The Role of the Monocyte in Atherogenesis

II. Migration of Foam Cells From Atherosclerotic Lesions

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A defined role in the atherogenic sequence is proposed for the circulating monocyte. The author has been able to demonstrate a "monocyte clearance system" in which large numbers of circulating monocytes invade the intima of lesion-prone areas in arteries, become phagocytic, and accumulate lipid. A fatty cell lesion results. Once lipid-laden, foam cells migrate back into the bloodstream by crossing the arterial endothelium. The ratio of penetrating monocytes to emerging foam cells decreases as fatty cell lesions develop until a one-to-one ratio is achieved in late fatty cell lesions, which do not progress further. Advanced fibroatherosclerotic plaques in the same animals do not show the same characteristics and have smooth muscle cell involvement. It would appear that advancement of the lesion is at least partially a result of failure of the monocyte clearance system to remove sufficient lipid. The invasion of monocytes and endothelial damage caused by foam cell clearance may, in late fatty lesions, contribute to plaque evolution by introducing growth factors from macrophages and platelets and allowing greater lipid influx. Elucidation of this system was facilitated by the examination of vessels from diet initiation onwards and by the observation of late nonprogressing fatty cell lesions. It is possible that this system exists in other models but has been overlooked by a predilection for the study of advanced lesions that prevails in the literature. (Am J Pathol 1981, 103:191–200)

THE DEPOSITION OF LIPID in the arterial intima plays a central role in atherogenesis. The initiation of this event is considered by many to be an alteration in the permeability of the endothelium[1,2] or endothelial injury,[3] either of which may facilitate the movement of blood lipid into the intima. Intimal lipid initially accumulates intracytoplasmically in foam cells, which in the mature plaque are considered to be largely derived from medial smooth muscle cells,[3,4] with a second cell type, the macrophage foam cell,[5] playing a more minor role. The relative proportions of macrophage and smooth muscle foam cells in the lesion is still a matter of argument, and the origin of some intermediate cell forms is unclear.[6] Regardless of the cause of lipid deposition in these cells, its accumulation in the intima must reflect a failure in lipid removal.[7] In this respect, the macrophage foam cell, which is suggested to be derived from the blood monocyte,[8,9] may be a better candidate for removal of lipid from the lesion than the smooth-muscle-derived foam cell.

In a previous study,[9] and in the accompanying paper, we have demonstrated that blood-borne monocytes, identified by their ultrastructure and histochemistry, penetrate the intima of lesion-prone (high permeability) areas in swine fed an atherogenic diet, both prior to and during lesion development. In the absence of smooth muscle cell involvement, they constitute the major source of foam cells in both early the late (nonprogressing) fatty streak lesions. In advanced plaques from the same animals, smooth-muscle-derived foam cells predominate. The present study examines the fate of these monocyte-derived foam cells, and, together with earlier studies[9] allows the formation of a hypothesis for a defined role of the monocyte in atherogenesis.

Materials and Methods

Twenty-two Yorkshire pigs, 6 weeks of age (15–20

kg body weight) were fed a Purina Pig Chow diet containing 1.5% USP cholesterol and 19.5% lard (C/L).[9] Nine age-matched pigs were fed the Chow diet alone. The animals were killed at 12, 15, and 30 weeks after diet initiation, and tissue samples were examined by light and electron microscopy. All animals were injected with Evans blue (0.1 mg/kg in isotonic saline) 3 hours before killing, exsanguinated, and perfusion-fixed with glutaraldehyde as previously described.[9,10] Samples of aortic arch, thoracic, and abdominal aorta from areas of Evans blue uptake (blue areas) and no dye uptake (white areas) were excised from standardized sites for electron-microscopic studies. Fatty streak lesions and nonlesion areas from the aortic arch and abdominal aorta were examined, as well as liver and spleen. Samples for transmission (TEM) and scanning (SEM) electron microscopic study were perfusion-fixed and processed as described previously.[9,10]

Samples of arterial blood and venous blood were taken from the femoral artery and jugular vein, respectively, with the use of heparinized syringes. Monolayers of buffy coat cells prepared on coverslips as described by Wetzel et al[11] for combined light and scanning electron microscopic examination were stained with Wright's stain and oil red O[12] and examined and photographed under the light microscope. Viewed areas were marked with a diamond stylus for location of the same area in the SEM. The coverslips were then fixed for 1 hour at 4 C with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.6, dehydrated in ethanol, critical-point-dried from CO_2, sputter-coated with gold, and examined in an Etec Autoscan SEM.

A total of 142 samples of lesion areas, each approximately 25 sq mm in surface area, from 22 pigs, were examined by SEM, with light-microscopic and TEM samples being taken from the same sampling sites for qualitative analysis. At the end of the qualitative SEM studies, a random sample of 220 scanning electron micrographs of various magnifications, but all from the surface of arch lesion areas, were selected for quantitative studies. A minimum of 70 micrographs each of 12-, 15-, and 30-week fatty streak lesions from the aortic arch were examined. The endothelial surface area of each micrograph was measured, and the numbers of adherent monocytes and migrating foam cells were counted on each micrograph. The results were pooled from each diet group (12, 15, and 30 weeks), and adherent cells and foam cells were expressed as cells per square millimeter of endothelial surface.

