METABOLISM AND ULTRASTRUCTURE OF THE ARTERIAL WALL IN ATHEROSCLEROSIS

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SPONTANEOUS atherosclerosis in man begins as a disease of the arterial intima. The morphologic and functional individuality of this intimal lining and the peculiarities of its metabolic requirements and cytologic components have been recently confirmed by findings on biochemical examination and electron microscopic studies.[1, 2]

Although the large arteries of a four-month-old human embryo show three layers, intima, media, and adventitia, their structural evolution occurs gradually and the intima of the aorta is not fully differentiated until the end of the second decade. Indeed its final development may be contemporary with early regressive changes usually considered as senescent alterations, particularly deposition of collagen and subendothelial thickening. These changes may be at first symmetric, but are later made more pronounced by incorporation of elastic elements. They will eventually modify the geometry of the vascular lumen and introduce significant variations in local laminar blood flow.[3,4]

It is therefore difficult to describe the characteristics of the normal intimal lining of large arteries of adult human beings without mentioning some of its earliest pathologic changes.

One of the functionally significant properties of the normal vascular intima is its lack of capillary vessels. Adventitial vasa vasorum do not penetrate farther than the middle portion of the tunica media. This means that both the intima, from 100 to 130 μ thick in the adult human aorta, and the inner portion of the media depend for their nutrition on metabolites filtered from the arterial lumen. Recent in vitro studies on the transport of sodium and chloride in the dog have shown flow gradients from intima to adventitia in the aorta and in the opposite direction in the vena cava.[5] There is also good experimental indication that labeled macromolecules are able to penetrate through the endothelial coat into the intima[6] and that phospholipids are transported fairly rapidly through the same layers.[7] These studies also suggested that concentration gradients of the colloidal constituents of the blood occur during transit through this avascular zone and that the elastic membranes encountered before reaching the capillary bed on the adventitial side of the artery provide further mechanical barriers to large plasma components. It is conceivable therefore that a number of factors extrinsic to the intima may play a most significant role in the regulation of the rate of transport of plasma constituents across this vascular lining.

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For the purpose of this discussion, the arterial intima, including its endothelial coat, is specially considered.

Cytologic Characteristics

The endothelial coat of the aorta of an adult human being is from 0.5 to 1.2 μ in thickness and consists of oval or polygonal cells usually elongated in the direction of the vascular axis and with central nuclei. They show under the electron microscope the usual cell constituents: mitochondria, Golgi zone, extensive endoplasmic reticulum with ribonucleoprotein granules and cytoplasmic vacuoles. Occasionally cell processes project either into the arterial lumen or toward the basement membrane.[8, 9] The typical mosaic configuration of endothelium stained by silver nitrate methods showing an intercellular "cement" has not been confirmed by electron microscopy which instead has shown extremely narrow spaces between cells with electron dense areas resembling *desmosomes* found in other cell types.[10, 11]

The subendothelial elements of the human arterial intima have been morphologically identified as fibroblasts, histiocytes, macrophages and isolated smooth muscle cells containing *myofilaments*, surrounded by a network of fibroelastic components consisting of elastic and collagenous fibers embedded in mucinous ground substance.

In recent years, experimental and clinical developments have provided two new technics for the study of isolated intimal cells both in human beings and in laboratory animals. One approach has been the use of tissue culture methods for the study of full-thickness arterial wall in vitro,[12, 13] and more recently of organ cultures of isolated vascular intima.[14] Organ cultures allow application of cloning technics, with isolation of clones of morphologically similar diploid cells and the preparation of cell suspensions in lipid-free chemically defined nutrients for biochemical and ultrastructural evaluation.[15] The other new procedure is the use of plastic prostheses implanted in the aorta and other large blood vessels of man and animals as a source of pseudointima. This layer has an identifiable endothelial coat and several different cell types, smooth muscle, giant cells, and polymorphonuclear leukocytes, in a framework of collagen, reticulin, and elastin.[10, 16] This pseudointima seems to originate from the deposition of blood cells rather than by the outgrowth of intimal elements from the host artery at the sites of anastomosis. The origin of these cells was demonstrated in dogs by the use of chromosomal markers in male recipients after total body irradiation combined with exchange transfusions of blood from females at the time of implantation of the vascular graft.[17] The pseudointima cannot be considered identical to "normal" intimal lining, not only because of its morphologic and histochemical characteristics (*Fig. 1*) but also because of accelerated atheromatous changes in experimental homografts[18] and plastic prostheses.[19] Similar changes have now been found in pseudointimal coating of long-term homografts and of plastic prostheses in human beings[17] and in thromboendoarterectomized segments.[20]

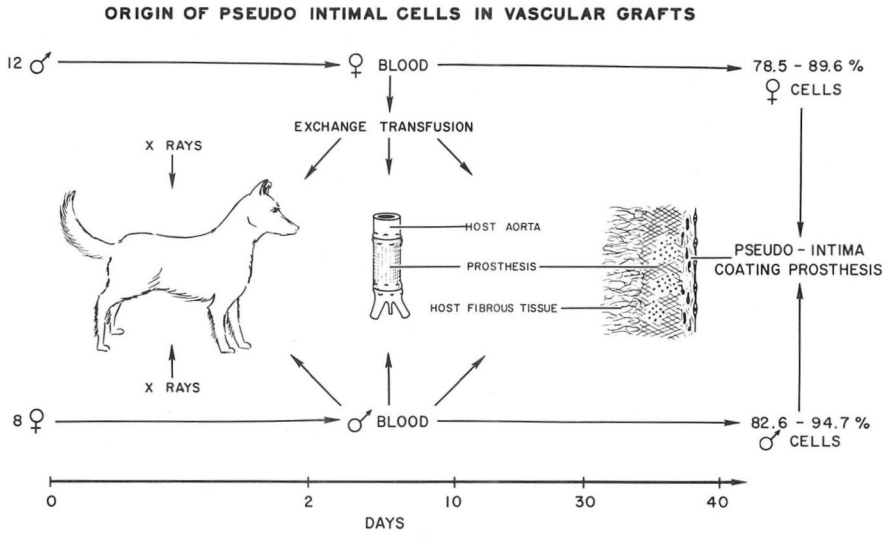

Fig. 1. Origin of pseudointimal cells coating vascular prosthesis. Dogs received total body irradiation preceding exchange transfusion with blood from opposite sex and replacement of a segment of abdominal aorta with a dacron prosthesis. The origin of the pseudointimal cells up to 40 days following transplantation was determined by their chromosomal characteristics. In both male and female hosts, the majority of intimacytes originated from blood cells.

