American Journal of Pathology, Vol. 141, No. 6, December 1992

Induction of Fatty Streak-like Lesions *In Vitro* Using a Culture Model System Simulating Arterial Intima

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In this study a two-compartment culture model of arterial intima was used for the* in vitro *induction of fatty streaklike lesions. The apparatus consisted of upper and lower compartments separated by a human amnion membrane stretched between them. Human umbilical vein endothelial cells (HUVECs) were cultured to confluence on the stromal surface of the amnion membrane. Maximal migration of blood mononuclear cells (MCs) through the HUVEC monolayer in response to a f-Met-Leu-Phe gradient was observed at 10^{-8} mol/l; the migration was 3.29 times greater than that observed under the condition of random migration (control). In the study of MC transformation into lipid-laden cells in the amnion membrane (foam cell formation in 'arterial intima'), 10^6 MCs were incubated, in the presence of freshly prepared low-density lipoprotein (LDL; 100 μg/ml). The lipid loading of MCs was time dependent. After 12 hours' incubation, 39% of the MCs that migrated into the amnion membrane contained a small number of lipid droplets, whereas the remaining 61% showed no lipid droplets. Only 1.7% of the cells contained a high number of lipid droplets in the cytoplasm and took on the appearance of foam cells. With time, the number of lipid-laden cells and the amounts of intracytoplasmic lipid droplets gradually increased. At 72 hours after incubation, 65.4% of the MCs were loaded with lipid droplets, and 20.9% of them, an eightfold increase over 12 hours of incubation, showed a foamy cell appearance. Because MCs consist of 70% monocytes and 30% lymphocytes, about 93% of the monocytes were filled with lipid after a 72-hour incubation. Ultrastructural examination showed that lipid-laden cells took on macrophage characteristics, such as wide and heterogeneous cytoplasm, indented nuclei, and abundant lysosomes. A minority of the MCs in the amnion were considered lymphocytes; they had scanty cytoplasm, round nuclei with abundant heterochromatin, no lysosomes, and no lipid vacuoles. In conclusion, the formation of an* in vitro *fatty streaklike lesion is demonstrated, and this is reminiscent of* in vivo *human atherogenesis. (Am J Pathol 1992, 141:1435–1444)

Atherosclerosis is one of the most important areas in pathology; there are, however, few *in vitro* studies using vessel wall models. Previous *in vitro* studies of monocyte migration and cell-to-cell interaction have been restricted to two-dimensional analysis because of the lack of simple and relevant models for the vessel wall. Recently, a number of studies have been performed in an attempt to elucidate the action of leukocytes using a three-dimensional culture system. Russo et al[1] were the first to design an *in vitro* amnion model system for studying the migration and chemotaxis of polymorphonuclear leukocytes through human native tissue barriers. Foltz et al[2] and Furie et al[3] improved the model system and developed an *in vitro* vessel model that consisted of amnion connective tissue lined by cultured endothelial cells (ECs) instead of amnion epithelium. Migliorisi et al[4] used the same model and showed monocyte migration across the endothelium in response to leukotriene B_4 and f-Met-Leu-Phe (fMLP).

The subendothelial accumulation of foam cells and the deposition of lipid components are important events in an early atherosclerotic lesion. Studies on humans[5–7] and on experimental animals[8–10] indicated that foam cells in atherosclerotic lesions were derived largely from blood monocytes. To initiate and develop the lesion, monocytes in the bloodstream must undergo at least two processes: migration into the subendothelium and lipid loading.[11] Monocyte adhesion to the endothelium and subsequent migration into and retention within the sub-

Accepted for publication June 15, 1992.

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endothelial space, a chain of events overlapping chronic inflammatory processes, have been considered early steps in the development of atherosclerosis.[9,11] Although these processes are regulated by various factors, including chemotactic agents,[12–14] divalent cations,[15] plasma lipoproteins,[16–20] cell surface molecules,[21,22] and extracellular matrix components,[23,24] certain biologically or chemically modified forms of low-density lipoprotein (LDL), particularly oxidized LDL, are major factors relevant to atherogenesis.[25–31] Oxidized LDL has some unique characteristics that are different in blood monocytes and in intimal macrophages[26]; oxidized LDL is chemotactic for blood monocytes but not for macrophages, and is retentive for macrophages in the intima. In addition, intimal macrophages can easily take up oxidized LDL through the high-affinity receptor-mediated pathway,[25,30,32,33] causing foam cell formation. Oxidized LDL is known to be generated biologically by the interaction of native LDL with ECs.[25,26,33]

We have developed an *in vitro* model of the arterial intima by a modification of the methods of Foltz et al[2] and Furie et al[3] to make a three-dimensional analysis of the vascular cell interactions relevant to atherogenesis. In this report, we attempted to induce fatty streaklike lesions by the use of blood mononuclear cells (MCs) and EC-modified LDL.

