Short Title:

Effects of Low Density Lipoproteins on Cells *In Vitro*

THE EFFECTS OF SERUM LOW DENSITY LIPOPROTEINS ON SKIN FIBROBLASTS AND AORTIC SMOOTH MUSCLE CELLS IN CULTURE

by

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ABSTRACT

Plasma lipoproteins appear to be major factors in atherogenesis.

The effects of human serum low density lipoproteins (LDL) were studied in cultures of human skin fibroblasts maintained in medium supplemented with human platelet-poor, lipoprotein-free serum (PPLFS). LDL increased the proliferation, the size, and the contents of cholesterol and protein of cells, and led to an enhanced accumulation of glycosaminoglycans (GAG) in the medium. The LDL effect on proliferation was abolished by ultrafiltration of PPLFS. Increases in cholesterol and protein were proportional as were increases in protein and cell size. It was inferred that cholesterol and protein accumulated in structural rather than storage forms. Elevations in cellular protein content were caused by a decreased rate of protein degradation.

No LDL effect on proliferation was observed in cultures of porcine aortic smooth muscle cells in PPLFS medium.

No abnormalities of diabetic cell responses to LDL or of the compositions of serum lipoproteins from diabetics were found.

RÉSUMÉ

Des facteurs majeurs dans l'athérogénèse semblent être les lipoprotéines plasmatiques.

L'effet des lipoprotéines sériques a faible densité (LDL) d'origine humaine fut étudiés dans des cultures de fibroblastes de peau humaines maintenues dans un milieu additioné de sérum humain pauvre en plaquettes et dépourvu de lipoprotéines (PPLFS). Les effets produits par les LDL furent une augmentation de la vitesse de réplication cellulaire, une augmentation du contenu cellulaire en cholestérol et en protéines, une augmentation de la grosseur des cellules et un accroissement de l'accumulation des glycosaminoglycanes (GAG) dans le milieu de culture. L'effet des LDL sur la prolifération cellulaire fut aboli par l'ultra-filtration du PPLFS. Les augmentations de cholestérol et de protéine étaient proportionelles ainsi que les augmentations en protéines et en grosseur cellulaire. Nous avons déduit de ces resultats que les augmentations en cholestérol et en protéines étaient de type structural plutôt que d'emmagasinement. Le contenu protéique élevé de cellules est dû à une diminution de leur vitesse de dégradation.

Aucun effet des LDL sur la prolifération cellulaire des cellules de muscle lisse de l'aorte porcine ne fut observé dans le milieu additioné de PPLFS.

La réponse des cellules diabétiques au LDL et la composition des lipoprotéines sériques des diabétiques apparaissent normales.

To Samira,

with love.

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ABBREVIATIONS

VLDL	-	very low density lipoproteins
IDL	-	intermediate density lipoproteins
LDL	-	low density lipoproteins
HDL	-	high density lipoproteins
SMC	-	smooth muscle cells
GAG	-	glycosaminoglycans
MEM	-	minimum essential medium
DME	-	Dulbecco's modification of Eagle's MEM
FCS	-	fetal calf serum
PPLFS	-	platelet-poor, lipoprotein-free serum
PPLFS-f	-	ultrafiltered PPLFS
PLFS	-	porcine lipoprotein-free serum
PBS	-	phosphate buffered saline
BSA	-	bovine serum albumin
HSA	-	human serum albumin
EDTA	-	ethylenediaminetetraacetic acid
TCA	-	trichloroacetic acid
SDS	-	sodium dodecyl sulphate
TRIS	-	tris-(hydroxymethyl)methane
c-AMP	-	cyclic 3',5'-AMP, 3',5'-adenylic acid
P-35	-	petri-dish, 35 mm diameter
T-75	-	flask, 75 cm^2 surface area
T-150	-	flask, 150 cm^2 surface area

