta were mounted on SEM stubs and coated with approximately 30 nm of gold as described above. This procedure highlights the outlines of the arterial lesion which become clearly visible. These specimens were then photographed using a 60 mm lens on a bellow extension with angle lighting. The last paired intercostal branches, coeliac axis, superior mesenteric and both renal branches of each aorta were photographed in this fashion. From the enlarged prints of these branches, the area of the lesion surrounding each branch was measured by planimetry. Any lesion not directly involved with the ostial lesion was excluded from this measurement.

Plasma Cholesterol Determination

At the end of each dietary period, a blood sample was taken at the time of death. The serum cholesterol values were then measured on a Technicon Autoanalyzer II (Technicon, Basingstoke, Hants, U.K.).

Results

a) Plasma Cholesterol Concentrations

The plasma cholesterol values are shown in Table 1. The mean levels increased with time and there was a statistically significant difference between groups with the exception of groups 2 and 3 where the plasma values were similar.

Table 1. Plasma cholesterol values of rabbits at time of death

Time on diet	Cholesterol concentration (mg/100 ml plasma)
Before diet (20 rabbits)	52± 16
4 weeks (Group 1)	702±131[a]*
6 weeks (Group 2)	1249±206[ab]
12 weeks (Group 3)	1252±199[ac]
20 weeks (Group 4)	1994± 60[abc]

* Each value is the mean for 5 animals in the group ± the standard deviation

Values with the same alphabetic superscript are significantly different at $P<0.001$ as calcualted by a one way analysis of variance.

b) Measurement of Size and Location of Aortic Lesions Around Ostia

The mean areas of the lesions around the major ostia are shown in Table 2. For all of the ostia studied, the lesions in rabbits fed the cholesterol-containing diet for 20 weeks were significantly larger than those in animals fed the diet for only

Table 2. Showing the area of certain aortic regions covered by lesions and expressed as a percentage of the total area measured

Time on diet (Weeks)	4	6	12	20
Site on Aorta				
Intercostal branch	1.0±1.8[a]*	4.8± 4.3[b]	12.1±4.4[c]	71.6±6.3[abc]
Coeliac axis	2.6±4.1[d]	23.5±10.1[e]	29.8±3.2[df]	86.0±5.1[def]
Superior mesenteric	4.1±3.4[g]	24.4± 9,9[h]	32.0±4.7[i]	84.3±6.8[ghi]

* Each value is the mean of 5 observations ± S.D.

Values with the same alphabetic superscript are significantly different at the 95% value as calculated by the Krusal Wallis analysis of variance and the Mann-Whitney test.