Results

In the SEM, lesion areas were visible even at low magnification, appearing as raised ridges (Figure 1), often at the periphery of areas of Evans blue uptake. The lesions were covered with a roughened endothelium, compared with adjacent nonlesion areas (Figures 1 and 2). Whereas the endothelium of nonlesion areas was flat, with well-defined cellular borders (Figure 2), the endothelial layer overlying fatty streak lesions was thrown into deep folds (Figure 2) with irregularly shaped raised mounds protruding luminally (Figures 2 and 3), even in perfusion-fixed specimens. In many areas, a globular subsurface structure was visible through the endothelium (Figure 3). In a few cases, the attenuated endothelium overlying the mounds was ruptured, revealing large subendothelial cells containing globular elements in their cytoplasm (Figure 4). These exposed cells were identified as foam cells and the globular elements as lipid droplets by transmission electron microscopic examination.

Monocytes were adherent to the endothelium over lesions in large numbers, generally in groups (Figure 5), as opposed to the more diffuse attachment previously reported at prelesion stages. A second cell type was observed overlying lesions at all three stages, although it was more frequently seen at 12 and 15 weeks. These cells were characterized by numerous flaplike lamellipodia and a globular substructure (Figure 6). Similar cells were infrequently found in buffy coat preparations from arterial blood samples (Figure 7), and only rarely in venous blood. When seen, they contained oil-red-O–positive droplets in their cytoplasm. In section, the adherent cells on the endothelial surface were identified as foam cells fixed while passing through the endothelium, trapped in endothelial junctions either singly (Figure 8) or, in some cases, with more than one cell passing through the junction simultaneously (Figure 9). That portion of the cell still in the intima was identical in ultrastructural appearance to other intimal foam cells, with a lipid-laden cytoplasm and prominent Golgi apparatus (Figures 8 and 9). This portion of the cell did not appear damaged or degenerate. That part of the cell extending into the lumen of the vessel was irregular in shape, with numerous cytoplasmic flaps (the lamellipodia and veil structures seen in SEM) and empty vacuoles, but reduced lipid content, compared with the intimal part of the cell (Figures 8 and 9).