Materials and Methods

Isolation of Human Amnion

Human amnion was prepared by a modification of the method of Foltz et al.[2] Normal term placentas were obtained immediately after delivery. The amnion, which had been peeled away from the chorion by blunt dissection, was immersed in sterile 0.25 mol/l NH_4OH for 2 hours at room temperature. Under aseptic conditions, the epithelial layer of the amnion was removed by gentle scraping with a rubber policeman, leaving behind the collagenous stroma. The amnion then was placed in the tissue-holding device of the two-compartment apparatus depicted in Figure 1. The tissue-holding device was composed of two concentric Lucite rings. The rings measure 28 mm in outside diameter and 14 mm in inside diameter. The upper ring is 7 mm in height, and the lower ring is 3 mm in height. The amnion, with the stromal surface facing the upper ring, was stretched over the bottom of the upper ring and held in place with a silicone rubber ring. The upper and the lower rings were screwed together with three side-stoppers. Thus, the chamber was divided into two compartments, upper and lower, by the amnion stretched between them. The chambers then were washed extensively with cold phosphate-buffered saline

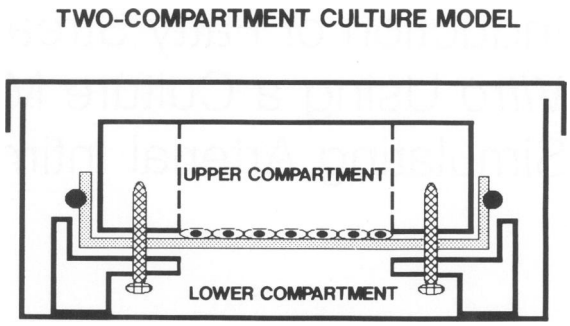

Figure 1. *Cross-sectional representation of the two-compartment culture apparatus. The amnion membrane is clamped between the upper and lower Lucite rings creating two separate compartments. The HUVEC monolayer is cultured on the amnion surface facing the upper compartment.*

(PBS) containing penicillin (500 U/ml) to remove debris and stored in PBS supplemented with an antibiotic cocktail consisting of penicillin (500 units/ml), gentamicin (30 μg/ml), and amphotericin B (3 μg/ml), at 4°C until use.

Isolation of Human Umbilical Vein Endothelial Cells (HUVECs) and Cell Culture

Human umbilical vein endothelial cells (HUVECs) were harvested from human umbilical vein and cultured by a previously described method.[34,35] Umbilical cords were obtained from full-term, natural delivery placentas soon after birth. The umbilical vein was rinsed thoroughly with cold PBS to wash away traces of blood. One end of the vessel was clamped, the lumen was filled with dispase solution (1000 units/ml; Sanko Phamaceutical Co., Tokyo), and then the other end was clamped for sealing. The umbilical cord was incubated at 37°C for 30 minutes. After incubation, the umbilical vein was perfused with 40 ml PBS. The effluent was collected in a sterile conical centrifuge tube. Human umbilical vein endothelial cells were harvested by centrifugation at 180*g* for 10 minutes. The cell pellet for primary culture was resuspended in RPMI 1640 medium (Nissui Phamaceutical Co., Tokyo) supplemented with L-glutamine (0.294 g/L; GIBCO, Grand Island, NY), 15% fetal bovine serum (FBS; Microbiological Associates, Logan, UT), 15% Nu-serum (Collaborative Research, Lexington, MA), 15 μg/ml of endothelial-cell growth supplement (ECGS; Collaborative Research, Lexington, MA), 100 μg/ml heparin, and antibiotics (100 U/ml penicillin and 15 μg/ml gentamicin). Human umbilical vein endothelial cells were seeded in the upper compartment of the chambers at a density of 2.0×10^5 cells/ring. The chambers were placed in six-well culture dishes (Terumo Co., LTD., Tokyo,) and incubated at 37°C in a humid atmosphere of 5% CO_2 and 95% air.

Silver Nitrate ($AgNO_3$) Staining

Human umbilical vein endothelial cell cultures were stained with silver nitrate by a modification of the method of Poole et al.[36] Cultures were washed twice with 5% glucose solution, flooded with 0.2% solution of $AgNO_3$ for 30 seconds, followed successively by 5% glucose for washing, 1% ammonium bromide for 30 seconds, 5% glucose for washing, 3% cobalt bromide for 30 seconds, 5% glucose for washing, and 10% formalin for fixing.