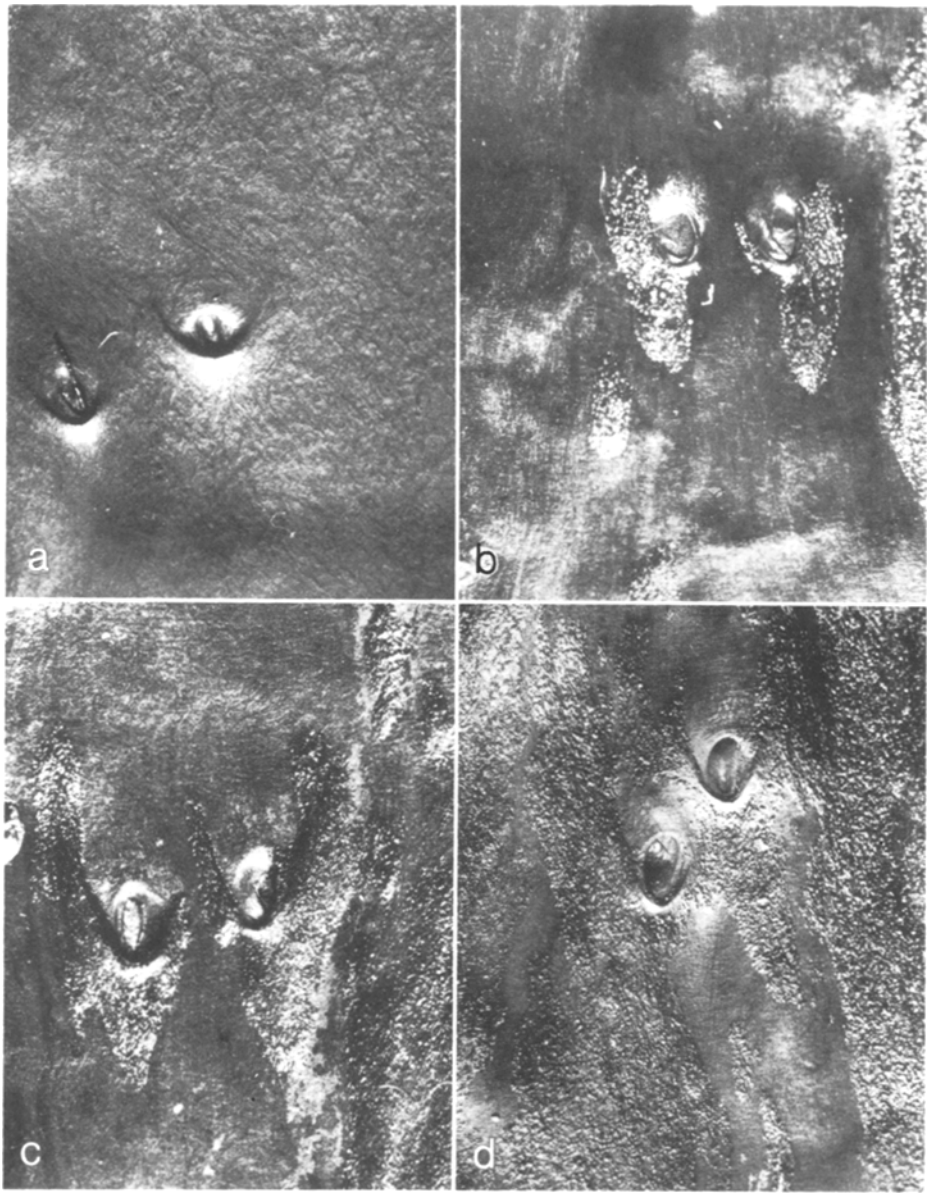

Fig. 1a–d. Macroscopic photograph of aortic fatty lesions around the intercostal branches of rabbits fed the cholesterol diet for: **a** 4 weeks; **b** 6 weeks; **c** 12 weeks; **d** 20 weeks × 5

4, 6, or 12 weeks. Figure 1 shows the location of typical lesions associated with intercostal, coeliac and mesenteric branches. In these photographs the lesions appear bright with a stippled surface. They were found distal and to the sides of flow dividers and in larger lesions extended up the aorta (Fig. 1b). In some instances, the lesion was skewed to one side (Fig. 1c). Areas proximal to openings of branches were always free of lesions.