The use of culture methods for isolation of intimal cells in vitro offers considerable advantages for interpretation of metabolic requirements and biochemical changes at cell level under laboratory conditions, particularly when, by employing a chemically defined medium, all metabolites are identified beforehand (*Fig. 2*). Such technics are not exempted from the natural shortcomings of all in vitro systems and the interpretation of data requires meticulous control and careful evaluation before comparison with the in vivo phenomena.

Organ cultures from full-thickness sections of the arterial wall of large and medium-sized arteries taken at autopsy or during reconstructive vascular surgery provide a biologic system for the isolation by growth of intimal cells from other elements of the arterial wall. In such cultures, the internal elastic membrane acts as a natural barrier, preventing contamination of the intimal outgrowth with cells from medial or adventitial layers.[14] Isolated intimal cells may then be harvested and transferred to surface cultures. This procedure supplies adequate cell yields for preparation of suspension cultures in chemically defined nutrients. Under such conditions, in man, pig, and monkey, two intimal cell types have already been identified. These *intimacytes* have considerable morphologic and biochemical differences that are maintained following isolation by cloning in culture flasks.

"Atherophils" are flat polygonal elements growing as close networks and contacting each other with abundant cytoplamic processes. The minimum generation time of these cells in man is from 28 to 39 hours. Under the electron microscope

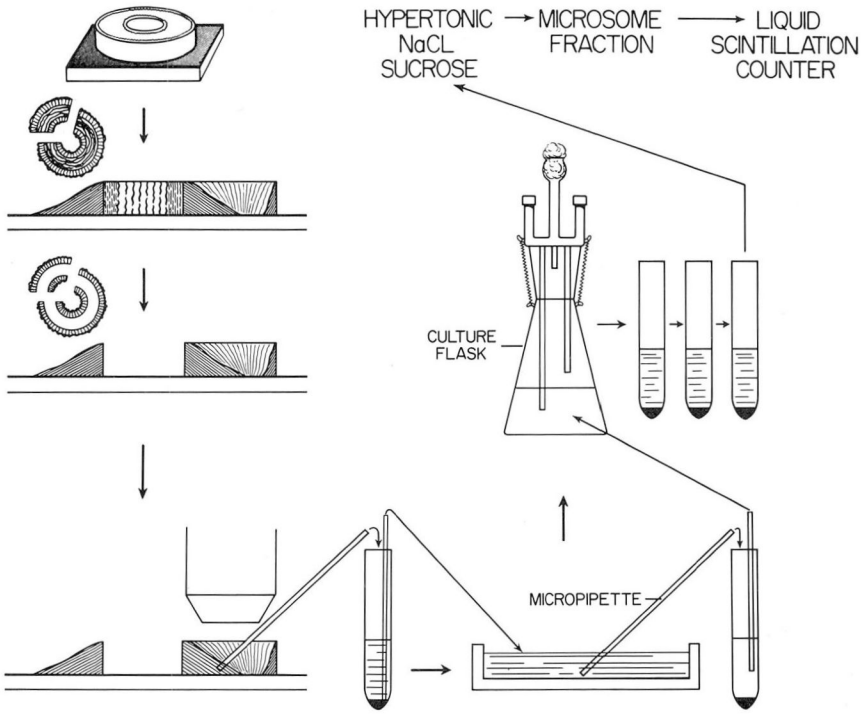

Fig. 2. Flow chart of technic used for isolation of arterial intimal cells (intimacytes) from cross sections of blood vessels in organ cultures. With the internal and external elastic membranes acting as natural barriers to prevent mixing of growing cells, intimacytes are harvested under a dissecting microscope following removal of the original explant. They are then resuspended in balanced salt solution and transferred to monolayer cultures on agar plates where isolated clones are allowed to grow. The two different cell types, atherophils and fibrophils, are separately transferred to suspension cultures in a chemically defined nutrient. Quantitative biochemical studies using labeled compounds may then be carried out using subcellular fractionation methods.

they show single or double oval nuclei and a cytoplasm characterized by the presence of pinocytotic vesicles, Golgi zone, abundant mitochondria, isolated myofilament-like elements, and an endoplasmic reticulum with abundant ribosomes (*Fig. 3*). Histochemically, they show alkaline and acid phosphatase, esterases, cholinesterases and cathepsin activities, respiratory enzymes of the Krebs cycle, and glycogen, all indications of a metabolically active cell.