Electron Microscopy

Cultures for electron microscopy were washed twice with PBS at the end of experiment, fixed in 3% cacodylate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated in a graded series of alcohols, and embedded in Epon 812 (TAAB Laboratories, Berkshire, England). Thin sections were stained with uranyl acetate and lead citrate before examination by electron microscopy.

Blood MCs

Mononuclear cells were prepared from 100 to 200 ml peripheral blood obtained from healthy volunteers by centrifugation on Ficoll-Hypaque gradients.[37] By the criteria of morphology and nonspecific esterase activity, the collected MCs were composed of approximately 70% monocytes and 30% lymphocytes.

Lipoproteins

Human LDL (density, 1.019 to 1.063 g/ml) was prepared by the discontinuous density gradient ultracentrifugation technique[38] from the serum of normal fasting volunteers. Blood samples contained Na_2 ethylenediaminetetraacetic acid (EDTA) at the final concentration of 4 mmol/l. Isolated LDL were dialyzed against 150 mmol/l NaCl containing 0.3 mmol/l Na_2EDTA, pH 7.4, at 4°C for 24 hours before use. The lipid peroxide contents of freshly prepared LDL were routinely determined by analyzing thiobarbituric acid-reactive substances and expressed as malondialdehyde equivalents. Low-density lipoproteins containing less than 2 nmol/ml malondialdehyde equivalents, was available for use. Protein concentration was determined by the method of Lowry et al,[39] using human serum albumin as the standard. Endothelial cell–modified LDL was prepared by incubating LDL (100 μg protein per ml) with HUVECs in 4 ml RPMI 1640 medium for 24 hours at 37°C. At the end of incubation, the medium was removed sterilely, centrifuged to remove cells and debris, and stored at 4°C. Control LDL was prepared by the same way in the absence of cells.

MC Migration Studies

Human umbilical vein endothelial cell monolayers grown on the amnion were washed twice with PBS before each experiment. Mononuclear cells (1.0×10^6) were introduced in the upper compartment and were allowed to migrate across the HUVEC monolayer in response to formyl-methionyleucylphenylalanine (fMLP) added in the lower compartment at the indicated concentrations. All migration studies were performed at 37°C in a 95% air-5% CO_2 atmosphere. At the end of the experiment, the HUVEC monolayers were washed twice with PBS, and fixed in 3% cacodylate-buffered glutaraldehyde for light and electron microscopic observations.

Quantitation of MC Migration

For each chamber, cross-sections (1 μ thick and approximately 4 mm in length) of four parts of the HUVEC monolayer were cut, stained with toluidine blue, and examined by light microscopy. The cells that had migrated across the monolayer were counted and were expressed by chemotactic index defined as the number of cells migrating in response to fMLP by that of negative control.

Induction of Fatty Streak-like Lesions

Human umbilical vein endothelial cell monolayers grown on the amnion were washed twice with PBS before each experiment. Mononuclear cells (1.0×10^6), suspended in the same RPMI 1640 medium used for HUVEC cultures, and freshly prepared LDL (100 μg/ml), were placed in the upper compartment, while the optimal concentration of fMLP (10^{-8} mol/l in this study) for MC migration was added in the lower compartment. The chambers then were incubated for the indicated time at 37°C in a 95% air-5% CO_2 atmosphere. At the end of the experiment, the HUVEC monolayer was washed twice with PBS, and fixed in 3% cacodylate-buffered glutaraldehyde for oil red O staining and electron microscopy. After fixation, the monolayer was bisected. One half was embedded in ornithine-carbamoyltransferase compound (Miles, Elkhart, IN) and dropped into a bath of hexane, precooled in a Cryocool immersion cooler (Neslab Instruments, Portsmouth, NH). Cryostat sections were cut at 6 μ in thickness, stained with oil red O, and examined by light microscopy. The other half was postfixed in osmium

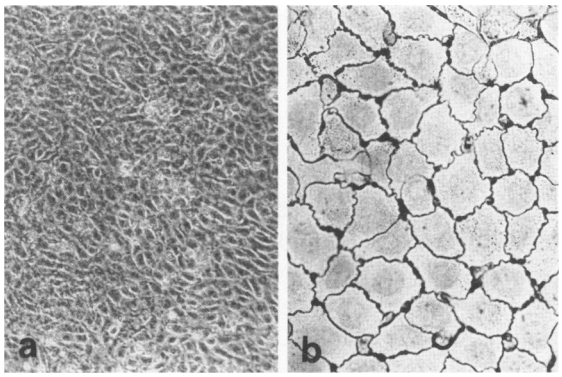

Figure 2. **a:** *Phase-contrast micrograph of HUVECs cultured for 8 days and grown to confluency on the amnion surface. The cultured HUVECs are a homogeneous population of polygonal cells with distinct cell borders (×120).* **b:** *Silver nitrate staining of a confluent monolayer of HUVECs cultured for 8 days. The edges of the HUVEs reacted with silver nitrate to produce the flagstone pattern (×350).*

tetroxide, and used for the examination by electron microscopy.