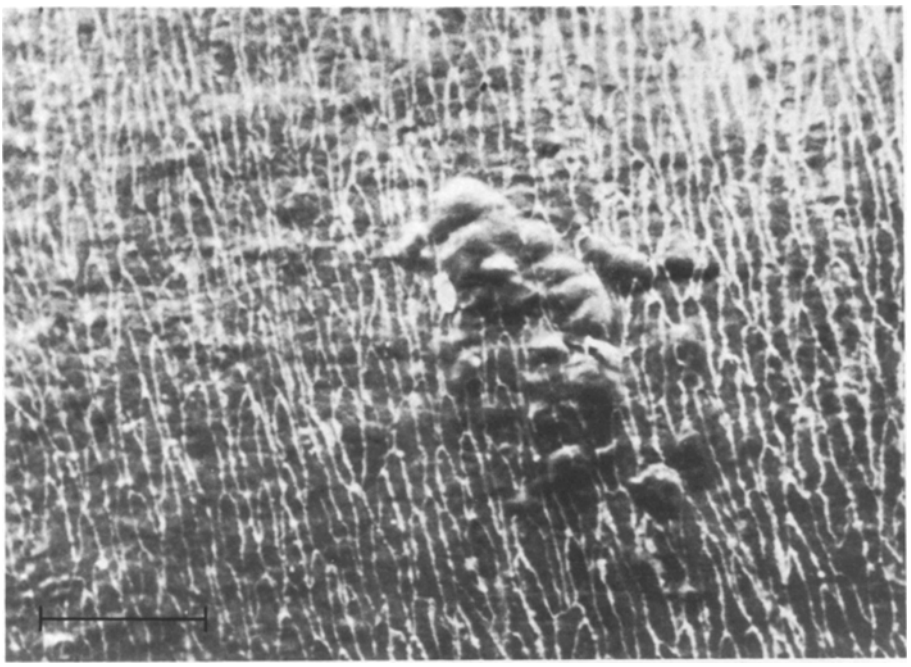

Fig. 2. Luminal surface of aorta from rabbit fed the cholesterol diet for 4 weeks. Scale Bar 100 μ

c) SEM Morphology

All figures show arteries with blood flow top to bottom.

Group 1. After 4 weeks on the semi-synthetic diet, the aortic luminal surface was completely endothelialised both at the ostia studied, and also in areas away from branches. Endothelial cell boundaries were stained with silver and the cells were orientated with their long axis in the direction of blood flow. The area immediately distal to the flow dividers stained weakly with silver. Small intimal swellings were observed around the flow dividers and the endothelial cells overlying them appeared enlarged (Fig. 2). The silver lines over these regions were thinner than those in surrounding normal tissue and occasional brightly-staining cells (argyrophilic cells) were found associated with lesions. Crystal-like structures were also found on the luminal surface of these aortas (Fig. 3), always in close association with the intimal swelling.

Group 2. The aortas from animals fed the diet for 6 weeks showed similar changes of the luminal surface to those in group 1. The area covered by each lesion was, however, larger and often involved many endothelial cells, forming a confluent lesion, especially near the flow dividers. Figure 4 shows the boundary of such a confluent lesion and it can be seen that the endothelial cells overlying the lesion were wider than the adjoining cells. They were also less argyrophilic (Fig. 5).

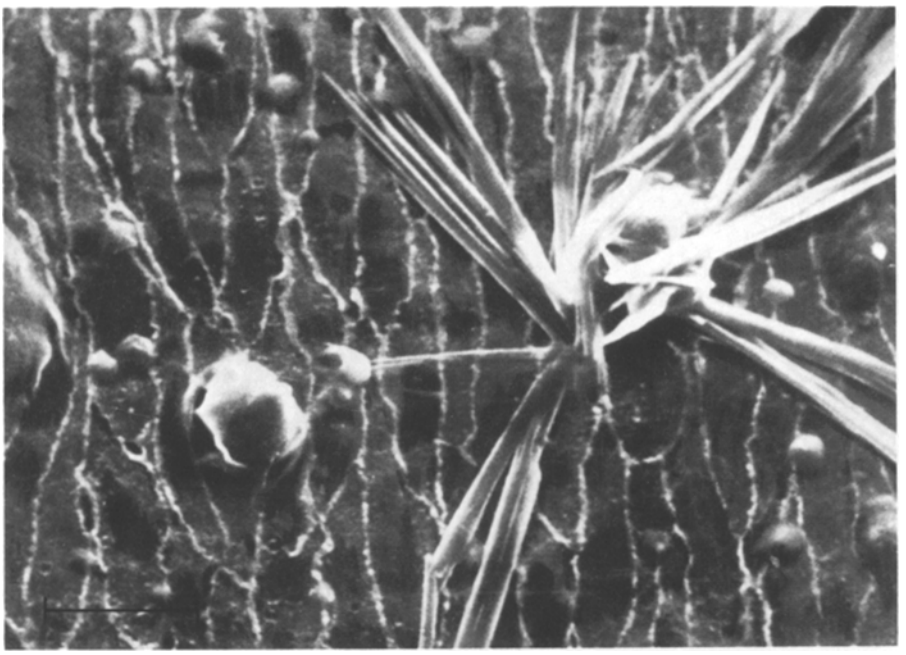

Fig. 3. Luminal surface of aorta from rabbit fed the cholesterol diet for 4 weeks. Crystal like structures and small focal swelling are present. Scale Bar 20 μ