"Fibrophils" in contrast are spindle-shaped cells, growing as radiating strands that establish contact between cells only by their poles (*Fig. 4.*) The minimum generation time of these cells ranges from 24 to 35 hours. Histochemically, they contain sulfated mucopolysaccharides and qualitatively less glycogen and fewer respiratory enzymes than the atherophils. The fibrophils are also easily distinguishable from atherophils by their reluctance to incorporate extracellular lipids from the

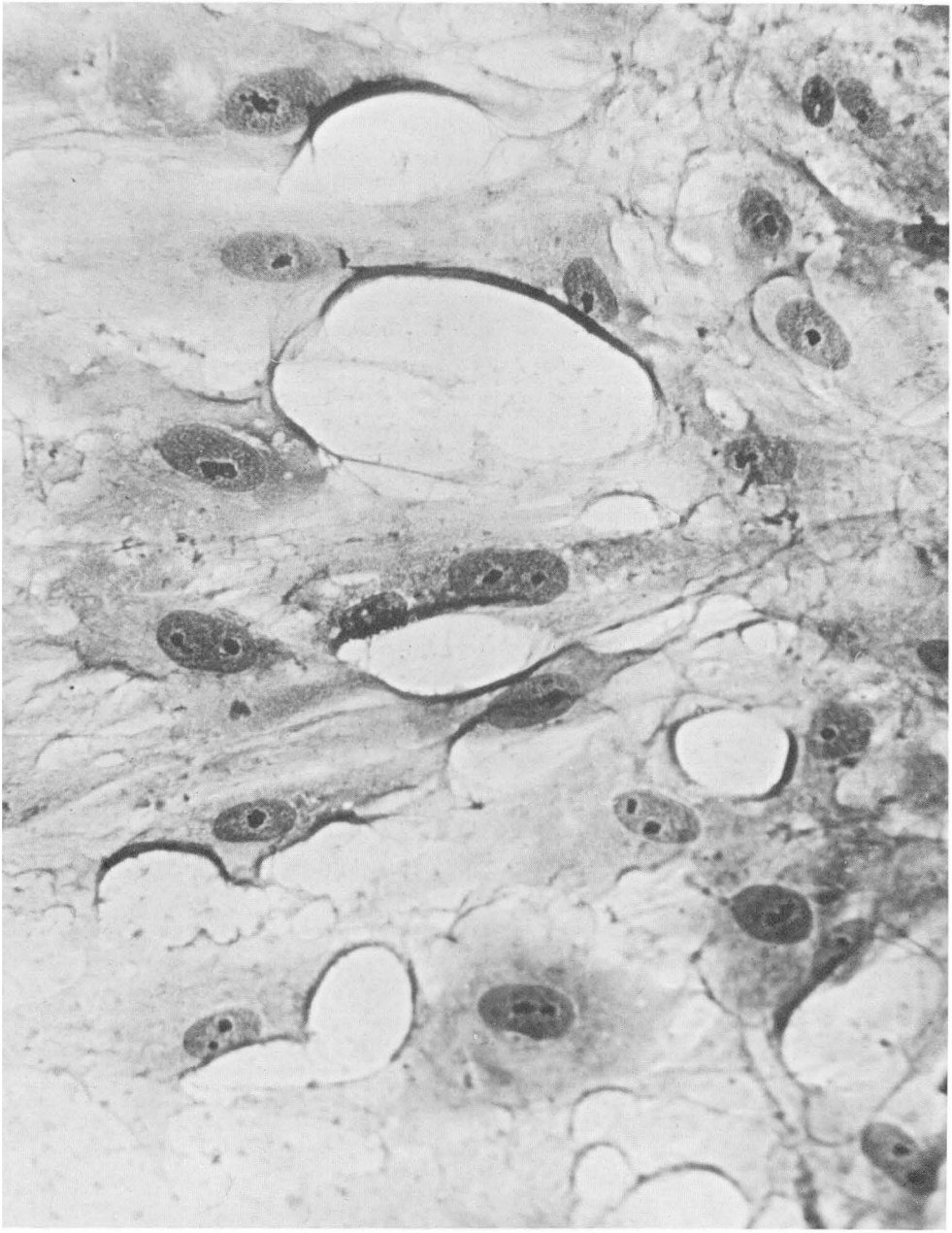

Fig. 3. Atherophils of human arterial intima growing on cell monolayer after isolation. Abundant mitochondria surrounding cytoplasmic vacuoles. Silver carbonate-Janus green stain; magnification X 1600. (From FUNDAMENTALS OF VASCULAR GRAFTING edited by Sigmund A. Wesolowski, M.D. and Clarence Dennis, M.D., Ph.D. Copyright, ©1963, McGraw-Hill, Inc. All rights reserved.)

Fig. 4. Fibrophils of human arterial intima after isolation. Spindle-shaped cells growing in long strands. May Grünwald-Giemsa stain; magnification X 1000. (From FUNDAMENTALS OF VASCULAR GRAFTING edited by Sigmund A. Wesolowski, M.D. and Clarence Dennis, M.D., Ph.D. Copyright, ©1963, McGraw-Hill, Inc. All rights reserved.)

culture medium at concentrations at which there is fast uptake by atherophils. Under the electron microscope fibrophils show a cytoplasm with scattered mitochondria, poorly defined Golgi zone, and absence of significant numbers of pinocytotic vesicles.

After isolation by cloning, the morphologic, histochemical, and biosynthetic characteristics of these two cell types in the human arterial intima are maintained in vitro for periods of up to 10 weeks. No evidence of transformation of one cell type into the other has been observed. Some cell lines derived from such cultures have been maintained for as long as 29 months, but only after significant morphologic and chromosomal changes, giant-cell formation, and profound variations in metabolic requirements.

Cells obtained by growing in organ culture segments of pseudointima of long-term vascular homografts and of plastic prostheses in man, dog,[17] pig, and baboon, are *not* histochemically identical to those mentioned above. These cells from pseudointima may be cloned and maintained as suspension cultures for short periods, have short cytoplasmic connections, one or two nuclei, and considerable acid phosphatase activity (*Fig. 5*). They show abundant pinocytotic vesicles and myofilaments under the electron microscope.

Lipid Metabolism and Biosynthesis

One of the most interesting biochemical parameters used to differentiate the cellular components of the arterial intima, is that of incorporation and synthesis of lipid. Accumulation of lipid is one of the earliest findings in the evolution of spontaneous atheroma in man, establishing a clear distinction between the early fatty streak and the fibroelastic plaque.

