Morphology and Cell Kinetics of Fatty Streak Lesion Formation in the Hypercholesterolemic Rabbit

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The rationale for this study was to determine whether in the hypercholesterolemic rabbit any evidence of endothelial injury could be detected prior to or during the early phase of fatty intimal lesion formation. The data presented showed that in the first 12 weeks of feeding a 0.1% cholesterol-rich diet, rabbit aortas were covered with an intact endothelium. Focal areas of increased endothelial cell replication were observed adjacent to the aortic ostia at 12 and 20 weeks. These replicating cells were almost exclusively located at the shoulders of large raised lesions. In a similar fashion, adherent leukocytes were observed adjacent to the aortic ostia, and at later times they were concentrated at the periphery of these intimal lesions. Smooth muscle cell replication, as assessed by autoradiography, was found to be significantly increased only after 20 weeks of feeding the lipid-rich diet. These data suggest that an increased endothelial cell turnover and leukocyte adhesion were the first detectable changes induced by cholesterol feeding and that smooth muscle cell proliferation occurred soon after these events. (Am J Pathol 1986, 125:450–459)

THE MORPHOLOGIC EVENTS in the formation of fatty streak lesions in experimental models of atherosclerosis have been extensively studied in many species.[1-6] Contrary to earlier reports that suggested that smooth muscle cell (SMC) proliferation in lesions was initiated by loss of endothelial cells, it has now been shown that evidence of endothelial cell loss does not occur prior to the formation of early intimal lesions.[1-4,6] These data, however, cannot be interpreted to mean that endothelial injury did not occur, because several studies have shown that endothelial injury and desquamation can occur without any detectable breaks in the integrity of the endothelium.[7-10]

With the above data in mind, the aim of this study was to reevaluate the effect of cholesterol feeding upon vascular endothelium of rabbits and to determine whether any changes were present prior to or during the formation of intimal fatty streaks. A similar approach has been undertaken by several other authors, who have reported some but not all of the parameters in differing species during fatty lesion formation.[11-16] For example, Scott et al[15] recently reported changes in medial cell proliferation of swine lesions without any change in endothelial cell replication, whereas Jerome and Lewis[13] have shown that pigeon leukocytes adhere to areas with replicating endothelium. Whether this was related to any smooth muscle replication was not determined. Likewise, Hansson and Bondjers obtained data on increases in endothelial cell replication and leukocyte adhesion but did not relate these findings to proliferation of vascular smooth muscle cells.[11] In this study, our aim was to try to seek evidence of the sequence of cellular events that are important in intimal lesion formation. In particular, we wish to report on the integrity and replication of the vascular endothelium, the location of adherent leukocytes, and the proliferation of SMCs in areas of the aorta that are known to develop fatty intimal lesions.

Materials and Methods

Twenty-five New Zealand White rabbits (Redfern strain) were used in this study. Animals were fed a semi-

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synthetic diet[6] containing 0.1% cholesterol for 0, 4, 6, 10, and 20 weeks; 5 animals were observed at each time point. Serum cholesterol levels were determined every second week[17] and high or low responders to the diet were discarded from the experimental group. A total of 33 animals were placed on the diet, 5 animals were found to be high-responders and 3 were low-responders (abnormal responders were defined as those whose serum cholesterol was equal to or greater than 40% from the mean). Serum cholesterol levels of all animals were maintained below 450 mg/dl (Table 1) by decreasing the cholesterol supplement as required (2 animals at the 20-week time point were fed the semisynthetic diet without added cholesterol for weeks 12–14; they were then put back on the 0.1% cholesterol supplement for the remainder of the experimental period).

The animals were given intravenous injections tritiated thymidine (0.5 mCi/kg body weight; 6.7 mCi/mmol, New England Nuclear) 17, 9, and 1 hour prior to death. They were perfused with fixative (2% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) via the left carotid artery at 37 C, 100 mm Hg for 1 hour. The aorta was then excised and immersed in fresh fixative for a minimum of 12 hours.

The thoracic aorta was cleaned of adventitia and opened longitudinally. Segments of tissue were prepared for scanning and transmission electron microscopy and autoradiography (both cross-sections and *en face*) as shown in Figure 1.

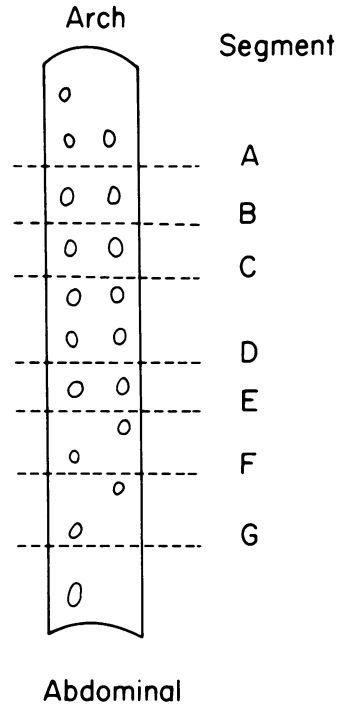

Figure 1—Diagrammatic view of the thoracic aorta. Segments A and D were prepared for scanning and transmission electron microscopy. Segments B, E, and G were used for *en face* autoradiography; segments C and F were used for cross-sections.