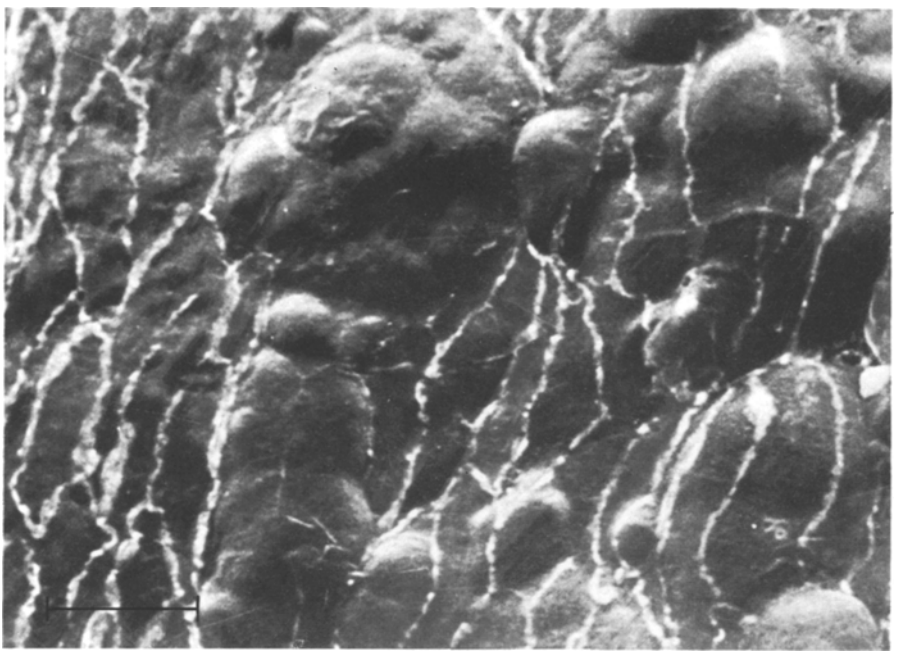

Fig. 4. Luminal surface of aorta from rabbit fed the cholesterol diet for 6 weeks. Scale Bar 100 μ