Two other cellular structures were observed by SEM on lesion surfaces at 15 weeks, and in particular, at 30 weeks. Round cells (15–40 μ in diameter) with a turgid appearance and globular substructure were found in large numbers, either singly (Figure 10) or in clusters (Figure 11) at a single focus. When viewed at a high angle (>80°) in the SEM, the base of these globular cells was often seen to emerge from beneath the en-
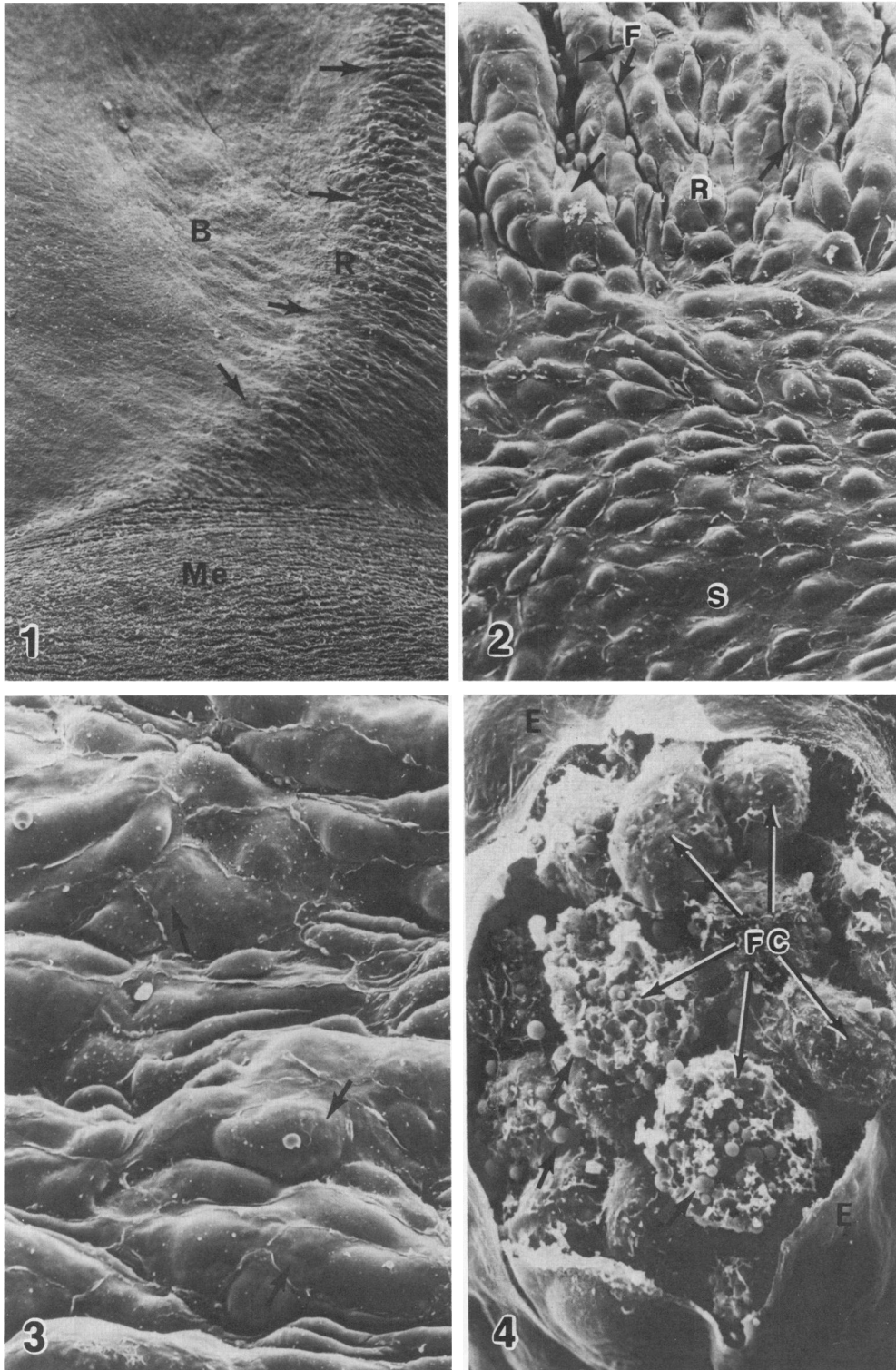
Figure 1—SEM of raised fatty cell lesion (arrows) at periphery of arch blue area (B) in a 15-week C/L-fed pig. Endothelium overlying lesion (R) is rough in appearance, compared with nonraised areas. Cut edge of media (Me) is visible at bottom. (×60)   Figure 2—SEM of area at base of lesion ridge from Figure 1 showing smooth endothelium (S) of nonlesion area with well-defined cellular borders. Adjacent area shows transition to rough surface (R) overlying lesion, with deep folds (F) and irregularly shaped mounds protruding luminally (arrows). (×600)   Figure 3—SEM of endothelium overlying arch lesion in 30-week C/L-fed pig showing cellular borders of endothelium and globular subsurface structure (arrows) underlying protruding mounds. (×1000)   Figure 4—SEM of mound from 15-week arch lesion in which the endothelium (E) has ruptured, revealing numerous foam cells (FC) immediately below. Lipid droplets (arrows) are visible in ruptured foam cell cytoplasm. (×1600)
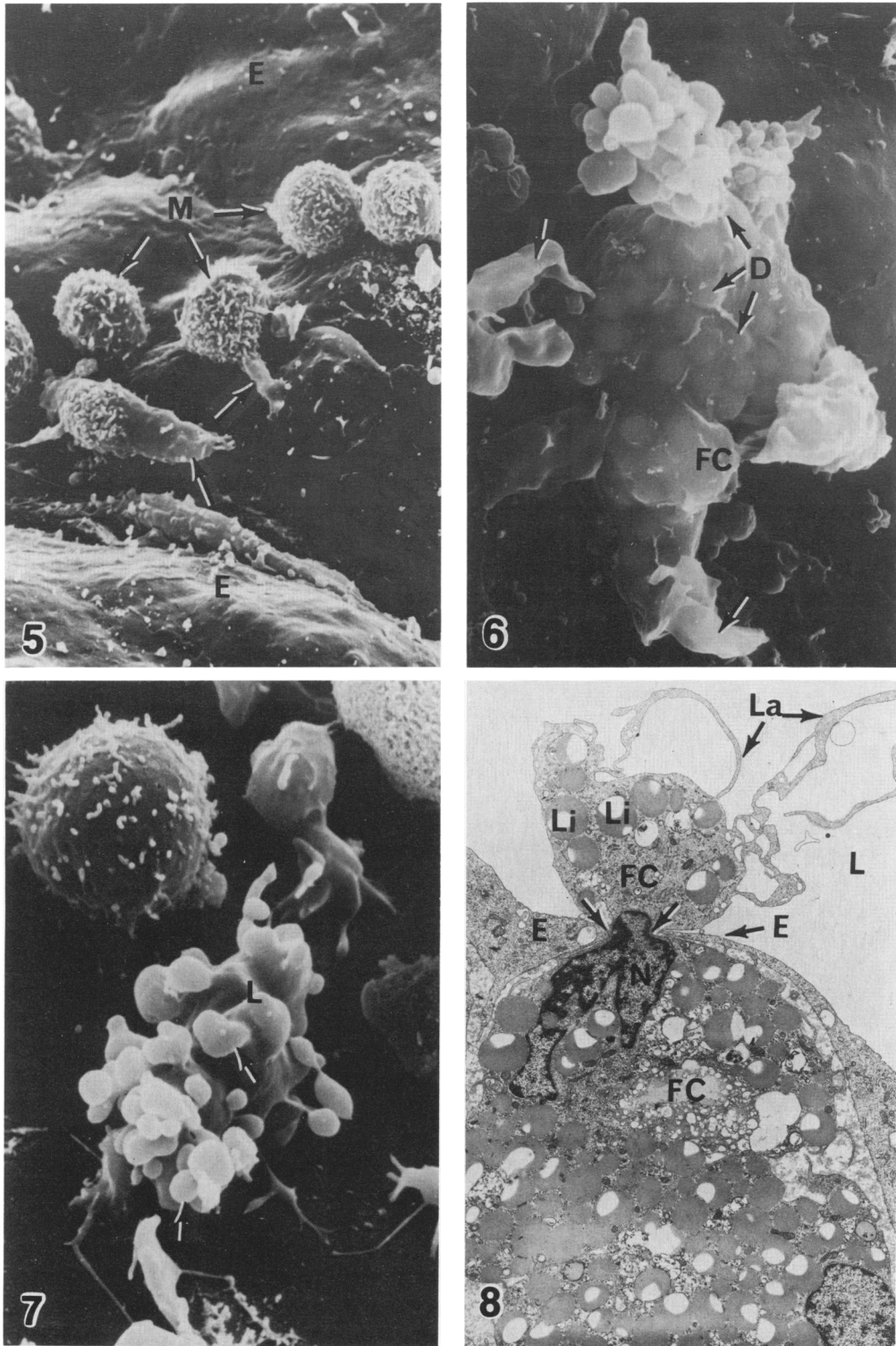
Figure 5—SEM of monocytes (*M*) adherent to the endothelium (*E*) overlying a 12-week arch lesion. Clusters of monocytes around single foci are more common during early lesion stages. Some spreading of pseudopods from monocytes can be seen (*arrows*). (× 2600)   **Figure 6**—SEM of foam cell (*FC*) on surface of 15-week arch lesion, showing globular substructure indicative of fat droplets (*D*) and peripheral cytoplasmic flaps or lamellipodia (*arrows*). (× 4800)   **Figure 7**—SEM of buffy coat cells from femoral blood sample on coverslip circulating lipid-laden cell (*L*) characterized by globular structure (*arrows*). When viewed by light microscopy, this cell contained oil-red-O–positive droplets in its cytoplasm. (× 5600)   **Figure 8**—TEM of foam cell (*FC*) trapped in an open junction (*arrows*) of endothelium (*E*) overlying a 15-week arch lesion. Lamellipodia (*La*) and cytoplasmic lipid droplets (*Li*) analogous to those in similar cell viewed by SEM in Figure 6 are visible in portion of cell in lumen (*L*). Nucleus (*N*) is trapped in junction. (Uranyl acetate and lead citrate, × 4000)