Isolated intimal cells provide ideal conditions for the study of early atheromatous changes at cell level. The electron microscope has shown that the earliest stage of fatty streak formation in man is characterized by deposition of lipid in the intima as *intracellular* vacuoles and that it is only after degeneration and lysis of these intimal "lipophages" that lipid deposits appear between cells.[9]

Recent in vitro studies utilizing suspension cultures of atherophils in lipid-free chemically defined nutrient in the presence of labeled serum-bound cholesterol or β-lipoproteins have shown higher incorporation rates (from 65 to 79 percent) by the microsomal fraction of cells obtained from vessels with advanced disease than from control specimens taken from arteries of persons of the same age groups without gross evidence of atheroma (*Fig. 6*).[21] These findings suggest a metabolic inability at cell level to control temporary overloads of lipid, which is sufficiently severe to be transmitted as a phenotypic metabolic abnormality for several cell generations. These lipid-loaded cells or "atherocytes" functionally resemble the behavior of cells found in other metabolic cellular dysfunctions, such as the inability of cells from patients with galactosemia to metabolize galactose in vitro[22] or glucose in those from glucose-6-phosphate deficient mutants.[23]

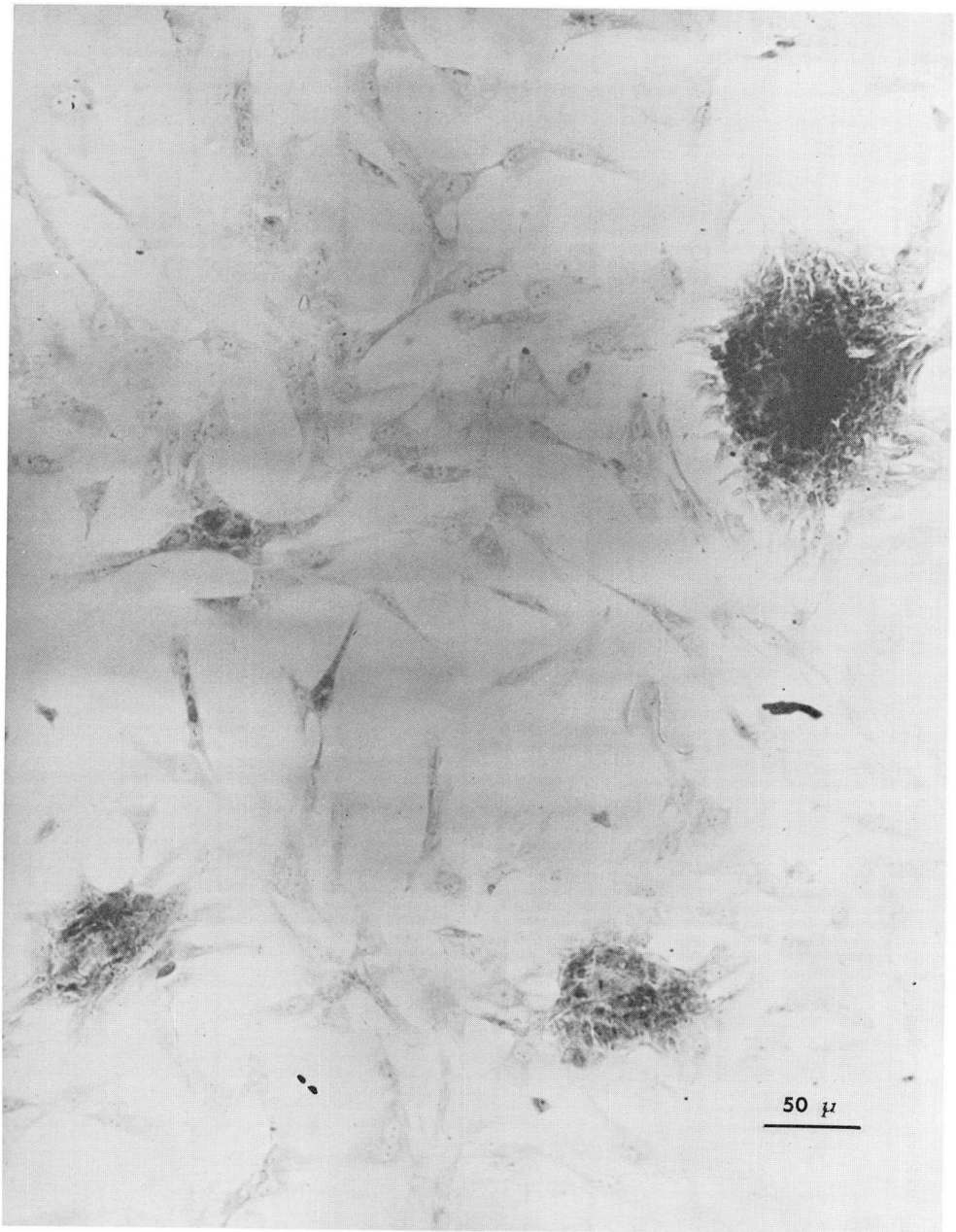

Fig. 5. Clones of atherophils following isolation from pseudointimal coating lumen of woven dacron prosthesis implanted in the abdominal aorta of a patient for 38 months. May Grünwald-Giemsa stain; magnification X 340.