Scanning Electron Microscopy

Two pieces of tissue from each animal (segments A and D, Figure 1) were prepared for scanning electron microscopy. Tissue for scanning electron microscopy was prepared with a modification of the osmium–thiocarbohydrazide–osmium procedure.[18] In brief, tissue was treated with 1% osmium tetroxide for 2 hours, 1% thiocarbohydrazide for 30 minutes, followed by 1% osmium tetroxide for 1 hour. Tissue was then critical-point-dried from liquid CO_2 after dehydration in ethanol. Tissue was mounted on aluminum stubs, sputter-coated with 40 Å gold, and viewed in a JEOL JSM35C scanning electron microscope at 15 kv.

Table 1—Serum Cholesterol Levels of Rabbits Fed a Semisynthetic Diet Containing 0.1% Cholesterol

Time on diet (weeks)	Serum cholesterol mg/dl (± SD)
0	87.3 ± 12.2
4	280.0 ± 35.1
6	218.1 ± 47.3
10	320.5 ± 89.5
20	333.4 ± 62.0

The number of leukocytes adhering to endothelium was also assessed from two pieces of tissue from each animal after 20 weeks on the diet (A and D Figure 1). The number of adherent cells in a microscopic field (×400) was quantitated only for areas with raised intimal lesions. In four separate segments of aortic tissue, the location of these cells was plotted with respect to the outline of the intimal lesion.

Transmission Electron Microscopy

Two intercostal flow dividers from each animal were prepared for transmission electron microscopy (one from segment A and one from segment D, Figure 1). Care was taken to ensure that orientation of blood flow was known for each piece of tissue. Tissue for transmission electron microscopy was embedded in Spurr low-viscosity resin, and sections were cut downstream of the flow divider. Several sections were obtained at each of a series of 150 μ intervals up to the edge of the flow divider. Sections were stained with uranyl acetate and lead citrate and were viewed in a Philips EM300 electron microscope at 80 kv.

Autoradiography of Cross-sections

Two pieces of tissue from each aorta (segments C and F, Figure 1), which included tissue approximately 2 mm

above and below the ostia, were used for preparation of cross-sections. Tissue was embedded in methacrylate resin, and sections were cut at 200-μ intervals. For quantitation, a section from each of three 200-μ intervals was used from each block of tissue, so that six sections per animal were prepared. Sections were dipped in Kodak NTB2 emulsion and developed after storage for 2 weeks at 4 C. Sections were then counterstained with hematoxylin. The numbers of labeled and unlabeled SMCs, above and below the internal elastic lamina (intima and media, respectively), were counted for each section. For all autoradiographic studies, a labeled cell was defined as one that had more than three grains of silver over the nucleus. The labeling index for cross-sections was calculated as follows:

$$\text{labeling index} = \frac{\text{number labeled cells}}{\text{total number cells}} \times 100\%$$

En Face Autoradiography

Three pieces of tissue from each animal (segments B, E, and G, Figure 1) were prepared for *en face* autoradiography. Two different methods of tissue preparation were used for *en face* autoradiography, namely, Hautchen preparations and scanning electron microscopy autoradiography (SEM-autoradiography). Hautchen preparations of the endothelium, prepared as previously described,[19] were exposed to NTB2 emulsion, developed, and counterstained with hematoxylin. Labeled cells were then mapped by use of a microscope with a graticule. One field was the area covered by a 10 × 10 reticule at ×400 magnification, and the number of labeled cells per field was plotted on a map of the Hautchen. To estimate total cell number, we determined the mean cell number (average cell density) from 15 random fields and this was then multiplied by the total number of fields observed. A 10 × 10 field area was counted and mapped around two ostia per animal observed.

Tissue for SEM-autoradiography was washed in glycine (0.1 M) in phosphate-buffered saline (PBS) for 2 days and was then critical-point-dried after dehydration in ethanol. Dried tissue was exposed and developed as described above, and after development the tissue was allowed to air-dry. Tissue was then mounted for scanning electron microscopy, sputter-coated with gold, and observed at 15–20 kv. Tissue was counted and