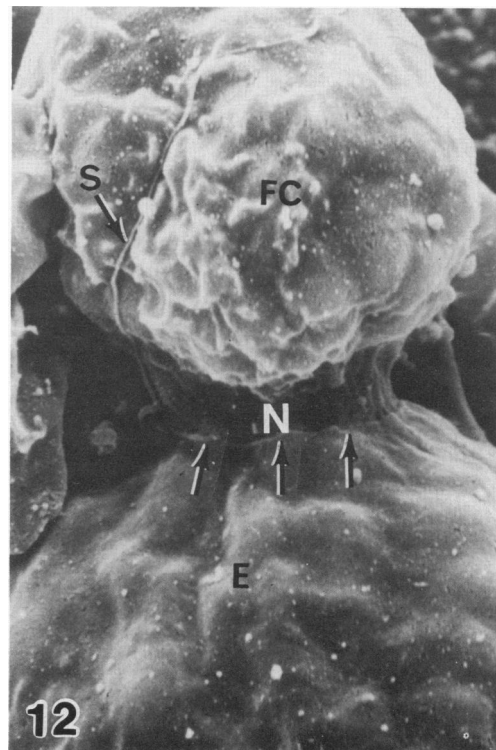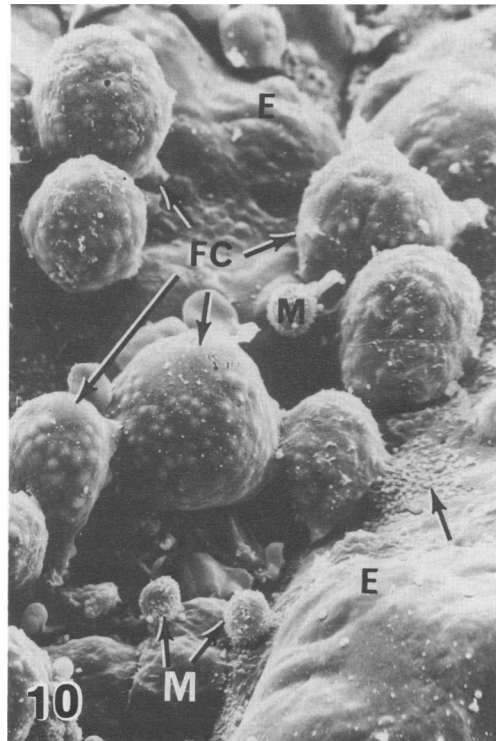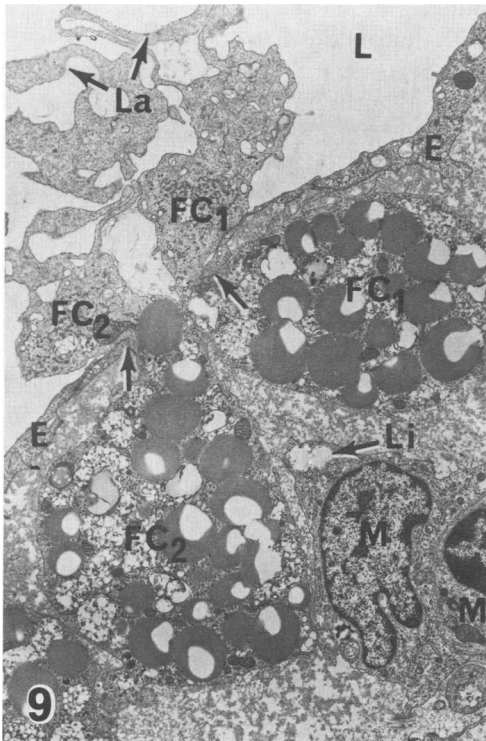Figure 9—TEM of two foam cells (*FC₁*, *FC₂*) trapped in an open junction (*arrows*) in endothelium (*E*) overlying a 12-week arch lesion. Foam cell cytoplasm devoid of lipid droplets and lamellipodia (*La*) are visible in the lumen (*L*). Portions of two monocyte-derived cells (*M*), one containing a lipid droplet (*Li*), are visible in the intima. (Uranyl acetate and lead citrate, × 4500)   Figure 10—SEM of 15-week arch lesion showing foam cells (*FC*) with globular substructures at different origin sites on the endothelial surface (*E*). The latter is rough with microvilli in some areas (*arrows*), and adherent monocytes (*M*) are also present. (× 1400)   Figure 11—SEM of 30-week abdominal lesion showing a cluster of globular cells (*FC*) seemingly originating at a single focus point on the endothelial surface (*E*). (× 1400)   Figure 12—SEM of surface foam cell (*FC*) (taken at high angle) overlying abdominal lesion at 30 weeks. Cytoplasmic neck (*N*) of foam cell can be seen emergent from craterlike edge (*arrows*) of endothelial cell (*E*). A strand of cytoplasm (*S*) continuous with endothelium extends over the foam cell. (× 6800)