Foam Cell Transformation of MCs in a Culture Dish

Mononuclear cells in 35-mm dishes were incubated with 2 ml PBS for 6 hours and washed twice, and then incubated with 2 ml of the same RPMI 1640 medium used for HUVEC cultures containing native or EC-modified LDL (100 μg/ml) at 37°C in a humid atmosphere of 5% CO_2 and 95% air. After 48 hours' incubation, the dishes were washed twice, fixed in formalin, and stained with oil red O.

Results

Culture of HUVECs on the Amnion Membrane

Primary cultures of HUVECs grown on the stromal surface became confluent between 6 and 7 days after plating. The cells were homogenous, closely opposed, large, and polygonal, with a centrally located nucleus and distinct cell borders (Figure 2a). The monolayers remained quiescent over 14 days. The cultures were determined to be free of contaminating cell types by immunofluorescent localization of Factor VIII-related antigen (not shown).

$AgNO_3$ Staining

The monolayers of HUVECs stained by $AgNO_3$ after 8 days in culture on the amnion produced an *en face* appearance of continuous flagstone (Figure 2b). In contrast,

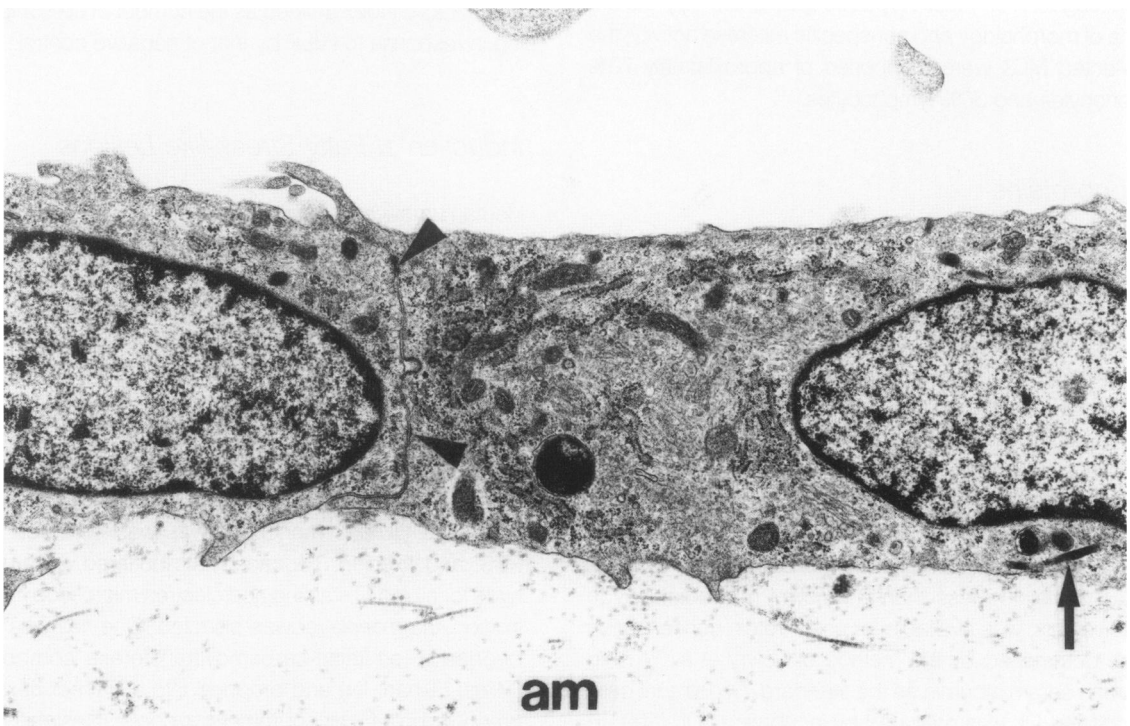

Figure 3. *Transmission electron photomicrograph of HUVECs grown for 8 days on the amnion. Two flattened HUVECs are in direct contact by junctional specializations (arrowheads). The cytoplasm contains a number of vesicles, prominent Golgi complex, abundant smooth and rough endoplasmic reticulums, bundles of fine filaments, and mitochondria. Arrow indicates a rod-shaped Weibel-Palade inclusion body. am: amnion (×13000).*