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I. SURVEY OF THE LITERATURE

I.A. The Lesions of Atherosclerosis

1. The Lesions in Humans

The lesions of atherosclerosis are situated in the arterial intima. They are characterized by a localized thickening of the intima that appears to be caused by proliferation of smooth muscle cells and an accumulation of the extracellular connective tissue matrix. Both the cells and the surrounding matrix (which comprises collagen, elastin, glycoproteins, and proteoglycans) contain lipid, most of which is free and esterified cholesterol (1). For practical purposes, the lesions have been classified according to the severity of disorganization of the intimal structure. Three types are referred to most commonly: the fatty streak, the fibrous plaque, and the complicated lesion. The exact relation among them, in a developmental sense, remains to be established.

a. The Fatty Streak

Fatty streaks appear on human arterial luminal surfaces as early as three years after birth (2). These lesions are yellow, and flat or somewhat elevated relative to the surrounding intimal surface. The intima is thickened by a proliferation of cells and the accumulation of collagen fibres, elastic fibres, and possibly proteoglycans also. Lipid is present as fine droplets in association with elastic fibres, as small aggregates of these droplets, or as larger aggregates occupying a significant volume of the cytoplasm of stellate

and fusiform intimal cells. In its extreme form, the lipid-laden cell is called a "foam cell", the origin of which is accepted generally as being the intimal smooth muscle cell although some "foam cells" form apparently from mononuclear hematogenic cells (3).

b. The Fibrous Plaque

The fibrous plaque is a more advanced lesion and is probably unrelated to the fatty streak. Smith has found that these two lesions appear to differ in chemical composition (4). Haust has also found difficulty in relating the fibrous plaques, that are more numerous in the abdominal aorta in adults, to the fatty streaks, that are predominant in children in the thoracic aorta (5). The fibrous plaque is characterized by a band of fibromusculoelastic tissue that forms a cap over the lesion. The cells of this cap are smooth muscle, are generally long and slender with few cytoplasmic processes extending into the surrounding tissue, and contain numerous mitochondria, a central endoplasmic reticulum, and peripheral myofilaments (6). "Foam cells" are seen often near the luminal surface of the cap. The deeper regions contain smooth muscle cells in various stages of atrophy and the core of the lesion is composed of electron-dense debris and clefts presumed to be cholesterol crystals (5, 6).

c. The Complicated Lesion

The so-called complicated lesion is presumed to be derived from the fibrous plaque by a progressive involvement of blood elements. Some authors make a further distinction between the complicated lesion (in which there is evidence of hemorrhage, of ulceration, and of thrombosis with or without calcium) and the calcified lesion (in which calcium is visible without overlying hemorrhage, ulceration, or thrombosis) (7). Complicated lesions show evidence of intimal disruption by the presence of red cells, leucocytes, fibrin, and calcium in a thin layer that appears to bridge a defect in the intimal surface. A mass of red cells in the upper intima that has been covered by a fibromuscular cap indicates that hemorrhage has occurred also. In common with the fibrous plaques, these lesions contain masses of lipid-rich grumous material, cholesterol clefts, and flecks of dense material that is probably calcium. While it is recognized that the fibrous plaque in its complicated form is responsible for the occlusive and life-threatening event, it is uncertain what promotes the actual development of an occlusion. Thrombosis is a principal factor although it is sometimes undetectable. Intimal hemorrhage with rapid expansion of the lesion, atheromatous embolism, multiple plaques in major arteries, and vasospasm may each play a role (2). Uncertain also is the reason for the unequal development of lesions in the major arteries: severe atherosclerosis develops more frequently

in the aorta than in the coronary arteries, that in turn are more susceptible than the cerebral, the mesenteric, and the renal arteries (8).