dothelial layer (Figure 12). The surrounding endothelium was pushed luminally into a craterlike shape from which the compressed neck of the globular cell emerged (Figure 12). In many cases, thin strands of endothelial cytoplasm protruded over the luminal portion of the globular cells (Figures 12 and 13), giving the endothelium a ruptured appearance. Adherent monocytes were invariably present in these areas and the endothelial surface was rich in microvillus projections (Figures 10 and 13).

When viewed in section by TEM, these globular cells were readily identified as lesion foam cells again fixed while passing through the endothelial cell layer (Figure 14). In all cases, the attenuated endothelial cells on either side were pushed luminally (Figure 14), and strands of endothelium extending luminally adjacent to the foam cell were frequently observed. The possibility that these strands might be fibrin was excluded because they were continuous with the endothelium as seen both in SEM (Figures 12 and 13) and TEM (Figure 14). Fibrin was not seen in association with any of the lesions studied. Globular cells in the endothelium were much more degenerate in appearance than those bearing lamellipodia seen at 12 and 15 weeks (Figures 8 and 9). Lipid droplets were less osmiophilic, myelin forms in residual bodies were numerous, the Golgi was less prominent, and the plasma membrane was always attenuated (Figure 14). Frequently, one or more such cells were pressed in at the base of a foam cell already emergent from the endothelium (Figure 14). This membranous type of foam cell was more characteristic of 30-week than 15-week lesions, and was frequently observed ruptured in the intima.

The remaining cellular structure observed on lesion surfaces was found in large numbers on those lesions that also contained numerous globular cells. These structures consisted simply of pieces of membrane-like material that dotted the lesion surface in some areas (Figure 15). These veillike structures occasionally showed some globular substructure in the SEM and could be seen by TEM to be globular cell remnants emerging from the endothelium. Their appearance, numbers, and proximity to globular cells indicated that they were formed by the rupture of the latter after emergence, with loss of cytoplasmic contents.

The results of quantitation of surface monocytes and emergent foam cells overlying nonprogressing fatty cell lesions are expressed in Table 1. The results show wide variation, due to the focal nature of cell cluster attachment. Nevertheless, the data indicate that the number of monocytes adherent to nonprogressing fatty lesion surfaces is significantly elevated between 15 and 30 weeks; and more importantly, the

number of surface or emergent foam cells increases with time on the diet, so that by 30 weeks, the ratio of monocytes to foam cells is 1:1. In contrast, white areas show 39 ± 15 monocytes mm/sq of surface area, less than 50% of that found on 15-week lesions (with a mean of 25 sites from 6 pigs each at 15 and 30 weeks).