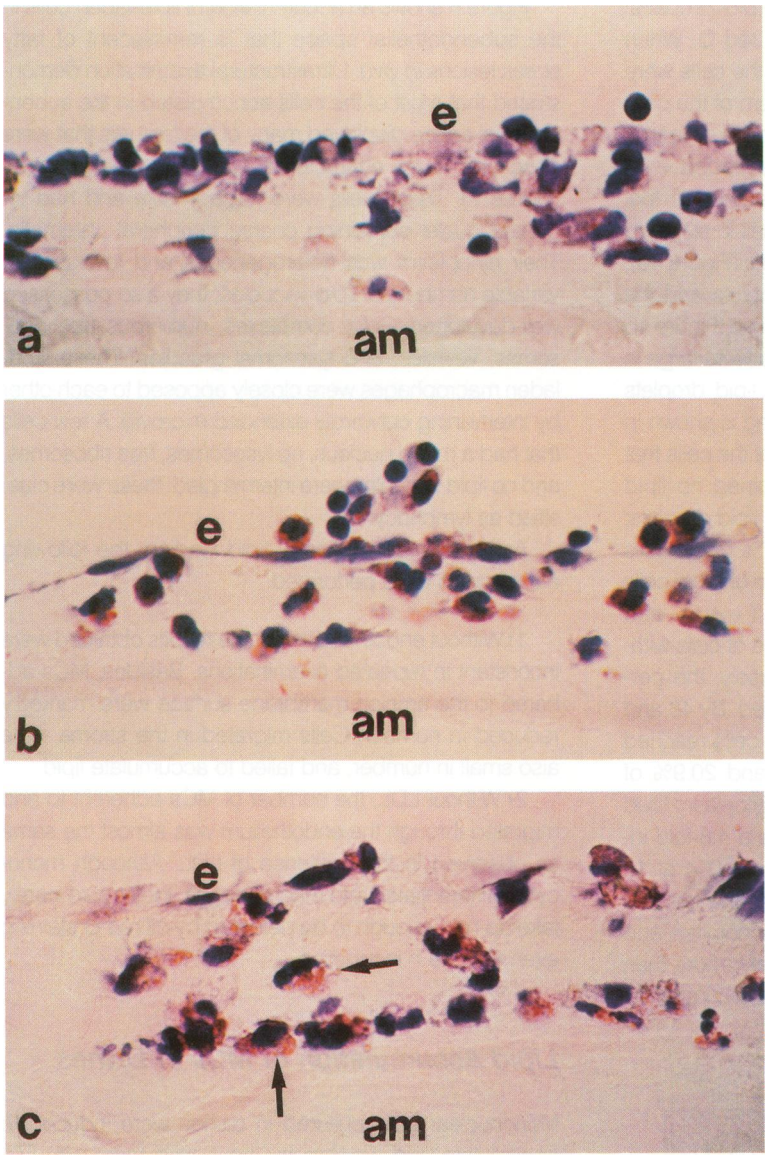

Figure 4. *Light micrograph of MCs in the lipid loading study. Incubation of MCs (10^6/ml) and native LDL (100 μg/ml) for 12 hr* **(a)**, *24 hr* **(b)**, *and 48 hr* **(c)**. **a**: *A small number of MCs accumulated in the subendothelial space contain oil red 0 positive lipid droplets. Note no macrophages taking on an appearance of foam cells (×1080).* **b**: *The number of lipid-laden MCs and the number of lipid droplets are increased. HUVECs also contain a few lipid droplets.* **c**: *MCs are enlarged and loaded almost completely with lipid droplets, often taking on an appearance of foam cells (arrows). am: amnion, e: HUVECs. Oil red 0 stain, ×1080.*

in monolayers after 5- to 6-day culture, the cell borders were stained irregularly or only weakly by $AgNO_3$.

Ultrastructural Observation

Ultrastructural observation of HUVECs that were grown on the amnion for 8 days showed many of the features characteristic of arterial endothelium (Figure 3). Rod-shaped cytoplasmic inclusions, known as Weibel-Palade bodies, were demonstrated in about 70% of cells.

Transendothelial Migration of MCs

In response to fMLP (10^{-6} – 10^{-9} M), MCs attached to the EC monolayer and began to migrate across the endothelium in 10 minutes; MCs accumulated in the subendothelial tissue with time, reaching a maximum at 2 hours. The rate of MC migration was optimal at 10^{-8} mol/l fMLP with a chemotactic index of 3.29.