2. The Arterial Lesions in Experimental Animals

Much of the present information on arterial disease is the result of work in animals. The obvious advantage of studying lesions in animal arteries requires no elaboration; the disadvantage does, however, because of possible confusion. It is clear that some species develop a type of arterial disease related only remotely to human atherosclerosis. The dog, for example, develops a degree of medial involvement found rarely, if at all, in humans. A combination of thyroid function suppression and dietary cholesterol has produced proliferation of medial smooth muscle cells, lipid infiltration, "foam cell" production, and disruption of elastic tissue followed by intimal changes. The dog is resistant, however, to the intimal thickening seen in humans (9a). Without the addition of cholesterol to the diet, some frequently-studied animals develop lesions that are quite different from those observed in human atherosclerosis. Thus, changes in rabbit arteries are similar to those changes seen in Monckeberg's medial sclerosis (9b), and lesions in rat arteries appear to have resulted from an acute arteritis (9c). Adding cholesterol to the diet of these animals produces intimal "foam cells" without apparent

vascular injury or regeneration. Prolonged feeding of cholesterol at high levels is often fatal in rabbits, but by alternating diets of high and low cholesterol content, Constantinides was able to produce lesions in the rabbit that resembled the fibrous plaque in humans. The complicated lesion that resulted in myocardial infarction developed rarely, however (9d).

There are available a few species of animal that are prone to a type of arterial disease which has many features in common with human atherosclerosis. Among these are the killer whale and the hippopotamus although, not surprisingly, other species such as sub-human primates and swine have attracted more attention. Atherosclerosis in the monkey and in the pig involves smooth muscle cell proliferation, migration of the cells into the intima, proliferation of fibrous tissue, and the formation of plaques. Moreover, the arteries that are most affected in these animals are the same as in humans (Section I.A.1.); and in the pig, also as in humans, lesions in the aorta appear first in the thoracic aorta. Dietary cholesterol and/or high-fat diets accelerate the progress of the disease in both the sub-human primates and swine (9e, 9f).

Atherosclerosis in humans develops usually over periods measured in decades; studies in animals have to compress the development into much shorter periods. In consequence, it remains uncertain how much of the information that is obtained from this work in animals is useful in the study of the human

disease. With the choice of an appropriate species, however, it is undeniable that the study of arterial disease in animals is potentially of great value in offering insights into human atherosclerosis.

I. B. The Arterial Smooth Muscle Cell

The predominant cell type in the aortic tunica and media is the smooth muscle cell (SMC). In the normal aorta, this cell is spindle-shaped, and partially or completely wrapped by a basement membrane. The nucleus is elongated and has an irregular outline; the cytoplasm contains variable numbers of mitochondria often situated in the perinuclear area and contains a variable amount of endoplasmic reticulum. Numerous myofilaments (100 Å and 40 Å in diameter) are longitudinally arranged in the cytoplasm as are irregularly-scattered fusiform densities. The latter are resolved as condensed myofilaments by electron microscopy. Triangular densities are present, often regularly spaced along the cell membrane and numerous pinocytic vesicles are also seen (10, 11).

The SMC in atherosclerotic areas of arteries often have different fine structure. Parker called these: "modified smooth muscle cells" (12). Also referred to as the myointimal cell, the modified version shows fewer myofilaments that have frequently been displaced to the periphery of the cell; fusiform densities are decreased; basement membrane is either discontinuous or absent entirely; and Golgi, lipid inclusions, mitochondria, and both the smooth and the rough endoplasmic reticulum are increased in size and quantity. Increases in the rough endoplasmic reticulum have been associated with increased activity in the synthesis of components of the connective tissue matrix (3 , 11).

The focal accumulation and fatty metamorphosis of intimal SMC are fundamental to atherosclerosis (1, 5, 13, 14, 15). The cause of SMC proliferation in the development of human atherosclerosis is unknown; dietary cholesterol can cause, however, an increased mitotic rate in intimal cells in pigs within three days (16). This increased proliferation appears to be accompanied by an increased rate of cell degeneration (17). This does not mean that the effect of the cholesterol on cell division is necessarily a direct one since cholesterol may injure the endothelial cells and thereby enhance the permeability of the endothelium to other agents in the blood which may promote proliferation. Intimal cell proliferation contributes to a thickened intima, which arises not only from more cells but from an accumulation of components of the extra-cellular matrix: collagen, elastic fibres, and proteoglycans (13). Several reports have indicated that arterial SMC have the ability to synthesize these components in vivo (18, 19) and in vitro (20-27) and also to take up extra-cellular sulphated glycosaminoglycans by absorptive pinocytosis in vitro (28).

