INVOLVEMENT OF MACROPHAGES IN ACCUMULATION AND ELIMINATION OF CHOLESTEROL ESTER IN ATHEROSCLEROTIC AORTA

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A striking characteristic of atherosclerosis is the accumulation of a large amount of lipids, mainly cholesterol ester, in the arterial wall. Recently, the involvement of macrophages in cholesterol ester accumulation in atherosclerosis has been emphasized by many workers (2-4, 8, 11, 12, 14). Histochemical and ultrastructural observations showed that the lipids mainly accumulate in the cytoplasm of foam cells in early atherosclerotic lesions (6). These lipid-laden foam cells have been thought to be derived from either macrophages or arterial smooth muscle cells. Studies by the quick-freeze, etching technique showed that the lipids accumulated in foam cells were present as membrane-bound droplets in lysosomes and membrane-free droplets with a concentric lamellar structure surrounded by 10 nm filaments (Amanuma *et al.* submitted for publication). We also suggested that foam cells probably derived from both macrophages and smooth muscle cells, according to monoclonal antibody against foam cells (Kimura *et al.* submitted for publication) and time lapse observation of cultured foam cells by chinemicrophotography (Amanuma *et al.* submitted for publication).

During progress of atherosclerosis, lipids also accumulate in the extracellular connective tissue space. Extracellular lipids have previously been observed with lipid stain by light microscopy, and we recently demonstrated their fine structure by the quick-freeze, etching technique (Amanuma *et al.* submitted for publication). In this paper, we review our new findings on foam cells in atherosclerotic aorta of cholesterol-fed rabbits and WHHL (Watanabe-Heritable Hyperlipidemic) rabbits and consider the role of macrophages in the process of accumulation and elimination of cholesterol ester within and outside the cells.

FINE STRUCTURE OF FOAM CELLS IN ATHEROSCLEROTIC AORTA

There have been many morphological studies on the origin of foam cells. Foam cells have long been thought to be derived from macrophages and, or smooth muscle cells. For the last 20 years, modified smooth muscle cells have been considered to be more important in atherosclerotic plaque formation (6, 9). But recent histochemical and cytochemical studies have shown the presence of foam cells of macrophage origin and the importance of macrophage foam cells has again become recognized (1, 5, 7, 10). To gain further insight into the origin of foam cells and the accumulation of cholesterol ester, we examined atherosclerotic aorta by three new

FIG. 1. Electron micrograph of the cytoplasm of foam cells prepared by the quick-freeze, etching method. The cytoplasm is filled with numerous lipid droplets with an onion-like lamellar structure (arrows). Some lipid droplets surrounded by a membrane (arrowheads) among onion-like droplets may be lipid-laden lysosomes. Few organelles such as the ER and mitochondria are seen in these cells (from Amanuma *et al.* submitted for publication). $\times 6,500$

FIGS. 2a, b. Lipid droplets prepared by floatation sucrose density gradient centrifugation.

a. Scanning electron micrograph. $\times 17,000$

b. Polarized micrograph. $\times 2,500$

techniques; the quick-freeze, etching technique (Amanuma *et al.* submitted for publication) cinemicrophotography (13), and monoclonal antibodies recognizing atherosclerotic lesions (Kimura *et al.* submitted for publication).

Fig. 1 shows a quick-freeze replica of the profiles of typical foam cell. Many

granules ($0.7\text{--}1.7\text{ }\mu\text{m}$ in diameter) with onion-like concentric lamellae are seen in the cytoplasm. These granules are certainly lipids, because of following reasons. For examination of droplets in foam cells, these droplets were isolated on floatation sucrose density gradient centrifugation (13). The particles appeared spherical ($1\text{--}3\text{ }\mu\text{m}$ in diameter) by scanning electron microscopy (Fig. 2-a), and anisotropic cross images were observed by polarized microscopy (Fig. 2-b). These droplets consisted mainly of cholesterol ester (about 95%) and their fatty acid moieties were rich in oleic acid (about 55%). Some lipid droplets surrounded by membranes, which are probably lipid-laden lysosomes, are also seen among the onion-like droplets.

Some of these foam cells may be derived from macrophages. Typical smooth muscle cells containing lipid droplets were also found in the layer of fibrous plaques (data not shown). The longitudinal fractured face of the cytoplasm of these cells consisted mainly of many myofilaments and onion-like droplets, with the same appearance as those in Fig. 1.