Lipid-laden cells were observed in sections of both spleen and liver (Figure 16) from 15- and 30-week animals. Such cells were often extremely degenerate and fragmented but in many cases resembled those within and emerging from 30-week lesions (Figure 14).

Discussion

The presence of large lipid-laden foam cells in intimal lesions is one of the most prominent and consistently found features of the atherosclerotic lesion both in humans[4] and in experimental animals.[13,14] The source of these cells remains a point of controversy, although there is little doubt that smooth-muscle–derived cells predominate in mature plaques.[3,4] The studies of O'Neal and co-workers[12,13,15-17] examined the possibility that foam cells arose from blood lipophages. They demonstrated the accumulation of lipid in blood cells, particularly mononuclear cells, and demonstrated an increase in the numbers of circulating monocytes in hypercholesterolemic rats.[12,15] Since they could demonstrate that foamy cells could cross the endothelium[13] and that circulating monocytes contained appreciable lipid,[15] they postulated that blood lipophages already containing lipid crossed the endothelium and became lesion foam cells.[17] They suggested, however, that further lipid accumulation must occur after penetration of the intima, since the ultrastructural morphologic features of the two cell types differed.[17]

The present data confirm our earlier suggestion of an alternative hypothesis[9] in that they describe the movement of foam cells through vascular endothelium overlying fatty lesions in the aortic arch of hypercholesterolemic swine. The results clearly demonstrate this movement and show that it involves large numbers of cells per unit area of endothelium. Two distinct types of foam cell can be identified: one that is more frequent in 12- and 15-week lesions and a second, more degenerate cell, which predominates at 30 weeks. In both cases, the migrating foam cell is identical to the foam cells in the underlying lesion.

Although we are aware of the dangers inherent in interpreting cell movement from static electron-micrographic images, all our results would indicate that the migratory foam cells are moving from the lesion into the bloodstream. In all instances where globular foam cells were observed by TEM or SEM, the under-
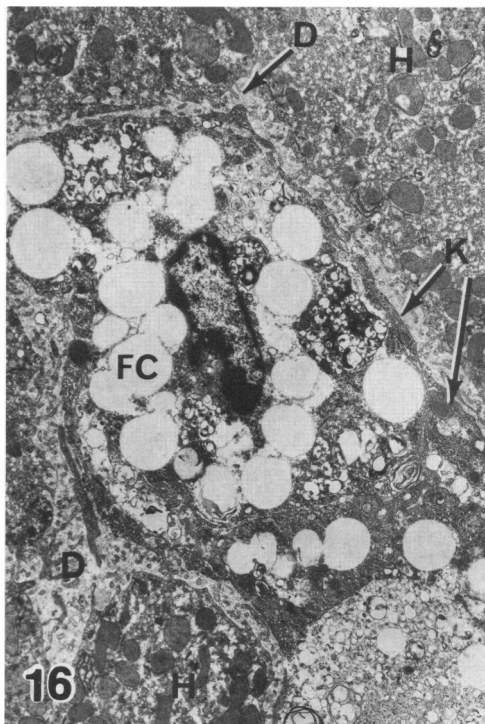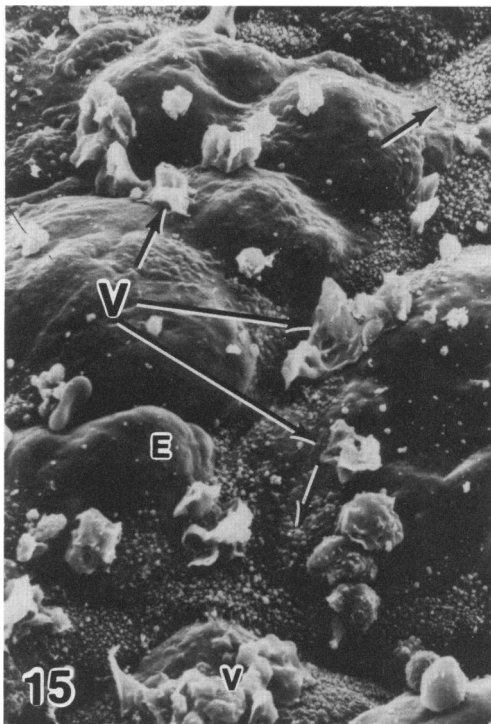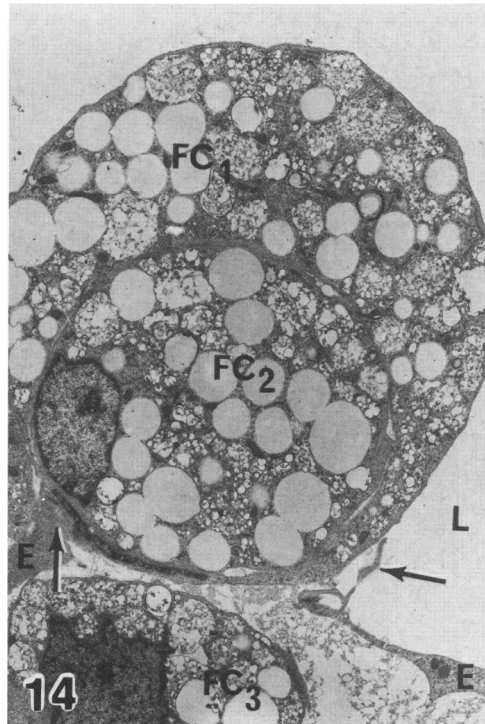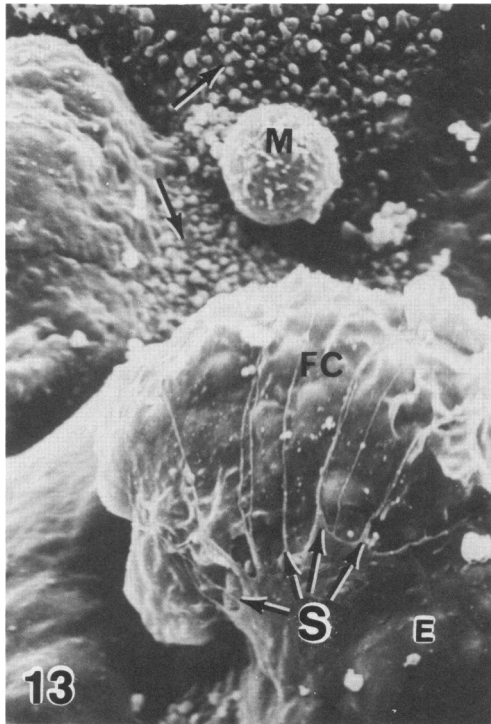
Figure 13—SEM of foam cell (*FC*) emergent from endothelium (*E*), over a 30-week arch lesion, showing numerous strands (*S*) of endothelial cytoplasm extending over foam cell. Note adherent monocyte (*M*) and microvilli (*arrows*) on endothelial surface. (× 3500)   **Figure 14**—TEM of two foam cells (*FC₁, FC₂*) emergent from endothelium (*E*) overlying 30-week abdominal lesion. Endothelium is pushed into lumen (*L*) on either side of foam cells (*arrows*). A third foam cell (*FC₃*) lies in the intima directly below. (× 3700)   **Figure 15**—SEM of surface of 30-week abdominal lesion showing numerous veil-like structures (*V*) on surface of endothelium (*E*), which is roughened by numerous microvilli (*arrows*). (× 2300)   **Figure 16**—TEM of liver from 30-week C/L-fed pig showing degenerate lipid-laden cell (*FC*) in sinusoid, space of Disse (*D*), Kupffer cell cytoplasm (*K*), and hepatic parachyma cells (*H*). (× 4400)