In lesions of atherosclerosis, intracellular fat is seen in droplets, granules, myelin-like figures, and vacuoles. Droplets are round, ovoid, or irregularly shaped, and appear either singly or in a mass which appears to have formed from a coalescence of single droplets. Many more vacuoles are seen in the SMC in advanced streaks than in the small yellow

dots; these cells assume a foamy appearance and are sometimes referred to as myogenic "foam cells" (10). In advanced lesions, the cellular border of the foam cell is disrupted and vacuoles appear in the portions of cytoplasm in the extracellular space. Haust believes that at this point the lesion is irreversible (5); some of the "sloughed" cytoplasm is removed, however, by macrophages (10). A portion of the intracellular lipid is observed to be enclosed in membrane-bound bodies; these have been identified as lysosomes by their positive reaction with acid phosphatase stains (29).

The arterial smooth muscle cell is thus of primary importance in the pathogenesis of atherosclerosis as the major cell type in the arterial intima that may proliferate in a localized area, that may accumulate massive quantities of lipid to form a foam cell, and that is the primary site of metabolism of the components of the connective tissue matrix. More details on each of these factors in atherosclerosis shall appear in later sections of this thesis.

I. C. Plasma Cholesterol and Atherosclerosis

1. Evidence Linking Elevated Plasma Cholesterol Levels with Atherogenesis

A knowledge of the causes of cholesterol accumulation in arterial walls is essential to a complete history of atherosclerosis. It is generally accepted that the risk of developing atherosclerosis increases with elevations in plasma cholesterol levels. Several lines of evidence support this theory:

(a) The addition of a sufficient amount of cholesterol to the diet of experimental animals increases the concentration of cholesterol in the plasma and can produce arterial disease. In some species the disease produced is similar to human atherosclerosis (Section I.A.2.).

(b) Mechanical injury to the arterial endothelium results in the proliferation of smooth muscle cells. This occurs in both normo- and hyperlipidemic animals but only in the latter can one observe the presence of "foam cells" and lipid deposited into the extracellular space. A reduction in platelet survival times and a faster turnover of platelets are also seen in the hyperlipidemic group. It has been suggested that a cause of these effects on platelets is a slower rate of repair of the arterial endothelium (30), a factor which would promote the development of atherosclerosis (13).

(c) Among human populations the incidence of coronary heart disease (C.H.D.) is highest in populations with the highest serum cholesterol levels (31). Conversely, a virtual absence of C.H.D. has been noted in a population in which the mean cholesterol level was relatively low (less than 160 mg/dl) (32).

(d) In any single population a greater risk of developing C.H.D. is associated with elevated plasma cholesterol levels (33-36). In one study, the risk to middle-age men increased four-fold as their cholesterol levels increased from 180 to 300 mg/dl (36).

(e) Gene-determined disorders that elevate plasma cholesterol levels are associated with premature atherosclerosis (37). Individuals who have inherited the homozygous form of familial hypercholesterolemia and who may have cholesterol levels of as high as 1,000 mg/dl frequently develop clinically significant coronary atherosclerosis before twenty years of age (38).

The weight of this evidence provides a firm support for the theory that plasma cholesterol levels predict the rate of development of C.H.D. It has been pointed out, however, that C.H.D. occurs throughout the range of cholesterol levels and that hypercholesterolemia, while undoubtedly a risk factor, cannot be considered to be an essential precursor of atherosclerosis (36).

A direct evaluation of the effect of hypercholesterolemia on atherogenesis depends on determining the origin of the bulk of the cholesterol which forms the lipid core of atheroma. If most of the cholesterol in arterial lesions is derived from cholesterol biosynthesis in arterial smooth muscle cells, then hypercholesterolemia would be only a coincidental abnormality and of no interest regarding the events that lead directly to atherosclerosis. If, on the other hand, most of the cholesterol in lesions comes from plasma, then plasma cholesterol levels would be expected to influence the rate of development of arterial disease.