ACCUMULATION OF LIPIDS IN EXTRACELLULAR MATRIX AND THE ROLE OF MACROPHAGES

With the progress of atherosclerosis, droplets or granules staining positively for lipid are seen in the extracellular space by light microscopy. We also demonstrated the presence of extracellular lipids by the quick-freeze, etching technique (Fig. 3-a) (Amanuma *et al.* submitted for publication). These extracellular lipids appeared as irregular-shaped vesicles, and some of them were seen to have a membrane on their surface. These extracellular lipids corresponded to vesicles and electronlucent areas among collagen fibers in thin sections (Fig. 3-b). Some of

FIGS. 3a, b. Electron micrograph of lipids accumulated in the extracellular space of atherosclerotic aorta.

- a. Prepared by the quick-freeze, etching method. Large amounts of lipids with a vesicular structure (arrowheads) are observed among collagen fibers. $\times 23,000$
- b. prepared by the thin section method. Lipids are observed as vesicles (arrowheads) and electron-lucent regions among collagen fibers. $\times 17,000$

FIGS. 4a, b. Electron micrograph of rupturing foam cells.

a. prepared by the quick-freeze, etching method. An aggregation of vesicles in the lower right may be disrupted foam cells (arrowheads). A plate crystal is also seen. (from Amanuma *et al.* submitted for publication) $\times 23,000$

b. prepared by the thin section method. A rupturing foam cells (arrowheads) is seen in the lower left. $\times 6,100$

FIG. 5. Electron micrograph of a lipid-laden cell in atherosclerotic aorta. Myofilaments are seen in the cytoplasm. This cell seems to be endocytizing extracellular materials. $\times 5,700$

the extracellular lipids were aggregated into clusters of similar size to the cells (Fig. 4-a). Furthermore, lipid-filled cells that had ruptured (or were destined to collapse) were seen in atherosclerotic aorta (Fig. 4-b), suggesting that some of extracellular lipids are derived from ruptured foam cells. Other possible sources

of extracellular lipids could not be excluded; for example, lipoprotein or denatured lipoprotein may be deposited in the extracellular space.

These extracellular lipids seem to be endocytized and to accumulate in lysosomes, since the appearance of extracellular lipids (Fig. 3-a) was very similar to that of lipids in lysosomes of foam cells (Fig. 1). This possibility was supported by the observation of endocytosis of foam cells in thin sections by electron microscopy (Fig. 5). Thus, foam cells, whether derived from macrophages or modified smooth muscle cells, have high endocytic activity and seem to act as scavengers of lipids accumulated in the extracellular space. Brown and Goldstein (2) proposed a scavenger system in atherosclerotic aorta, since, using modified low-density lipoprotein as a substrate for the scavenger system, they observed marked accumulation of cholesterol ester in cultured macrophages. The extracellular lipids observed in this work may correspond to denatured lipoprotein.

MONOCLONAL ANTIBODIES RECOGNIZING LIPID-LADEN CELLS

Monoclonal antibodies against lipid-laden cells in atherosclerotic aorta were prepared (Kimura *et al.* submitted for publication). Mice were immunized with a delipidated homogenate of atherosclerotic aorta of WHHL. Hybridomas were obtained by fusion and cultured in HAT (hypoxanthine aminopterin and thymidine) selection medium. Specific antibodies were selected by indirect immunohistochemical staining of frozen sections of atherosclerotic aorta. Two clones (FCR1a/201F and FCR1b/904B) producing antibodies specific to lipid-laden cells were established. Two types of immunofluorescence-positive lipid-laden cells were observed: one type was round and was located beneath the endothelium (Figs. 6-a, b). The necrotic cells located at the bottom of the intima, shown were not stained.

Two types of antibody-positive cells, round and elongated, were recognized (Figs. 7-a, b). The round lipid-laden cells, distributed beneath the endothelium and recognized by these antibodies (Figs. 6-a, b, 7-a, b) may originate from macro-

FIGS. 6a, b. Correlation of immunofluorescence of FCR1a/201F antibody staining and lipid deposits. (a) Both intracellular (arrows) and extracellular (arrowheads) lipid deposits in atherosclerotic lesions are stained with oil-red O. (Oil-red O and hematoxylin staining) $\times 400$ (b) Only intracellular lipid deposits (arrows) show immunofluorescence with FCR1a/201F antibody. (Indirect immunofluorescent staining of the adjacent section to that for (a)) $\times 400$