Induction of Fatty Streak-like Lesions

After incubation with fresh LDL in the upper compartment, MCs continued to traverse the EC monolayer and accumulated to form clusters in the underlying connective tissue, where they began to be loaded with lipid vacuoles (Figure 4). The rate of MC transformation into lipid-laden cells was studied as a function of time. In sections stained by oil red O, 300 of the cells that migrated into the subendothelium were divided into grades 0 to + +, ac-

cording to the quantity of cytoplasmic lipid droplets. Grade 0 represented no staining with oil red O. When there were a few to several lipid droplets, the cells were graded +. In the case where the cytoplasm of the cells were filled with numerous lipid droplets and the cells took on the appearance of foam cells, they were graded ++. Mononuclear cells accumulated in the subendothelial space after 12 hours' incubation were small in size and loaded with a small number of lipid droplets (Figure 4a). Both the number of cytoplasmic lipid droplets and the number of lipid-laden cells increased with time (Figure 4b and c). After 48 hours' incubation, the cells were large in size, and their cytoplasm was filled with lipid droplets (Figure 4c). The time course of lipid loading is shown in Figure 5. After 12 hours' incubation, 61% of the cells that migrated into the subendothelium contained no lipid droplets, whereas the remaining 39% had lipid droplets; most cells were classified + and only 1.7% of the cells showed a foamy cell appearance. The ratio of foam cells (++ cells) to the total lipid-laden cells (++/+ and ++) was 0.044. With time, the percentage of cells without lipid droplets decreased and, conversely, the percentage of cells with lipid droplets increased. By 48 and 72 hours, total percentages of lipid-laden cells reached 62.3% and 65.4%, respectively; 16.5% and 20.9% of them transformed into foam cells with the cytoplasm filled with lipid droplets. At 72 hours, there was a 4.8-fold increase in foam cell generation (++ cells, 0.044 at 12 hours *versus* 0.209 at 72 hours). Because the isolated MCs consisted of approximately 70% monocytes and 30% lymphocytes, as mentioned previously, more than 90% of monocytes showed lipid droplet loading after 72 hours.

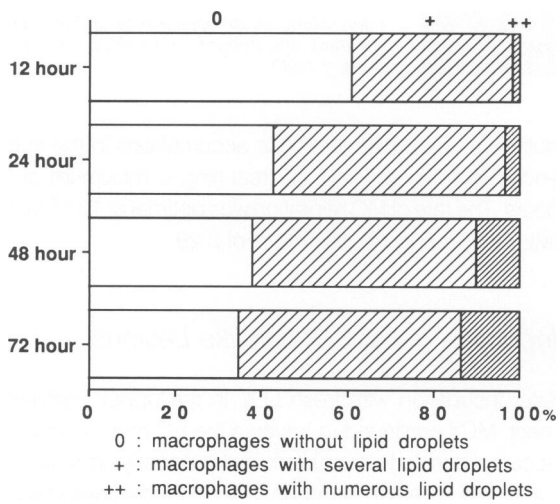

Figure 5. *Time course of MC lipid loading. 300 MCs found in the subendothelial space were graded according to the number of lipid droplets. Percentages of lipid loading MCs (+, ++) show a time-dependent increase.*

Figure 6 shows an accumulation of lipid-laden cells in the subendothelial space that is reminiscent of fatty streak lesions *in vivo*. Ultrastructural examination demonstrated that most of the cells accumulated in the subendothelial space displayed many of the features that were described to be characteristic of tissue macrophages (Figure 7). These cells were large in size and had indented nuclei containing coarse peripheral chromatin. Their cytoplasm was heterogeneous and loaded with variable numbers of lipid vacuoles; they also possessed well-developed Golgi complexes, numerous free ribosomes, vesicles, and lysosomal granules. These lipid-laden macrophages were closely apposed to each other by intertwining outwardly extended microvilli. A few cells that had a round nucleus, no lysosomes, free ribosomes, and no lipid vacuoles were intermingled; these were classified as lymphocytes.

As the control studies of lipid loading, the following experiments were performed.

1) Without endothelial lining, the results obtained were inconstant in repeated examinations. Besides, MCs adhered to the amnion membrane surface were markedly reduced in number. Cells migrated in the stroma were also small in number, and failed to accumulate lipid.

2) Without LDL, the number of MCs adherent to and migrated through the endothelium was almost the same as observed in the presence of LDL. Although monocytes differentiated into macrophages, they could barely take up lipid enough to be detected by oil red O staining even after 48 h incubation.

Lipid Accumulation of MCs in Dishes

Mononuclear cells cultured in dishes were induced to accumulate lipid droplets by incubating them with EC-

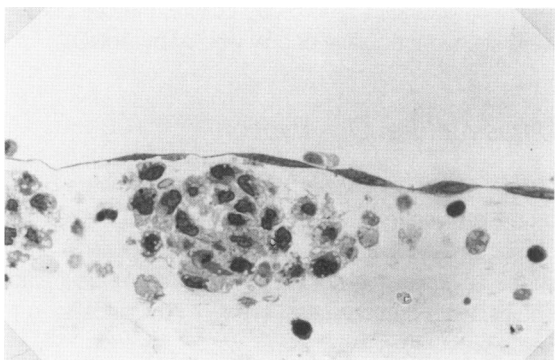

Figure 6. *Light micrograph of a 48-hr incubation of MCs (10^6/ml) and native LDL (100 μg/ml). MCs accumulated in the subendothelial space contain many lipid vacuoles in their cytoplasm (×770).*