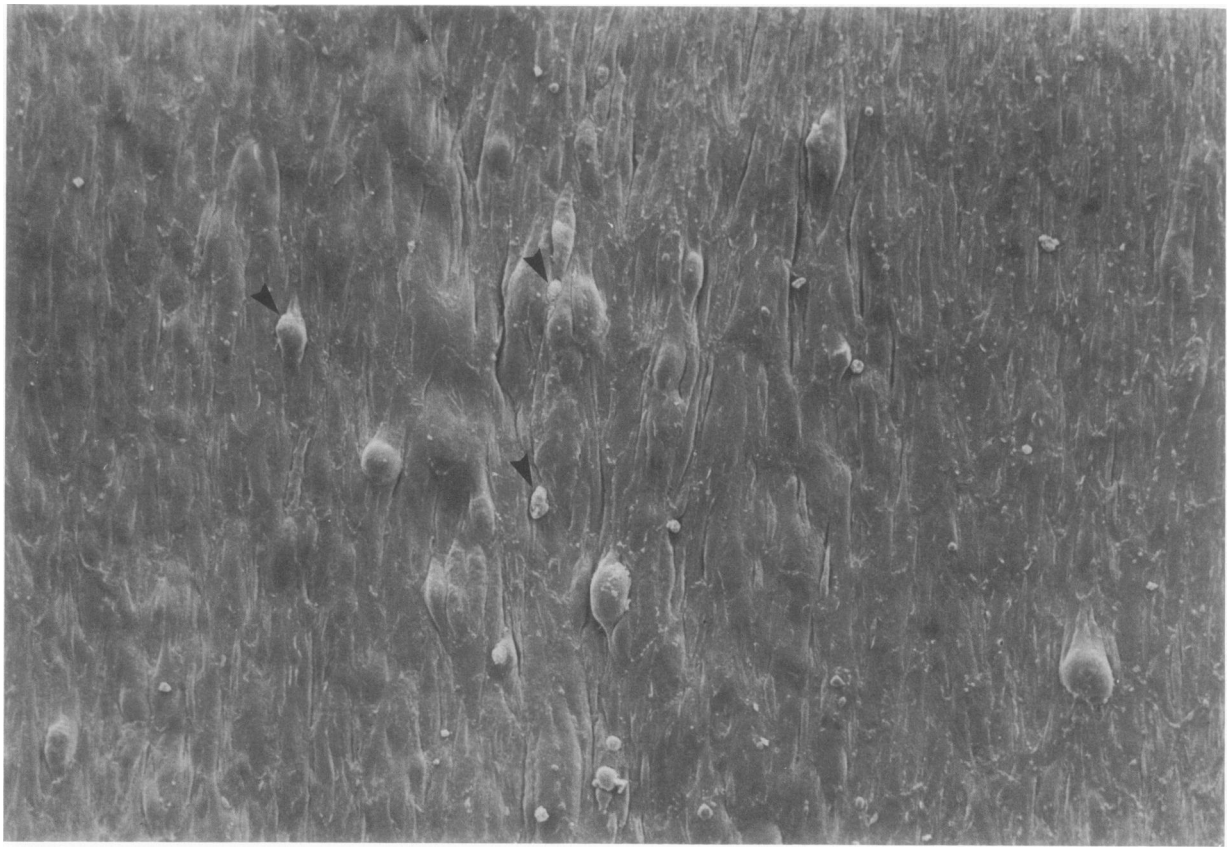

Figure 2—Scanning electron micrograph of thoracic aorta 10 weeks after initiation of the cholesterol-supplemented diet. The area shown is immediately down stream of a flow divider. Small raised areas with adherent leukocytes (*arrowheads*) can be seen adhering to the luminal surface. (×580)

mapped as for Hautchen preparations, one field being the area of the scanning electron microscope viewing screen at ×600 magnification. A labeled cell for SEM-autoradiography was defined as one that had 10 or more grains of silver over the nucleus.

Results

Morphology

Observation of tissue from control animals showed that the endothelium was a continuous monolayer of cells with overlapping junctions. None of the control tissue examined showed evidence of platelet or leukocyte attachment. Cross-sections of control tissue showed the occasional isolated cells within the intima of the vessel which were presumed to be smooth muscle cells by transmission electron microscopy as determined by the presence of dense bodies, microfilament bundles, and a basement membrane.

No changes in endothelial cell surface morphology were observed at 4 and 6 weeks after initiation of the diet. At 10 weeks, small raised areas involving two to three endothelial cells (Figure 2) with occasional adherent leukocytes were seen around the ostia. Transmission electron microscopy revealed isolated lipid-laden cells within the intima of the vessel (Figure 3), which did not show any of the morphologic characteristics of SMCs and frequently possessed cytoplasmic extensions. Although morphologic criteria alone do not allow positive cell identification, these isolated intimal cells resembled leukocytes as described elsewhere in other studies of hypercholesterolemia.[3,4,20,21] A further change at this time was the appearance of intimal thickening around some ostia, with no cells adherent to the endothelium. Cross-sections through these areas showed increased numbers of intimal cells, which did not contain lipid droplets (Figure 4) and which were tentatively identified as SMCs with the use of the morphologic criteria described above.

Twenty weeks after initiation of the cholesterol-supplemented diet, focal fatty streak lesions were observed around flow dividers in 4 of the 5 animals observed. The lesions were covered by a morphologically intact endothelial cell layer as determined by scanning and transmission electron microscopy (Figure 5). Only 1 animal showed any evidence of loss of endothelial integrity, and the area covered by lesions in this animal