FIGS. 7a, b. Staining with FCR1a/201F antibody of foam cells sitting in subendothelial cells and extending from the internal elastic lamina toward the lumen. a. Assembled foam cells sitting in subendothelial intima (arrows) and intimal smooth muscle cells extend from the internal elastic lamina toward the lumen (arrowheads). (Hematoxylin eosin staining.) $\times 200$ b. Immunofluorescence on staining with FCR1a/201F antibody is restricted to the cytoplasm of both subendothelial foam cells (arrows) and these extend from the internal elastic lamina (arrowheads). Endothelial cells, smooth muscle cells and necrotic area are not stained. (Indirect immunofluorescent staining of the adjacent section to that for (a)) $\times 200$

phages, because their shape and the density of their cytoplasm were similar to those of macrophages. The other lipid-laden cells stained by these antibodies were elongated and extended from the internal elastic lamina toward the lumen (Figs. 7-a, b). These cells are unlikely to be derived from macrophages, because recently Watanabe *et al.* (2) found that monoclonal antibody against macrophages did not stained these elongated lipid-laden cells, although it stained round lipid-laden cells. These elongated cells may originate from smooth muscle cells. Thus, the antibodies of clones FCR1a/201F and FCR1b/904B seem to stain two types of foam cells originated from macrophage and smooth muscle cells.

CULTURED LIPID-LADEN CELLS OBSERVED BY CINEMICROPHOTOGRAPHY

Arterial lipid-laden cells prepared by enzyme digestion from cholesterol-fed rabbits were cultured and observed by time-lapse cinemicrophotography to examine their behaviour. To elucidate whether these lipid-laden cells are derived from macrophages or not, normal arterial smooth muscle cells were introduced into pre-cultured lipid-laden cells. Two types of cells were also observed in this case: one type was incorporated into the monolayer of normal smooth muscle cells and did not locomote freely (Fig. 8, arrowheads). The other type was locomoted freely on the cells layer or through intercellular space (Fig. 8, arrows, a nuclei is migrating between normal smooth muscle cells). These observation suggest that foam cells incorporated into normal smooth muscle cell layer are derived from smooth muscle cells, and foam cells moving freely are derived from macrophages.

To confirm existence of foam cells derived from macrophages atherosclerotic intima were scrapped and maintained in culture. Some lipid-laden cells were

Fig. 8.

Fig. 9.

FIG. 8. Time-lapse cinemicrophotography of lipid-laden cells in the presence of normal arterial smooth muscle cells at 5-minute intervals (from top to bottom). A large lipid-laden cells was migrating through smooth muscle cells monolayer. A nuclei was migrating from right to left between the cells (arrows). A foam cell sat between smooth muscle cell layer was also seen (arrowheads). (Phase contrast microscopy) $\times 450$

FIG. 9. Time-lapse cinemicrophotography of cultured lipid-laden cells scrapped from atherosclerotic intima at 4-minute intervals. A large lipid-laden cells (arrows) was moving freely on the lipid-laden cell monolayer. (Phase contrast microscopy) $\times 450$

spread confluent to form monolayer and the other moved freely on the cell layer (Fig. 9, arrow). These findings indicate that lipid-laden cells were derived from two different species; macrophages and smooth muscle cells.

CONCLUSIONS

In this article, the role of macrophages in cholesterol ester accumulation in cells and elimination of the lipids deposited in the extracellular space were discussed. The cholesterol ester accumulating in atherosclerotic aorta may be derived mainly from circulating low density lipoprotein and from ruptured foam cells. These lipoproteins and denatured lipoproteins are endocytized and degraded by macrophages and modified smooth muscle cells in intimal space of the arterial wall. The cells probably both macrophages and modified smooth muscle cells are transformed to foam cells during progressive accumulation of cholesterol ester. After excessive endocytosis, the foam cells may become overloaded with cholesterol ester and rupture (Fig. 10). Extracellular lipids, which may be harmful to living tissue because of their hydrophobicity, are possibly endocytized and segregated by macrophages as scavenger cells to maintain a hydrophilic environment. In foam cells, lipid droplets are surrounded by either membrane or 10 nm filaments, and so these hydrophobic droplets may be controlled by the cells. Thus, foam cell formation seems to be a mechanism for preventing accumulation of lipids in the extracellular space of atherosclerotic plaques. Macrophages seem to act as scavengers to eliminate extracellular lipids. In this respect, macrophages probably play an important role in cholesterol ester elimination in atherosclerosis.

FIG. 10. Working model of foam cell formation and elimination of cholesterol ester deposited in the extracellular space.

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