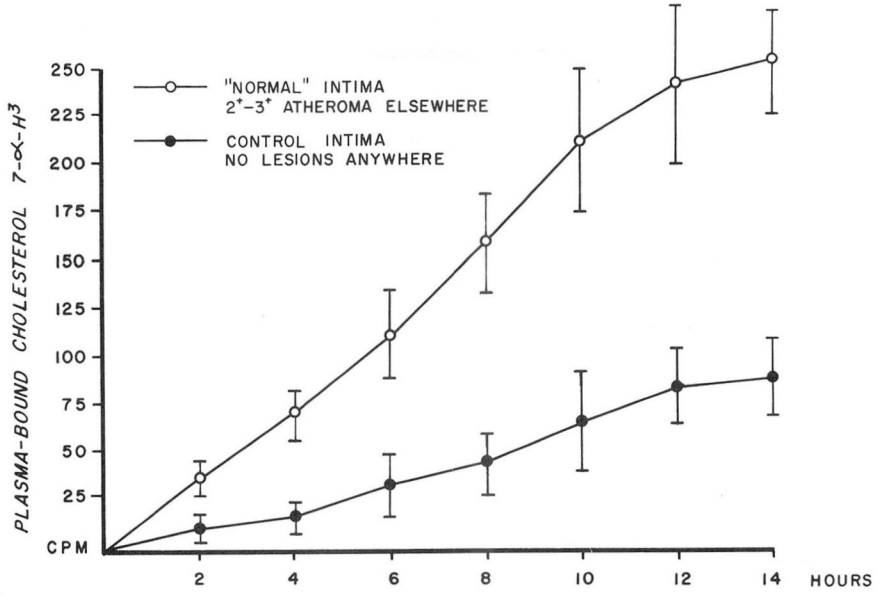

Fig. 6. Differences on intracellular incorporation (microsomal fraction) human coronary intimal cells in vitro 18-26th generation—36 cultures—chemically defined nutrient HACHD 6.

The increased incorporation of extracellular lipids by intimal cells becomes an irreversible process once cytoplasmic lipid droplets large enough to be seen by the optical microscope appear, but it is still reversible if cells are removed from the lipid-containing nutrient at earlier stages of incorporation.[17]

These findings agree with the concept that if the physiologically "masked" micellar state of dispersed cytoplasmic lipids is altered by changes in their physicochemical characteristics as well as in their total lipid-cell content, becoming globular fat, microscopically visible and metabolically inactive, the cell may be so impaired in its functions as to suffer irreversible regressive changes.[24]

The uptake rates of labeled lipid by microsomal fractions of atherophils vary with species, and human and pig cells from normal arterial intima show higher values than those of most laboratory animals with no evidence of spontaneous lesions (*Fig.* 7). These findings are in agreement with evidence that swine are also affected by spontaneous atherosclerosis[25, 26] and valuable in experimental atherogenesis.[27] Pigs are also suitable for studies on the fate of long-term vascular replacements.[28]

The implantation of vascular prostheses in reconstructive vascular surgery has today reached sufficient technical refinement as to require evaluation of their atherogenic properties in long-term implants. In an in vitro study, the incorporation of labeled lipoproteins by microsomal fractions of human cells harvested from the pseudointimal coating of long-term homografts was from 75 to 81 percent and

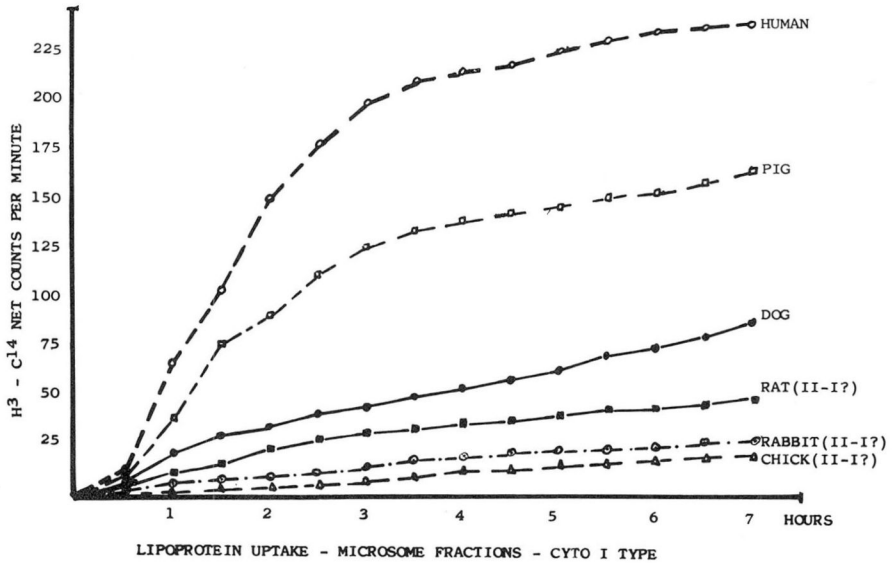

Fig. 7. Species differences in labeled-lipoprotein uptake by microsomal fraction of atherophils (man, pig, and dog). Intimacytes from rat, rabbit, and chick do not provide precise cytologic or histochemical differences for accurate cell isolation after cloning. (From FUNDAMENTALS OF VASCULAR GRAFTING edited by Sigmund A. Wesolowski, M.D. and Clarence Dennis, M.D., Ph.D. Copyright, ©1963, McGraw-Hill, Inc. All rights reserved.)

that of plastic prostheses from 62 to 74 percent higher than that of cells from normal intima (*Table 1*). Since variations in incorporation of lipid were found to

Table 1.—*Cholesterol-4-C^{14} serum—average microsome fraction uptake atherophils*

	cpm*					
Hours	8	12	16	20	24	28
Control	8-12	18-24	31-39	46-51	62-71	74-82
Homografts	14-18	29-31	62-71	82-89	108-114	134-142
Synthetic substitutes	13-15	23-27	46-57	65-76	101-110	122-131

*Average net counts per minute of 16 cultures for each donor.

depend in part on the type of fiber used in the construction of the prosthesis, in vitro evaluation of the properties of intimal cells and of cytotoxicity of the plastic fibers themselves may be of value in the selection of the best prosthetic material.

Considerable evidence has been accumulated as to the ability of the aortas of experimental animals and of human beings to synthesize lipids in situ.[29, 30] Recent studies of the in vitro incorporation of extracellular lipid versus intracellular synthesis were carried out utilizing DL-mevalonic-2-C^{14} as cholesterol precursors. They

showed inhibition (from 48.6 to 85.2 percent) of synthetic lipid pathways after incorporation of large amounts of extracellular lipids.[31] These results indicate that local lipid biosynthesis by human arterial intima does not play a significant role in total lipid content of the cell when extracellular lipids are readily available.