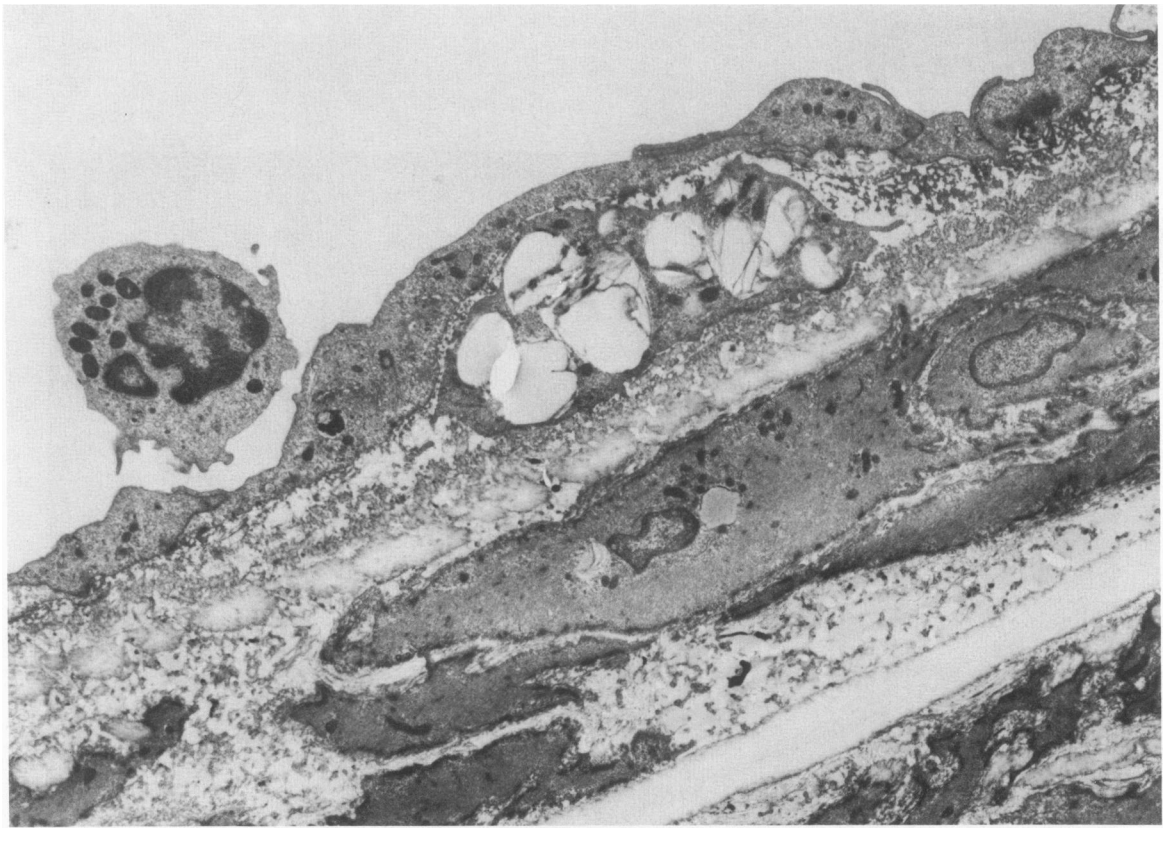

Figure 3—Transmission electron micrograph of a lipid-laden cell directly beneath the endothelium in an area with minimal intimal thickening after 10 weeks on the diet. The cell has no dense bodies, has many pseudopodia, and is heavily laden with lipid. (×4700)

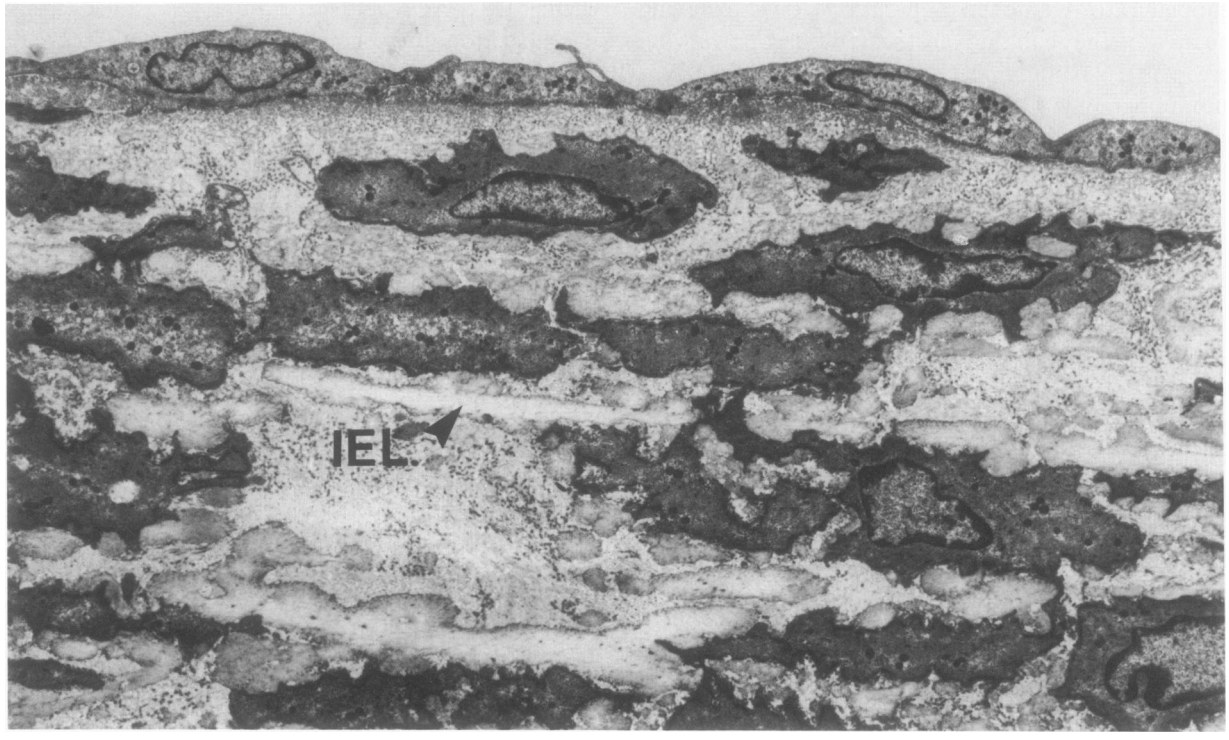

Figure 4—Transmission electron micrograph of thickened intima beneath intact endothelium after 10 weeks on the diet. The cells within the intima appear to be composed entirely of SMC and cells can be seen between fenestrae of the internal elastic lamina (*IEL*). (×5800)