Table 1—Occurrence of Monocytes and Foam Cells on Fatty Streak Lesion Surfaces in Swine

Cell type	Cells/sq mm endothelium (mean ± SE)		
	12-week†	15-week†	30-week†
Monocytes	923 ± 193	762 ± 237	1368 ± 115*
Foam cells	133 ± 76	401 ± 123*	1415 ± 152*
Monocytes: Foam cells	7:1	2:1	1:1

* Significantly different from preceding stage (P < .01).
† Time on C/L diet.

lying endothelium is pushed luminally at the point of migration, indicating a luminal movement of the foam cell. Secondly, endothelial strands were observed extending over the luminal surface of migrating foam cells, a morphologic observation impossible to explain if their movement was from the circulation into the vessel wall. Furthermore, if foam cells were moving from the blood into the vessel wall in the large numbers observed, one would expect to find significant numbers of them in the arterial circulation. Such was not the case, although large numbers of degenerate foamy cells were found in liver and spleen sinusoids. These results are consistent with migration of foam cells out of the lesion into the bloodstream, and subsequent clearance by the reticuloendothelial system. Outward migration is also consistent with the clustering of foam cells from a single focal point where an initial break in the endothelium has been made, as seen in the SEM. The presence of foam cells indented into the intimal aspect of overlying foam cells that have already ruptured through the endothelium further supports this argument. The occurrence of an attenuated, mounded endothelial surface overlying these lesions is apparently caused by the pressure exerted by large numbers of underlying foam cells closely apposed to the endothelium. The endothelium is stretched such that the globular structure of the underlying foam cells is frequently seen through the endothelial layer, which is subject to damage, revealing the underlying cells. A similar disruption of endothelial cells and exposure of underlying foam cells in rhesus monkeys was shown by Taylor et al,[18] who also questioned the vulnerability of attenuated endothelium overlying lesions. Their findings are remarkably similar to those of the current study with respect to the appearance of this damage. These authors have interpreted their findings as in vivo damage. They also observed the globular substructure of endothelium over lesions as seen in the SEM and, as in the present study, have interpreted it as due to the presence of foam cells pressed against the attenuated endothelium.[18] These authors also showed foam cells protruding from the endothelium, but interpreted the finding as being a re-