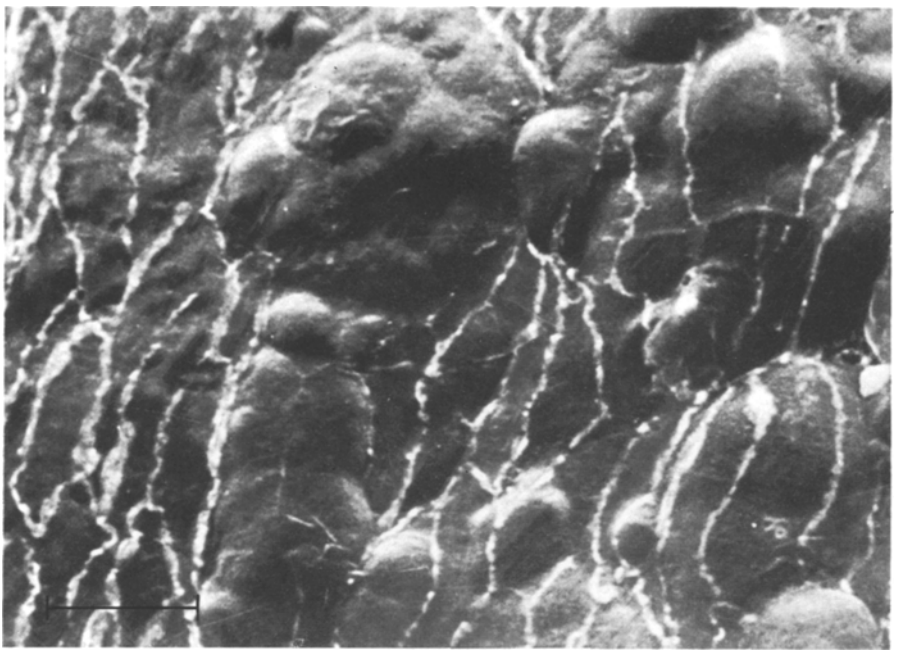

Fig. 5. as in Figure 4. Scale Bar 20 μ

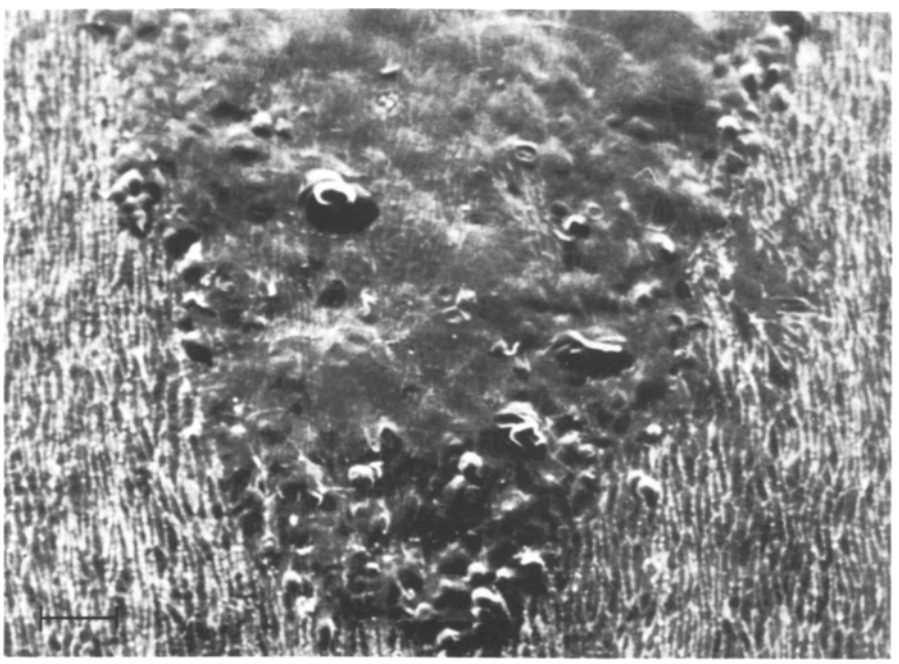

Fig. 6. Luminal surface of aorta from rabbit fed the cholesterol diet for 12 weeks. Scale Bar 100 μ