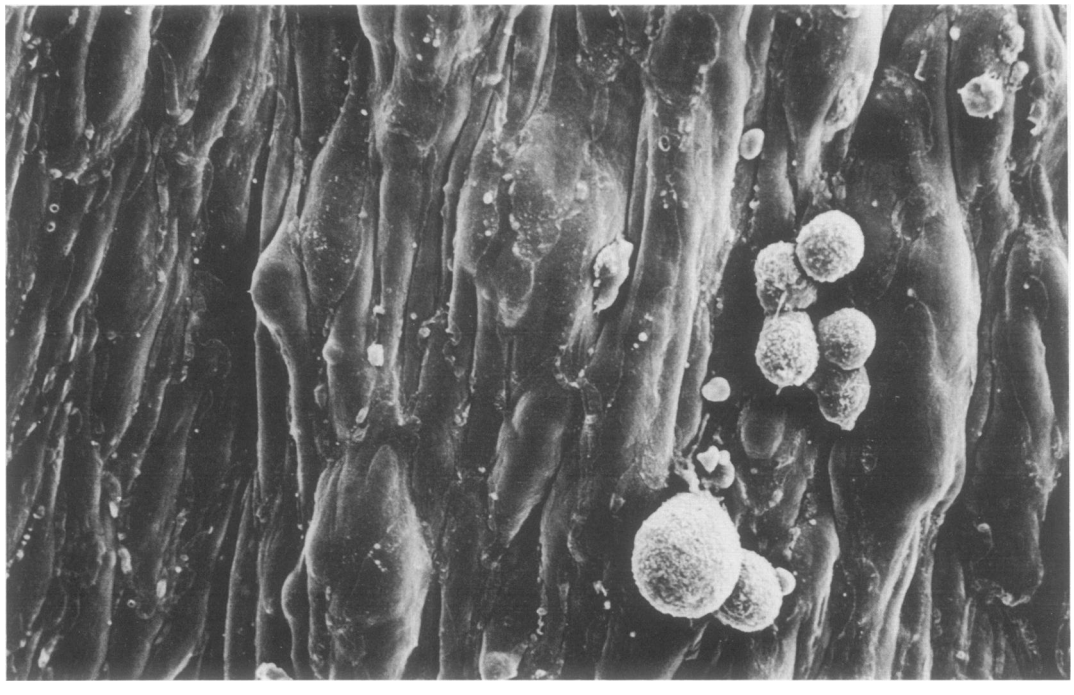

Figure 5—Scanning electron micrograph of the edge of a lesion after 20 weeks on the diet. There are adherent leukocytes on the surface, but no platelets. The endothelium is completely intact, with no exposure of the subendothelium. (×600)

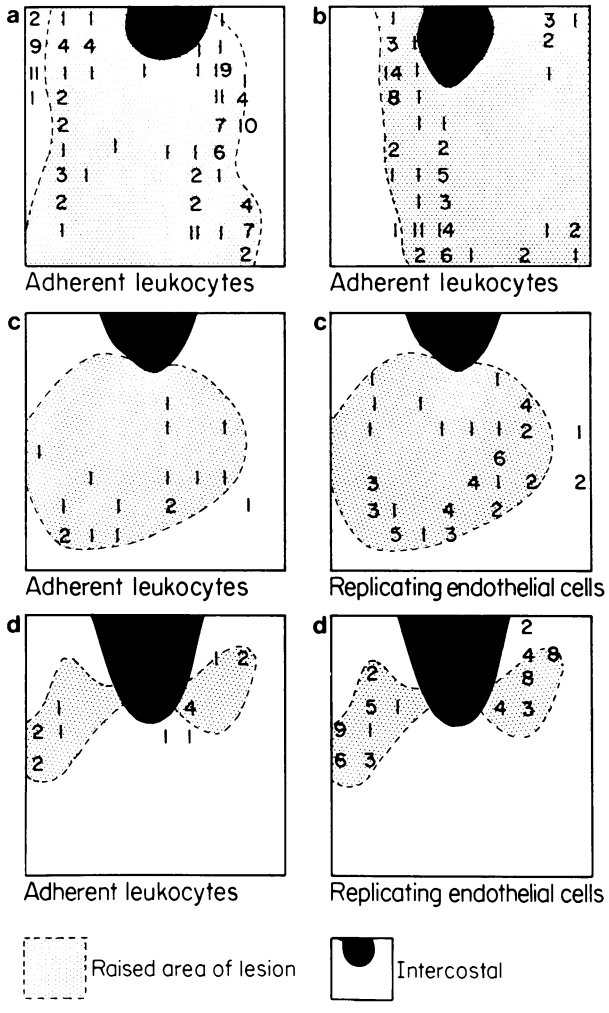

Figure 6—Map of leukocyte adherence (**a–d**) and endothelial cell replication (**c**, and **d**) on thoracic aorta after 20 weeks on a cholesterol diet. The areas shown are the region downstream of and around intercostal arteries. The number of adherent cells or replicating endothelial cells was counted at ×400, and the size of the field was approximately 5 × 5 mm as drawn. Areas with no adherent leukocytes nor replicating endothelium are left blank.