The lipid content of the individual cells of the arterial intima does not depend solely on actual biosynthesis or continuous incorporation from plasma lipids but also on that provided by "foam" cell lysis that contributes an extra source of readily available lipid material (*Table 2*) for local reutilization.[32] This sequential

Table 2.—*Lipid sources for deposition in early atheroma**

Perfusion of Blood Lipids
(hemodynamic and metabolic factors)
↓
LOCAL REUTILIZATION
↑
De novo Lipid Biosynthesis by the
Arterial Wall

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lysis of intimacytes does not require further supply of plasma lipids and in many clinical cases could explain the lack of correlation found between levels of blood lipids and degrees of atherosclerosis. Temporary postprandial hyperlipemia could thus suffice to produce the initial stimulus for intimal deposition in susceptible areas.

Metabolism of Ground Substance

The mucopolysaccharide content of metachromatically stained material in the ground substance of arterial intima has been confirmed by biochemical analysis showing the presence of hexosamine, uronic acid, and acid hydrolyzable sulfate fractions,[33] as well as by the use of S^{35}-labeled sulfate[34] in rabbit aorta. Labeled sulfate incorporation was particularly significant at the intimal surface, and in cholesterol-fed rabbits was found to be localized at the site of lipid-rich lesions. There was a close parallelism between S^{35} distribution and intensity of metachromatic staining.

Noble, Boucek, and Kao[35] showed that total hexosamine concentration of normal portions of aortic human intima is essentially similar to that of atheromatous

areas. These results were confirmed by Dyrbye[36] utilizing S^{35}-labeled sodium sulfate, who found decreased incorporation into hexosamine with age, and by Bottcher and Klynstra[37] who showed relatively constant concentrations of acid mucopolysaccharides in intima-media defatted preparations regardless of severity of atherosclerosis.

In vitro studies utilizing S^{35}-labeled sulfate as a precursor and determining activity of mucopolysaccharides have indicated increased uptake rates by fibrophils in man.[21] These cells also stained metachromatically with toluidine blue and showed considerable activity when studied by autoradiography.

Organ cultures have demonstrated that one of the functions of sulfated mucopolysaccharides in the ground substance is that of regulating transport of macromolecules, acting as polyanions or polyelectrolytes, across the cell plasma membrane[38] (*Table 3*). This function may be even more important for intimacytes than

Table 3.—*Effects of electronsurface charges on vascular cell lipid incorporation from plasma lipoproteins*

Cations	=	Enhancement
Amphoteric	=	No effect
Anions	=	Inhibitory

the effect of mucopolysaccharides as anticoagulants (α-heparin, chondroitin sulfate B and heparitin sulfate)[39] or as cofactors for tissue lipoprotein lipases. Indirect evidence of the opposite effects of cations and anions, including heparin lipid incorporation by cells has also been demonstrated by the electron microscope. In fact, micropinocytosis seems to play only a minor role in the incorporation of extracellular lipids by intimacytes although these cells are able to incorporate ferritin and other colloidal particles by cytosis (*Fig. 8*).

Subendothelial elements seem also to have phagocytic potentialities, as shown by electron microscopy of cytoplasmic components surrounded by a single "unit" membrane so called because it has half the thickness of the cell plasmolema. Many of these vacuoles are rich in acid phosphatase activity and closely resemble *lyosomes* or "digestive bags" described in other cell types by De Duve[40] and Novikoff.[41] In addition to acid phosphatase, these cell components possess a variety of acid hydrolases, such as cathepsin, glucuronidase, acid ribonuclease, acid desoxyribonuclease, phosphoprotein phosphatase and others known to be related both to phagocytosis and to pinocytosis. Several agents seem to mediate their effects on cells by modifying the permeability of lysosomes and thus the release of proteolytic enzymes.

It is suggested that the presence of these intracellular organelles in human intimal cells surrounding fatty streaks may be related to processes involved in intracellular incorporation of protein-coated lipid vacuoles, and the availability of