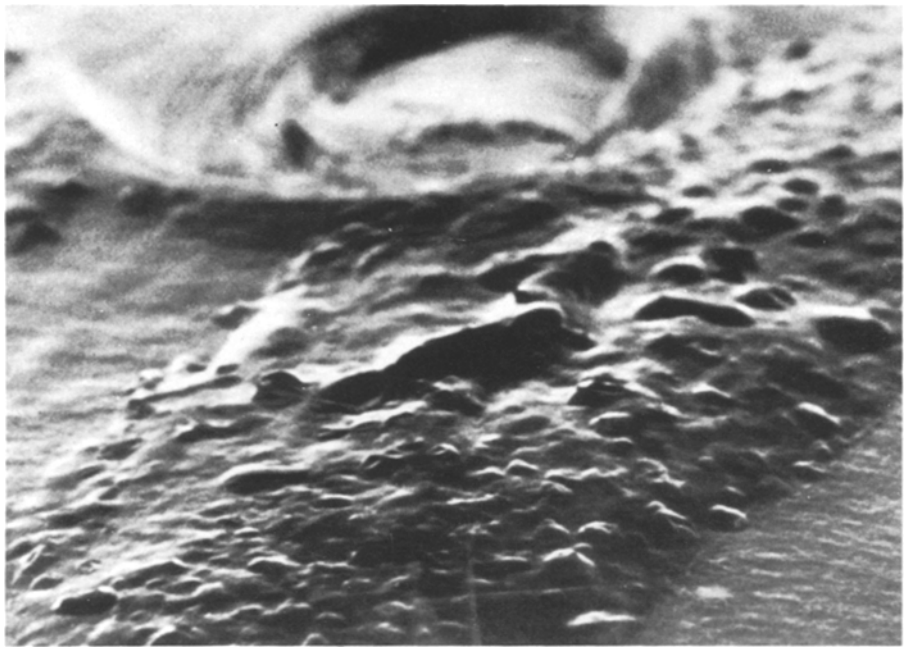

Fig. 7. Raised aortic lesion immediately distal of intercostal branch from rabbit fed the cholesterol diet for 12 weeks. ×120

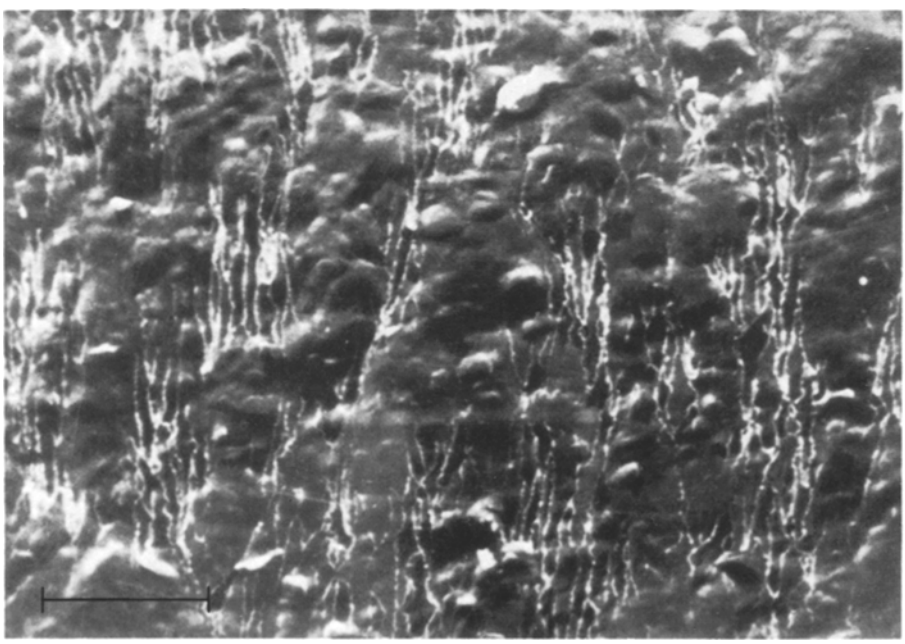

Fig. 8. Luminal surface of aorta from rabbit fed the cholesterol diet for 20 weeks. Scale Bar 100 μ

Group 3. In animals fed the diet for 12 weeks, large areas of aorta were covered with confluent lesions (Fig. 6). As in groups 1 and 2, the endothelium was still intact over lesions, but the cells were larger than normal and stained poorly. Areas around branches were more heavily involved than those distant from branches. Figure 7 shows the aortic surface distal to an intercostal ostium. In this picture, the stub was angled to 65° to the electron beam so that the raised surface of the lesion could clearly be seen.

Group 4. After 20 weeks of feeding the diet, aortic lesions were very pronounced and covered the majority of the aortic surface. A typical view is shown in Figure 8. All the lesions were covered by cells with silver-stained boundaries, although cells stained weakly and it was difficult to discern the complete boundary of some cells. The majority of cells were abnormally large. No difference in appearance was detected between cells overlying lesions away from branches and those over ostial lesions.