was more extensive than in any other. Breaks between endothelial cells in this animal contained leukocytes but did not expose any recognizable thrombogenic surfaces. It was common to find leukocytes adhering to apparently intact endothelium at this time. The location of these adherent cells, however, was not random and occurred at certain anatomic sites, namely, in close proximity to the raised intimal lesion. Occasional single leukocytes were observed throughout the aortas, but clusters of adherent cells (>2) were observed only in close approximation to the ostia. The number of these adherent cells was quantitated, and at 20 weeks the average number of adherent cells per field (×400) was 3.3; values ranged from 1 to 19 cells. These adherent leukocytes were almost invariably located over raised intimal lesions of the aortic ostia and most often were found preferentially at the edges of such lesions. An example of this distribution in four separate segments of aorta is shown in Figure 6.

At 20 weeks, the cross-sections of aortic lesions showed that many intimal cells contained lipid, whereas no lipid accumulation was evident within medial cells. Intimal cells closest to the lumen of the vessel showed the most extensive intracellular lipid accumulation, and it was not always possible to distinguish whether these cells were leukocytes or SMCs on a morphologic basis (Figure 7). Cells at the base of the lesion contained less lipid and were readily identified as SMCs on the basis of their morphologic characteristics (Figure 7).

Cell Replication

Endothelial cell replication was measured both from Hautchen preparations and from SEM-autoradiograms. SEM-autoradiography was particularly useful for quantitation of endothelial cell replication at 20 weeks; Hautchen preparations were difficult to prepare and count because of contamination with subendothelial cells and adherent leukocytes. In terms of obtaining endothelial cell replication, these *en face* techniques are identical. An example of such an autoradiograph is shown in Figure 8. Labeled endothelial and adherent leukocytes can be seen adhering focally to a shoulder of an intimal lesion.

The position and number of labeled endothelial cells within a 10 × 10 field area (approximately 11,000 cells) around and downstream of two intercostal flow dividers were quantitated for each animal at each time point (Table 2). One-way analysis of variance (ANOVA) of the endothelial labeling index in this area showed a significant increase in the labeling index after 20 weeks on the diet. Maps of labeled endothelial cells, however, suggested there were small focal areas of increased endothelial replication around the ostia after 10 weeks (Figure 6); yet the calculated thymidine indices of these entire areas were not significantly different from the controls (Table 2). Therefore, cell replication was expressed on a per-field basis. The number of fields containing two or more labeled endothelial cells was expressed as a percentage of the total number of fields for each 10 × 10 field area. ANOVA of the number of fields containing two or more labeled cells showed a significant increase at 10 and 20 weeks as compared with control animals (Table 2).

Observation of cross-sections showed that after 20 weeks on the diet there was a significant increase in DNA synthesis by medial and intimal smooth muscle cells (Table 3, analyzed by ANOVA). Because of intracellular lipid accumulation, it was not always possible to dis-