2. Synthesis of Cholesterol by Arterial Smooth Muscle Cells

When incubated in culture medium containing no cholesterol, mammalian cells synthesize cholesterol (39). The rates of cholesterol synthesis in the arterial walls of several species in vivo were found, however, to be low and generally insignificant compared with the rates of synthesis in the liver (40). It is not known if the rate of synthesis is increased in atherosclerosis.

It has been suggested that cholesterol esters in some types of lesions are formed by local synthesis but the evidence for this is indirect. Thus, several investigators have analyzed the proportions of the most abundant fatty acids present in cholesterol esters and have compared the results

from arterial tissue and from plasma. A similar distribution of fatty acids would imply that the cholesterol esters in the arterial wall were transported there from plasma; a different distribution would imply that the cholesterol esters were formed in situ. Smith found not only an increase in esterified cholesterol in normal intima with advancing age but observed also that the distribution of fatty acids in the esters increasingly resembled the cholesterol esters in the low density lipoproteins (LDL) of plasma (41). Thus, the ratio of oleic acid to linoleic acid in cholesterol esters from the normal intima was 1.5 in children and young adults, and 0.7 in adults over 40 years of age; the ratio in plasma LDL was 0.5. (These values were calculated from Smith's data). It was inferred that the proportion of plasma-derived esterified cholesterol in the intima increases with age. In fatty streaks, in which most of the esterified cholesterol is within fat-filled cells, the oleic acid : linoleic acid ratio was greater than 3.0. Thus in arterial lesions that contain fat-filled cells, it appears unlikely that the cholesterol esters originated in the plasma. Results consistent with Smith's results in fatty streaks have been reported by Portman (40). Rhesus monkeys were made hypercholesterolemic by the addition of butter and cholesterol to the diet and the fatty acids esterified with cholesterol were analyzed in plasma and aortic tissue samples that were taken at various times. The ratio of oleic acid to linoleic acid in cholesterol esters increased in both plasma

and aortic tissue in the hypercholesterolemic animals. After the onset of atherosclerosis this ratio was higher in the aortic tissue cholesterol esters than in the plasma cholesterol esters, a result that suggests that in atherosclerosis cholesterol esters are formed in situ.

Rigid conclusions concerning the origin of esterified cholesterol in atherosclerotic arteries may be drawn from the results above only if it can be demonstrated that the arterial cells possess no capacity for hydrolysis and re-esterification of cholesterol esters from plasma. In the absence of this evidence it may be argued that the arterial wall takes up esterified cholesterol from the plasma and after hydrolysis and esterification with a different fatty acid, produces a pattern of cholesterol esters unlike that in the plasma. The evidence for ester hydrolysis and the esterification of free cholesterol in the arterial wall is summarized below.

3. Cholesterol Ester Metabolism in the Arterial Wall

There is evidence that the vascular wall has the capacity for both the esterification of free cholesterol and the hydrolysis of cholesterol esters. Although the incorporation of fatty acids into cholesterol esters was not detected in cell-free preparations of arterial tissue from rabbits (42, 43), dogs (42), and monkeys (44) that were normal (42-44) and atherosclerotic (43, 44), incorporation was demonstrated in isolated arterial cells in culture (45, 46) and in intact

arterial tissue from atherosclerotic rabbits (47), from normal and atherosclerotic pigeons (48), and from humans (49). Lofland et al. reported that the incorporation of acetate into fatty acids that were then esterified with cholesterol was greater in perfused aortas from atherosclerotic pigeons compared with control pigeons (50). These investigators suggested that the lack of cholesterol-esterifying activity in homogenates, reported by others, resulted from both a period of incubation that was too short and the absence of a structured system which prevented the sequence of labelling of complex lipids necessary in the formation of cholesterol esters. Very little esterifying activity is detectable in the aortas of atherosclerotic rabbits in vivo or in vitro when ^{3}H-cholesterol is used as a precursor (51). Since labelled fatty acids are readily incorporated into cholesterol esters in these systems, it is apparent that cholesterol reaches the site of enzymic activity only with difficulty (52).