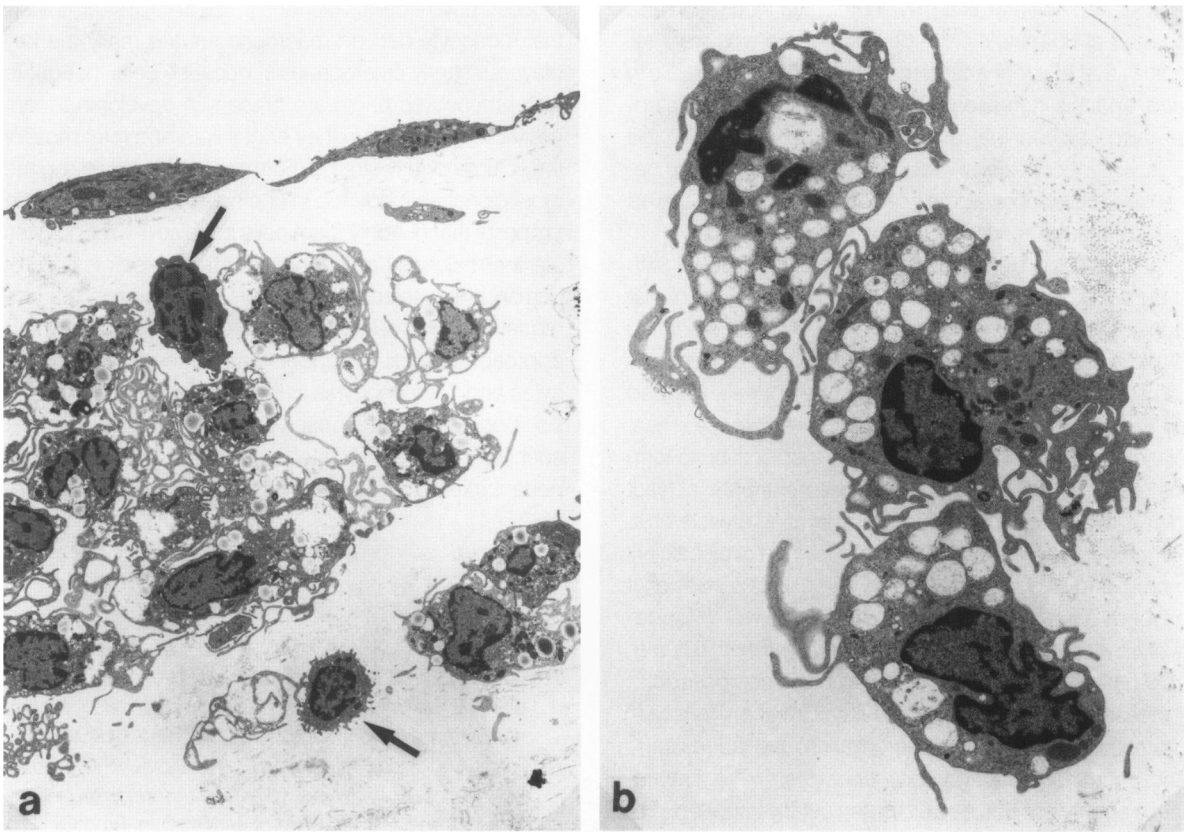

Figure 7. *Transmission electron photomicrograph after 24 hr* (a) *and after 48 hr* (b) *incubation of MCs (10^6/ml) and native LDL (100 μg/ml).* a: *MCs containing lipid vacuoles display many features of macrophages such as wide cytoplasm, indented nuclei and abundant lysosomes. Their surface ruffles are prominent, outwardly extended, and intertwining one another. Cells considered lymphocytes are intermingled with them (arrows) (×2900).* b: *MCs with macrophage characteristics are filled with lipid vacuoles and take on the appearance of foam cells (×6700).*

modified LDL (100 μg/ml) or with native LDL (control) for 48 hours. Most of MCs cultured in the presence of EC-modified LDL showed oil red O–positive fine lipid droplets. In contrast, MCs failed to accumulate lipid droplets in the control study incubated with native LDL (not shown).