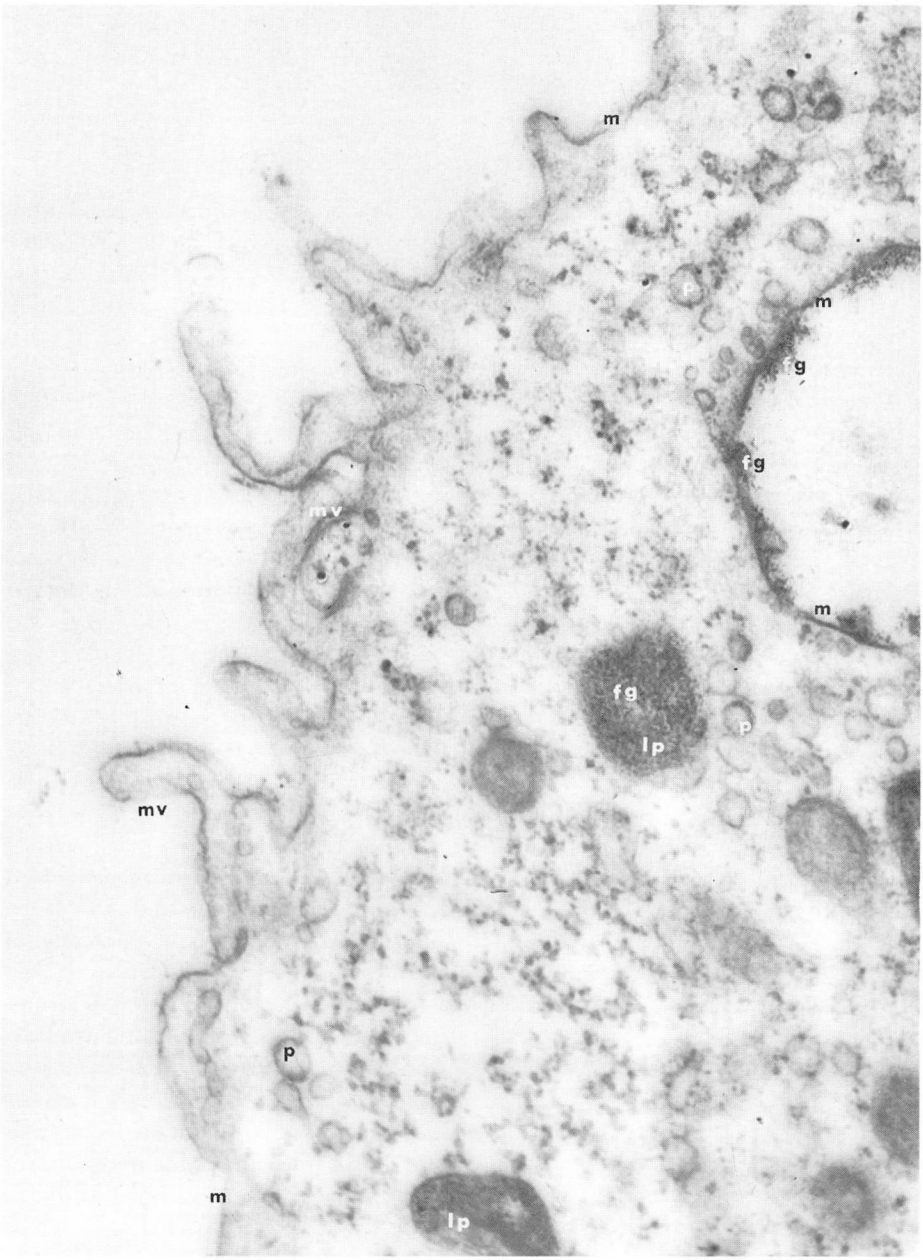


Fig. 8. Electron micrograph of human atherophil after incubation in chemically defined nutrient containing as electron marker a ferritin conjugated serum β-lipoprotein. Abundant microvilli (mv) on cell membrane (m) surface show a few pinocytotic vesicles (p) but considerable amounts of clumped ferritin granules (fg) are already present in lipoprotein-rich cytoplasmic vacuoles (lp). Magnification X 65,000.

compounds that have direct effect on the mechanisms releasing lysosomal enzymes may play a significant role in atherogenesis.

Oxygen Requirements of the Arterial Intima

The arterial intima is no exception to the double pattern of catabolic reactions for energy release: (1) the aerobic or oxidative pathway involving the Krebs citric acid cycle, and (2) the anaerobic or glycolytic cycle for the conversion of glucose or glycogen to lactic acid. It is well known that the oxygen requirements of full-thickness arterial wall of laboratory animals are low compared with those of other tissues such as liver or kidney. However, a relatively high rate of production of lactic acid is found in the presence of oxygen, indicating a deficiency of the regulatory mechanisms that prevent excessive accumulation of lactic acid under normal concentrations of oxygen.

The demonstration of succinic dehydrogenase and cytochrome oxidase in human aortic tissue[42, 43] has now been extended to several other enzymes of the Krebs cycle studies in vitro by a modification of the method proposed by Kaufman and Hill,[44] utilizing cobalt chelated monotetrazolium salts as proposed by Pearse, Scarpelli, and Hess.[45]

Measurements of direct oxygen requirements by isolated intimal cells have been achieved by using cloned human intimacytes either from vein or artery in the cartesian diver respirometer. Oxygen consumption rates were shown to be higher for the aorta with severe atheroma than either for normal aorta or vena cava; even higher oxygen requirements were found for internal cells from diseased human coronary arteries.[46] These results are in agreement with observations of higher rates of respiration by isolated intima of atherosclerotic rabbits.[47]

In vitro studies utilizing suspension cultures under reduced oxygen tensions have shown that human atherophils also are susceptible to hypoxia, and those taken in the vicinity of atheromatous lesions had increased rates of incorporation of extracellular labeled lipoproteins.[46] In those experiments, hypoxia was produced by reducing oxygen concentration below 5 percent and the incorporation of serum-bound C^{14} cholesterol in a chemically defined nutrient was found to be inversely proportional to oxygen level (*Fig. 9*).

Low oxygen tensions inhibited intracellular cholesterol biosynthesis from C^{14} acetate or H^3 mevalonic acid by these cells but caused a significant increase in intracellular squalene concentrations, because of insufficient oxygen necessary for cyclization of squalene into lanosterol.[48]

The effects of local hypoxia in atherogenesis may be represented as a vicious circle in which a number of extrinsic factors such as hemodynamic changes due to mural thrombi, vasomotion or alterations of the normal constituents of the intima, reduce normal flow of metabolites through the avascular zone of the arterial wall. This in turn reduces oxygen supply to such an extent that intracellular changes