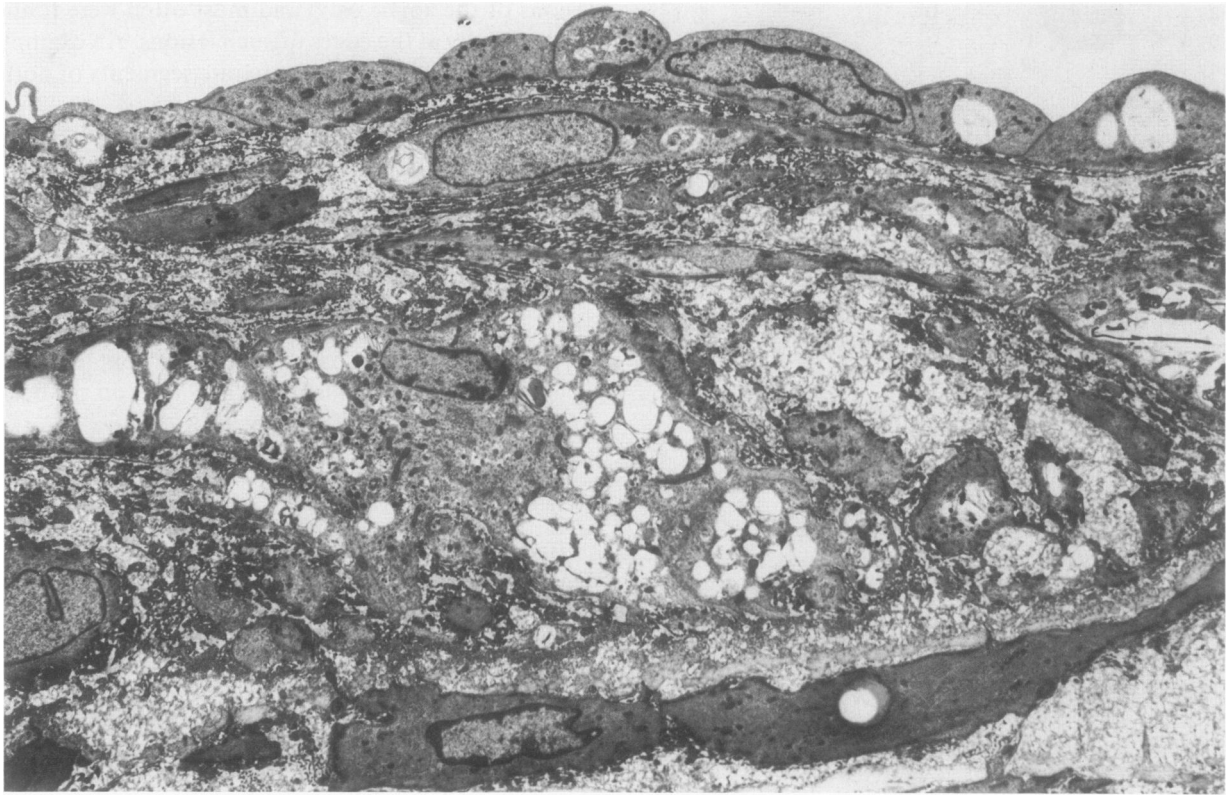

Figure 7—Transmission electron micrograph of a lesion at 20 weeks on the diet. Cells at the luminal side of the lesion contain large amounts of intracellular lipid. Cells at the base of the lesion contain little or no lipid and can be readily identified as SMCs (×6500)

tinguish between intimal leukocytes and SMCs close to the luminal surface of the lesion, but many of these labeled cells were at the base of lesions and resembled SMCs.

Discussion

Diet-induced hypercholesterolemia in the rabbit induces the formation of characteristic fatty streak lesions at predictable sites in the thoracic aorta.[5,6] The level of hypercholesterolemia in this study was controlled to eliminate extreme responses to dietary challenge which may produce different rates of morphologic change in fatty streak formation. Observations were made at the same position in the arterial tree for each animal, as different sites showed different rates of lesion progression.[22] These precautions enabled us to observe the sequence of events in the development of fatty streak lesions in the rabbit, namely, increased endothelial replication, leukocyte adhesion, and SMC replication.

We found that cholesterol feeding induced focal areas of increased endothelial cell replication downstream from intercostal flow dividers. This is where fatty streak lesions form with increasing duration of the cholesterol-supplemented diet. This focal increase in endothelial replication was observed prior to any autoradiographic evidence of either intimal or medial SMC proliferation and without any exposure of subendothelium. Other investigators have also found an increase in endothelial cell replication in close proximity to intimal lesions[11,12,14] and have suggested that a possible transient loss in the endothelial cell barrier might be responsible for this increase.[14] Although we did not ob-

Table 2—Labeling Index of Aortic Endothelial Cells Around Intercostal Ostia

Time on diet (weeks)	Mean labeling index* (± SD) (n = 10)	% field with† ≥2 labeled cells (± SD)
0	0.038 (± 0.027)	0.308 (± 0.496)
4	0.025 (± 0.025)	0.432 (± 0.563)
6	0.032 (± 0.044)	1.088 (± 1.990)
10	0.132 (± 0.181)	3.854 (± 5.297)‡
20	1.282 (± 1.318)§	23.381 (± 25.309)§

* Calculated as $\frac{\text{number of labeled cells}}{\text{total number of cells}} \times 100\%$.

† Calculated as $\frac{\text{number of fields containing} \geq 2 \text{ labeled cells}}{\text{total number of fields}} \times 100\%$.

‡ $P < 0.05$.

§ $P < 0.01$.