Discussion

We have demonstrated here the feasibility of the induction of an *in vitro* fatty streaklike lesion by the use of a three-dimensional culture system. In this system, the amnion membrane lined by a single layer of HUVECs was referred to as arterial intima. The amnion connective tissue, mainly composed of a loose fibroblast network embedded in a mass of reticulin and collagen fibers,[40] provides a natural autologous substrate for EC growth. The culture system was transparent enough to allow us to get a first-hand look at HUVEC growth on the amnion surface (Figure 2) and at MC adhesion to the HUVEC monolayer. In addition to *en face* observations, cellular events in the subendothelial space could be examined transversely, by light and electron microscopy, because of the ample thickness of the amnion membrane.

Studies on humans and on experimental animals demonstrated that foam cells in developing atherosclerotic lesions were derived largely from blood monocytes.[5–11] To better understand the involvement of monocytes in atherogenesis, we have attempted to develop an *in vitro* model system to identify and reproduce the early events of atherosclerosis, because monocyte-derived foam cell lesions (fatty streaks) may be the precursors to the occlusive atheromatous atherosclerotic lesions.[41,42] Monocyte adherence followed by migration through the vascular endothelium plays an important role in the recruitment of monocytes to potential lesion sites.[11] Monocytes are known to adhere more strongly to ECs than neutrophils and lymphocytes[15,21]; this adhesion could be further enhanced under various conditions. Hypercholesterolemia is one of the accelerating factors of the response.[8,9] Recent studies[18,19] have demonstrated that macrophages from hypercholesterolemic rats adhere more strongly to cultured ECs than those from normocholesterolemic rats. Adherence of macrophages to the

ECs started as early as at 15 minutes after incubation and peaked at 60 minutes.[19] Alderson and co-workers[16] reported that LDL enhanced monocyte adhesion to ECs *in vitro,* and this increase was dose dependent. Their studies also showed that an interaction between LDL and ECs was necessary to induce monocyte adhesion, because simultaneous adding of LDL with monocytes failed to increase the response.

It has been demonstrated that LDL incubated with cultured ECs undergoes an oxidative modification that generates lipid peroxides (oxidized LDL).[25,33] Because blood constituents are constantly exposed to endothelium, plasma LDL (native LDL) must be normally trapped by ECs before entering the subendothelial space. Thus, plasma LDL could undergo biologic modification to form the oxidized LDL that is suspected to contribute, at least in part, to the initiation and progression of atherosclerosis. Recently, evidence for the *in vivo* existence of oxidatively modified LDL was presented by the use of monoclonal antibody assays[27–29] and biochemical analysis.[30] Oxidatively modified LDL has some characteristics different from native LDL in respect to the fatty acid composition,[30] increase in electrophoretic mobility,[33] higher density,[30] promotion of monocyte differentiation and adhesion,[31] and chemotactic activity for monocytes.[26] The chemotactic activity of oxidized LDL is particularly important. For monocytes, oxidized LDL exhibits a chemotactic activity, whereas for macrophages, oxidized LDL inhibits the chemotactic response and shows retentive activity in the vessel wall.[26] In addition, oxidized LDL shows a high affinity for scavenger receptors on macrophages.[25,30,33] Macrophages can easily take up EC-modified LDL through this receptor-mediated pathway[32] and can rapidly accumulate intracellular lipid. In *in vitro* studies, EC modification of LDL appears to be dependent on time, temperature, and concentration. The modification starts as early as 4 hours and reaches a maximum at 24 hours.[33] In our findings, 12-hour incubation of monocytes in the presence of native LDL and HUVEC monolayer was not enough to induce a fatty streaklike lesion. This would be concerned with LDL modification by HUVECs. Because most LDL remains native within 12 hours' incubation and only a small amount of it undergoes EC modification, macrophages cannot accumulate intracellular lipid. In contrast, after 48 hours of incubation, a large amount of LDL undergoes EC modification and can be easily taken up by macrophages, leading to formation of fatty streaklike lesions.

The fatty streaklike lesion demonstrated here may represent a simplification of lesion formation *in vivo.* Atherosclerosis is a complex disorder because of its mixed cell population, composed of endothelial cells,[35,43,44] monocytes/macrophages,[5–11] T lymphocytes,[7,45,46] and smooth muscle cells.[47] Among these cells, it seems likely that monocyte-derived macrophages are really the key players in early atherogenesis, because cells thought to be of monocytic origin are present in developing foam cell lesions,[5–11] where they engulf lipid and form most of the volume of fatty streaks. Further studies employing the monoclonal antibody technique have recently led to the proposal that not only foam cells but also T lymphocytes are the major cell components of fatty streak lesions of human atherosclerosis.[7,45,46] It is our assertion that the model system described here provides a sophisticated approach to assist in understanding the role of monocytes and T lymphocytes in the initiation of atherosclerosis, because recent observations[11,45] suggest that a specific *in situ* cell-mediated hypersensitivity plays an important role in the pathogenesis of human atherosclerosis.

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