Discussion

In recent years evidence has accumulated to underline the importance of integrity of endothelium in atherogenesis and numerous studies have shown that mechanical and chemical injury to this layer of cells can exacerbate the formation of atherosclerotic lesions (Bjorkerud, 1974; Friedman and Byers, 1965; Harker et al., 1976; Helin et al., 1971; Lee and Lee, 1975; Minick and Murphy, 1973; Moore, 1973; Spaet et al., 1974). It has also been shown that hypercholesterolaemia leads to endothelial damage. Thomas et al. (1968) demonstrated endothelial injury and regeneration within 3 days of feeding a cholesterol-rich diet and have suggested that this is caused by a necrogenic contaminant of the cholesterol.

Early en face observations of atherosclerotic lesions, by the "Häutchen-technique", revealed that endothelial cells were intact over lesions and although they contained sudanophilic material were a normal shape (Poole and Florey, 1958). Other Häutchen studies have confirmed these findings and although unusually shaped cells were found by Silkworth et al. (1975), no loss of endothelium or obvious injury was reported. In contrast, studies by SEM have shown a wide spectrum of changes in the endothelial surface and suggested that endothelium is often absent (Weber et al., 1974; DeBruijn and van Mourik, 1975; Nelson et al., 1976).

To try to resolve why two en face techniques for viewing the endothelium should produce such contradictory results we recently investigated the possibility that early techniques for preparation of arteries for SEM might produce misleasing artefacts (Davies et al., 1976; Goode et al., 1977). These studies showed that the endothelial surface overlying fatty lesions was grossly distorted if vessels were dehydrated with organic solvents prior to drying either by air-drying or critical-point drying. When diseased tissue was dried without the use

of organic solvents, by simple air drying, however, then viewed by SEM the endothelial cells were found to be intact and did not show any obvious signs of cellular injury or necrosis.

In this study arteries were again dried without the use of solvents and an intact endothelial cover was observed. The endothelial cells, however, differed from the cells in surrounding lesions-free areas in that they were larger and often appeared swollen; similarly shaped endothelial cells over-lying lipid-filled lesions have been observed by Silkworth et al. (1975) using Häutchen preparations. The early intimal swelling may be caused by accumulation of lipid within endothelial cells, as shown by French (1966) and also by sub-endothelial oedema. With increase in severity of the lesions the swelling is obviously also due to intimal thickening by accumulating foam cells lying beneath the endothelium.

Cells overlying lesions often showed poor staining of the intercellular boundaries. This had also been demonstrated by Silkworth. It is known from studies in the rabbit by Weber et al. (1973) that the concanavalin A positive glycocallyx over endothelium varies in thickness during development of lesions at first becoming thicker, but eventually disappearing. Balint et al. (1974) have also demonstrated that in hyperlipaemic rats the endothelial surface coat is decreased in thickness and electron density as compared with controls. Such changes in glycocallyx thickness and composition may affect the binding and precipitation of silver at endothelial junctions.

The observations on the sites of development of lesions show that the earliest lesions appeared distal to the flow dividers of major branches, whilst the areas proximal were spared even in the animals fed the diet for 20 weeks. Haemodynamic forces have long been implicated as the injurious agent leading to focal lesions and Fry (1969, 1973) suggested that the lesions form in areas of high shear, such as areas distal to aortic ostia, because of damage to the endothelium. Our observations support this hypothesis for the induced atherosclerotic lesions in the rabbit. Indeed we have recently demonstrated discrete endothelial cell injury on and immediately distal to the flow dividers and the area of erosion increases with age (Reidy and Bowyer, 1977a). It should be noted, however, that as the raised lesion develops, the flow of blood distal to the divider will be disturbed. This may lead to injury to adjacent cells causing an accelerated and centripetal development of the lesion.

This study has shown that intact endothelial cells were present over fatty arterial lesions induced in the rabbit aorta by hypercholesterolaemia. The cells however were larger than normal and the cell boundaries stained poorly with silver. Furthermore the lesions were always observed on the distal aspect of aortic ostia and even with severe hypercholesterolaemia the proximal entrance to the branch was free of disease.

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