sult, rather than a cause, of endothelial damage. In other studies,[19,20] these investigators were able to isolate both macrophage- and smooth-muscle–derived foam cells from advanced lesions in rabbits and monkeys, identifying the former by Fc receptors. Foam cell lesions from which these cells were isolated showed adherent leukocytes, but they were not identified or commented on as a source of foam cells. In early lesions in the present study endothelial attenuation is not as pronounced, lesions are less raised, and foam cells appear capable of migrating through endothelial junctions without damaging the endothelium. In later lesions, globular foam cells appear to simply rupture the endothelium, perhaps because they themselves are so lipid-laden they cannot squeeze through junctions. Focal endothelial damage thus caused could conceivably lead to an enhancement of lesion development.[3] The increased clustering of monocytes on later lesions may also be a response to such focal injury, whereas monocyte adherence at prelesion stages is more diffuse.[9] It would appear from the number of surface veillike cells that the turgid globular cells are also fragile and frequently rupture as they emerge, leaving membrane veils and occasional lipid droplets. Whether these cells are ruptured in vivo by the shear stress of flowing blood or as an artifact during processing is uncertain; but their presence also suggests outward movement, since when viewed by TEM, the intimal portion of these cells was intact and contained lipid, whereas the luminal portion consisted of empty vacuoles and membranous veils.

The results of these studies combined with those of the accompanying paper and previously published results[19] clearly demonstrate that large numbers of blood-borne monocytes migrate into lesion-prone areas in the early stages of hypercholesterolemia and are the major source of foam cells in fatty lesions in this model. These cells are shown to be phagocytic in the intima and probably, therefore, accumulate lipid by phagocytosis. The present findings demonstrate that foam cells subsequently leave the lesion by migration back through the overlying endothelium and are possibly cleared by the reticuloendothelial system. The quantitative data demonstrate that the number of monocytes adherent to fatty lesions which do not advance to form fibroatherosclerotic plaques in the periods studied increases slightly with time on the atherogenic diet. Of greater interest, however, is that the ratio of adherent monocytes to emerging foam cells decreases from 7:1 in early lesions to 1:1 in late fatty streaks. In other words, in late nonprogressing fatty lesions, cell immigration equals cell emigration, assuming that all adherent monocytes enter the wall. At earlier stages, influx of cells is greater than efflux, thus

forming a lesion through increased intimal cellularity and increased cell size as these cells engulf lipid. Later stabilization of the lesion may be reached, however, if efflux equals influx. Not only does the cellularity of the lesion remain constant, but lipid is effectively cleared and the lesion does not progress.

The defined role of the monocyte in this system would thus appear to be one of clearance of lipid from areas of lesion formation. This concept has been expressed previously,[6,7,21] largely based on the premise that macrophage-derived foam cells have been demonstrated in various lesions, both ultrastructurally[5] and histochemically.[6,7,21] Since the traditional role of the macrophage is to remove unwanted material from the tissue, and since macrophage foam cells appear to be enzymatically more capable of processing lesion lipid than smooth muscle cells,[21] it is logical to assign it a role in lipid clearance. The current studies are the first, however, to clearly identify monocytes as the source of these cells and to assess foam cell clearance as related to lesion development. It would appear that if the monocyte clearance system is successful, fatty lesions may not progress. However, over a protracted period of hyperlipidemia, this system may become inadequate to clear sufficient intimal lipid such that medial cell involvement occurs. This proposed system may relate therefore to the still unresolved relationship between fatty streaks and advanced lesions. Obviously other factors may also be influential, including the degree of intimal cell necrosis, endothelial injury and permeability, site of the lesion and vessel geography, hemodynamic stresses, which may be greater in some areas than others, and arterial wall composition and compliance. The aortic wall in the arch is much thicker and has a greater elastin and lesser collagen content[22] than the abdominal aorta. As such, its compliance to hemodynamic stresses may be different, and the concentration of smooth muscle cells per unit volume, as well as their availability to the intima, is probably greater in the abdominal aorta, which may make abdominal lesions more prone to progression.

The hypothesis advanced by Ross and colleagues[3] indicates that endothelial cell injury or denudation on a continuing basis allows greater influx of plasma lipoproteins and platelet-derived growth factor. Both of these factors stimulate the migration of medial smooth muscle cells into the intima and their proliferation,[23-25] thus promoting the formation of advanced plaques. The demonstration in the present paper, as well as in the accompanying one and the preceding one[9] of a large-scale involvement of monocyte-derived macrophages may also play a role in subsequent smooth muscle cell proliferation and plaque advance-

ment in that a growth factor has also been shown to exist in macrophages.[26] If such is the case, movement of monocytes into lesion areas and migration of foam cells out of lesions may contribute to plaque advancement in two respects: by introducing macrophage growth factor into the lesion area and by damaging overlying endothelium. It is of relevance in this respect that the number of monocytes involved and the occurrence of endothelial damage by emigrating foam cells both increase with the time of hypercholesterolemia.

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