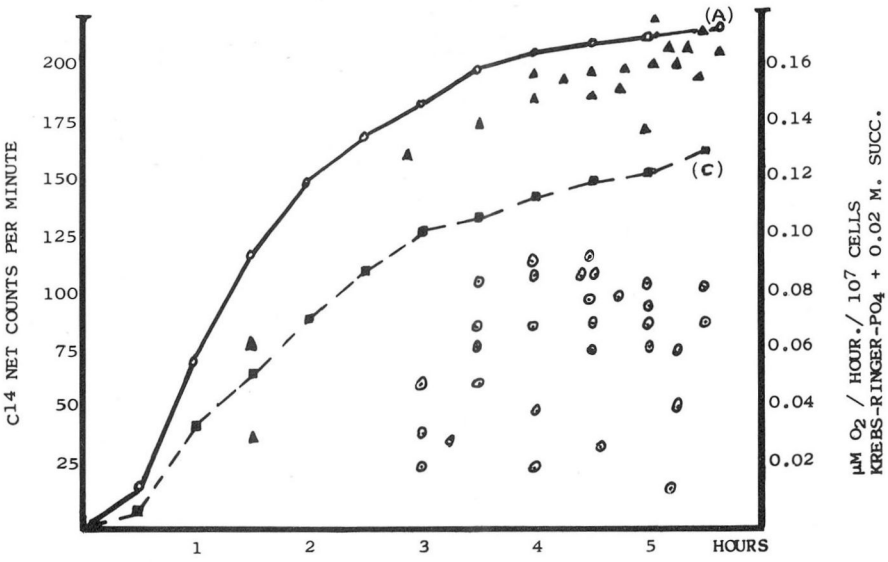
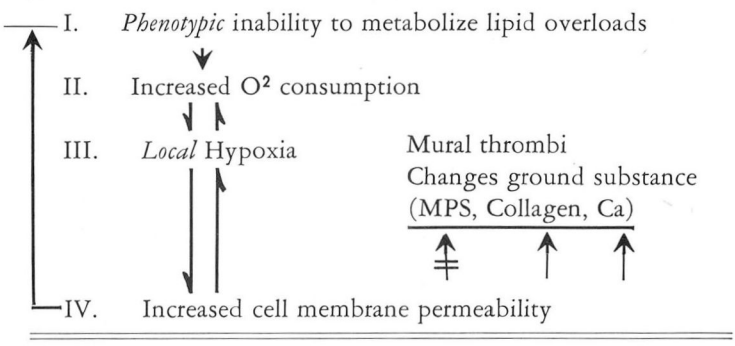


Fig. 9. Comparison between labeled lipoprotein uptake and oxygen consumption rates of human atherophils obtained from intima with severe atheroma (A) and from control disease-free arteries (C). Higher rates of lipoprotein uptake correspond to species with increased consumption of oxygen. (From FUNDAMENTALS OF VASCULAR GRAFTING edited by Sigmund A. Wesolowski, M.D. and Clarence Dennis, M.D., Ph.D. Copyright, ©1963, McGraw-Hill, Inc. All rights reserved.)

described above occur, creating oxygen demands that if not satisfied will produce further hypoxia (*Fig. 10*). The differences found in the oxygen requirements of intimal cells from the vena cava, the aorta, and the coronary arteries may thus represent a biochemical measure of the well-known variations in the incidence of atheromatous lesions in these vessels.

A summary of the present concept on cellular changes in early human atheroma is shown in *Table 4*.

Table 4.—*Cellular changes in* early *human atheroma*



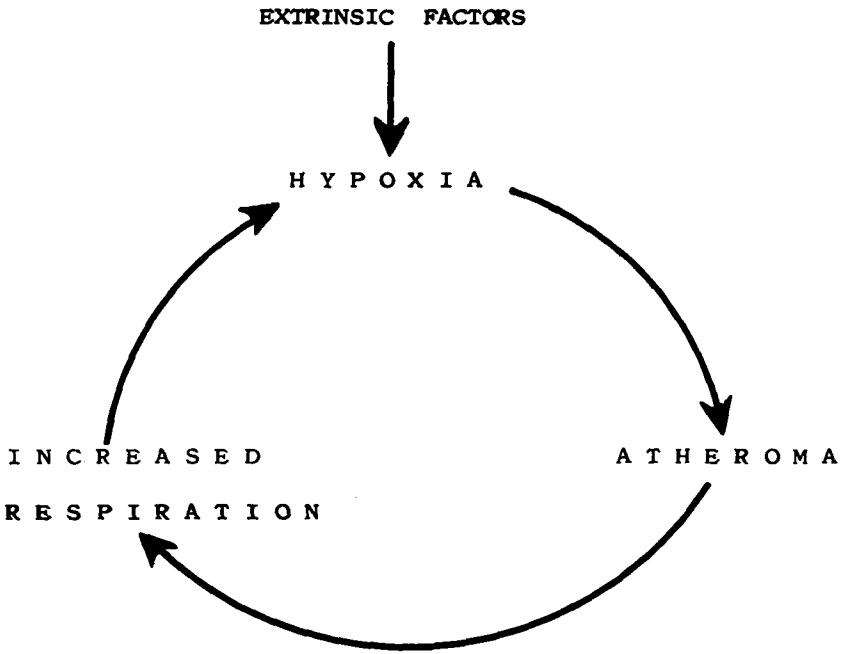


Fig. 10. Vicious circle inducing hypoxia and further atherogenesis. (From FUNDAMENTALS OF VASCULAR GRAFTING edited by Sigmund A. Wesolowski, M.D. and Clarence Dennis, M.D., Ph.D. Copyright, ©1963, McGraw-Hill, Inc. All rights reserved.)

Intimal cells from normal areas of arteries with atheromatous lesions elsewhere show in vitro inability to metabolize lipid overloads. This metabolic abnormality is maintained as a phenotypic character for several generations. Accelerated incorporation of lipid by atherophils and conversion to foam cells or atherocytes is characterized by increased oxygen consumption. If this is not satisfied, it will induce local hypoxia and initiation of a vicious circle. The net result will be increased permeability of the cell membrane of yet unaffected atherophils for serum lipids, and a significant acceleration in the rate of atherogenesis.

Conclusions

The vascular intima is a complex tissue formed by cellular elements that have distinctive histochemical and ultrastructural characteristics and are independent in their metabolic requirements from the rest of the arterial wall. Important species differences exist between man and laboratory animals which must be kept in mind in the evaluation of experimental data.

Some of the enzymatic and biochemical characteristics of incorporation and synthesis of lipid of the donor vessel are maintained in vitro by human and some

animal intimal cells for short periods, providing a useful model for direct evaluation of spontaneous atherogenesis in man and of experimental lesions in animals.

Cells harvested from the pseudointimal lining of long-term prosthetic vascular replacements in patients are not histochemically or metabolically identical to those from normal intima. A careful study of these differences will broaden our understanding of the physiopathology of the arterial wall as a whole as well as provide valuable data on cellular changes in human atherosclerosis.

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