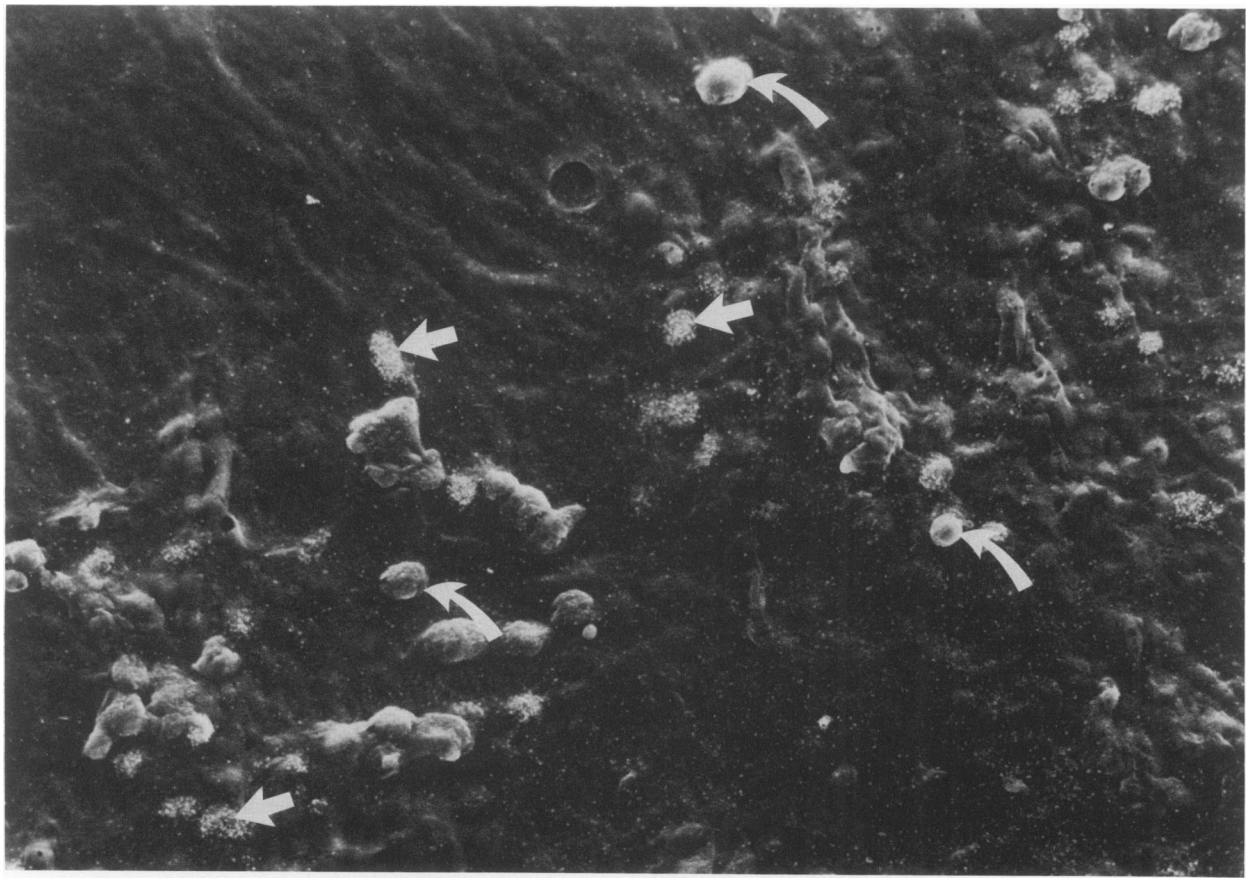

Figure 8—SEM autoradiography of the edge of a fatty streak lesion after 20 weeks on the cholesterol diet. Blood flow is from top right to bottom left; the lesion occupies the lower right portion of the micrograph. Labeled endothelial cells (*short arrows*) and adherent leukocytes (*arrows*) lie almost exclusively along the shoulder of the lesion. (×540)

serve any defects in the integrity of the endothelium, it is always possible that small focal defects could be missed with standard morphologic techniques. We considered this unlikely because in other models of vascular injury extensive endothelial cell loss can occur from the vessel wall without exposure of the subendothelium[9,10] and because not many other investigators[2-4,6,13,15,20] have observed any breaks in the integrity of the vessel wall in the early stages of lesion formation. These data would suggest that cholesterol feeding initially induces a desquamating, but not a denuding, endothelial injury.

Table 3—Labeling Index of Aortic Smooth Muscle Cells in Close Proximity to Intercostal Ostia

Time on diet (weeks)	Number sections	Intimal index (± SD)	Media index (± SD)
0	30	0.0 (± 0.0)	0.016 (± 0.024)
4	30	0.0 (± 0.0)	0.032 (± 0.051)
6	30	0.025 (± 0.137)	0.024 (± 0.044)
10	30	0.0 (± 0.0)	0.010 (± 0.057)
20	30	0.885 (± 1.611)*	0.073 (± 0.136)†

* $P < 0.005$.
† $P < 0.05$.

Coincident with increased endothelial cell replication around the ostial lesions was the finding of leukocytes present at these same sites. Other workers have also observed this fact,[11,12] although a recent study by Scott et al found no increase in leukocyte adhesion in cholesterol-fed swine.[15] In this study, it was not possible to determine whether endothelial cell replication or leukocyte adhesion was the initial event in lesion formation, but the results of this study did suggest that these events occur prior to any detectable increase in SMC replication. In light of the absence of any platelet-derived mitogen, these data have important implications, because they suggest that these two cell types, ie, endothelium and adherent leukocytes, may play a role in initiating the development of the fatty intimal lesion. Several possible pathways could be suggested whereby these cells participate. *In vitro* endothelial cells can synthesize relatively large amounts of a platelet-derived growth factor (PDGF)-like molecule,[23] and there is some evidence to suggest that this also occurs *in*

vivo.[24] Why endothelial cells would start to synthesize this mitogen is unclear, but work by Fox and DiCorleto[23] does show that at least *in vitro* an injurious event, such as produced by endotoxin, does stimulate PDGF-like production. Studies have shown that macrophages also synthesize a PDGF-like molecule.[25] PDGF is also known to be a chemotactic agent for monocytes[26] and SMCs.[27] Furthermore, there is evidence to show that endothelial cells themselves promote the adherence of monocytes by production of other chemotactic factors[28,29] or by unknown mechanisms.[30-32] Of recent interest is the study of Bevilacqua et al,[33] which shows that interleukin-1 strongly promotes leukocyte adherence to endothelium. Interestingly, several laboratories have now shown that endothelial cells can be induced to synthesize interleukin-1, again often by injurious agents.[34,35] Thus, there are several possible pathways both for the production of SMC mitogen within the intimal lesion itself and for the production of chemotactic agents which in turn recruit circulating cells, as well as SMCs, into the lesions. An important issue, as yet unresolved, is whether *in vivo* some form of injury to endothelium initiates all or some of these events.

In summary, this study has shown that the formation of fatty streak lesions in the rabbit is associated with endothelial cell injury, as demonstrated by increased endothelial cell replication. This injury does not involve exposure of the subendothelium or platelet adhesion. It is, however, associated with leukocyte adhesion followed by subsequent migration of SMCs from the media into the intima, intracellular lipid accumulation by intimal cells, and both intimal and medial SMC proliferation.

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