The hydrolysis of cholesterol esters has been demonstrated in homogenates of aortic tissue from rats and monkeys (53) and rabbits (54), and in intact rat and rabbit aortas (55). Esterified cholesterol was shown to be hydrolyzed also in cultures of established mouse cell lines (39), and of human diploid skin fibroblasts (56) but there appears to be no report in the literature of cholesterol ester hydrolysis in isolated arterial smooth muscle cells in culture.

It is evident, therefore, that a capacity for metabolism of cholesterol esters exists in the arterial walls. In the light of this, caution is required in the interpretation of results of the experiments referred to above which showed different proportions of oleic and linoleic acids in fatty streaks and in plasma. Since arterial cells can hydrolyze and re-esterify cholesterol esters, different ratios of the fatty acids cannot be used as evidence to rule out plasma as the origin of esterified cholesterol in arterial lesions. In fact, as the following shall show, results from a variety of investigations tend to implicate the plasma as the major source of cholesterol in the accumulations of lipid in atherosclerosis.

4. Transport of Cholesterol from Plasma into the Arterial Wall

The transfer of cholesterol into and out of the arterial wall has been demonstrated *in vitro* in the rat aorta (51), in the atherosclerotic rabbit aorta (51), and in the aortas of normal and cholesterol-fed pigeons (57). Influx of cholesterol was greater in diseased than in normal areas of rabbit (51) and pigeon aorta (57). Dayton and Hashimoto, measuring the transfer of free cholesterol into normal rat aortas, found no difference in uptake after boiling the tissue or introducing metabolic inhibitors (58). From this observation, they inferred that part of the transfer of free cholesterol from

plasma to aorta was due to a process of physicochemical exchange. Newman and Zilversmit (51) and Bell et al. (57) found that free cholesterol accumulated more rapidly than esterified cholesterol in the aorta even though the latter is more abundant in plasma. While this might imply a process of uptake of sterol from plasma that is selective, the physicochemical exchange mechanism referred to above could account for at least part of the additional uptake of free cholesterol. Furthermore, it is possible that some hydrolysis of labelled cholesterol ester may have led to an underestimate of the esterified fraction. Zilversmit suggested that the normal endothelium acts as a metabolic barrier to the influx of cholesterol, that plasma is, however, the main source of cholesterol in atherosclerotic rabbit aortas, and that cholesterol is delivered to the intima by a mechanism that involves its separation from plasma lipoproteins (59). Adams et al. (60) apparently found direct evidence for this mechanism by observing that radio-labelled cholesterol and plasma proteins labelled the vascular wall most heavily on opposite sides. Thus, cholesterol appeared to enter from the lumen while the plasma proteins entered from the adventitial side. In atherosclerotic vessels, the labelling of the walls by cholesterol and by plasma proteins were parallel and thus it was inferred that a barrier to plasma proteins that existed normally at the endothelium was no longer present in atherosclerotic arteries. The interpretation that plasma

proteins normally enter from the adventitial side has been questioned, however, by Bratzler *et al.* who have pointed out that the methods used may not have eliminated either of the possibilities that the radioactivity counted was due to residual blood in the adventitia or that it was non-protein bound (61). Other investigators have attempted to determine if cholesterol can enter the intima while remaining in association with its lipoprotein vehicle by examining directly the transport of LDL into the intima.

5. LDL as a Vehicle for the Transport of Cholesterol into the Arterial Wall

Examinations of the entry of LDL into the aortas of terminally-ill patients (62, 63) and of rabbits (61) have revealed that LDL can penetrate the endothelium and can accumulate in sub-endothelial sections of the vascular wall close to the lumen. Penetration of the endothelium by LDL has been demonstrated also in rat aorta *in vitro* (64). It is evident, therefore, that contrary to the results of Adams *et al.* referred to above, normal aortic endothelium is permeable to some plasma proteins and that since LDL is the major vehicle for cholesterol in the plasma of humans, cholesterol can be transported from the plasma into the vascular wall as part of a lipoprotein molecule.

Further evidence for the transport of LDL into the intima has been gathered from successful attempts to detect

immunologically-reactive LDL protein in human aortas. In one study in adults at autopsy, more immuno-reactive lipoprotein was detected in fibrous plaques than in normal tissue (65). No reactive lipoprotein was found in the aortic wall from a three-year old child or in the "gruel" of advanced lesions in adult aortas. Another study revealed the presence of lipoprotein in intimal sections but not in medial or adventitial sections of human aorta (66). The protein detected in both of these studies was probably apolipoprotein B, the major protein of LDL, although the antisera used were raised against both VLDL and LDL, and VLDL contains significant amounts of apoproteins other than apo-B. Walton and Williamson used an antiserum, monospecific for human β-lipoprotein, to detect, by immunofluorescence, LDL protein in sections of aorta and of other large arteries from humans (67). In combination with a histological technique for detecting lipid, immunofluorescence revealed two distinct lipid pools in atherosclerotic lesions. Positive reactions to the lipid stain and to the antiserum were observed in uncomplicated plaques at all stages of development while only lipid-staining was found in lesions containing large, fat-filled cells. No immunofluorescence was detected in normal sections of the aorta and of the larger arteries of children or of young adults. Immuno-reactive LDL has been isolated from human aortas by Smith and Slater. Aortic tissue was incubated in saline and the extract was ultracentrifuged at the density used to purify LDL (1.063 gm/ml). By using a

quantitative immunoprecipitation technique, they found that intact LDL protein was detectable in fibrous lesions and in fatty streaks at levels higher than in surrounding tissue in the former but at levels lower than surrounding tissue in the latter (68). Although these results may reflect nothing more than the relative ease of extraction of LDL protein from the various tissue samples, the results are in agreement with those of Walton and Williamson referred to above.

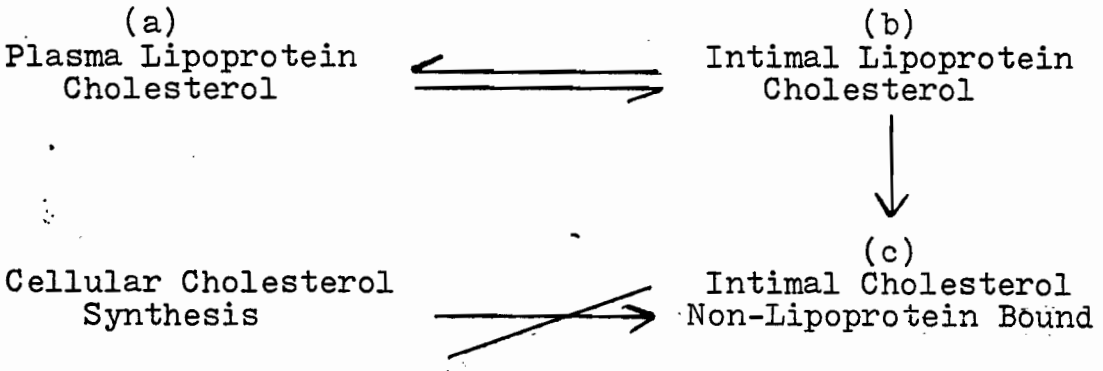
After extracting LDL protein from aortic intimal tissue by electrophoresis, Smith and Slater were then able to estimate the amounts of apo-LDL in the intima of a number of autopsy cases. A correlation was found between the value of intimal apo-LDL and the serum cholesterol level estimated in a sample taken one week before death and it was inferred from this correlation that a constant amount of plasma enters the intima and that a fraction of the LDL in that volume is retained (69). Their data indicated that at plasma levels of 200 mg cholesterol per ml, about one-half of the intimal cholesterol would be present in association with LDL protein and about one-half would be free of LDL, present presumably in cells or in extracellular lipid deposits. Although LDL was isolated from intimal extracts at its hydrated density of 1.063 gm/ml and in other experiments was characterized partially by electrophoresis, the estimate of LDL-cholesterol in the intima was based, nonetheless, on an assumption that the intimal LDL and plasma LDL were identical in cholesterol composition. The amount of cholesterol in

intimal LDL was not estimated directly and thus the calculated distribution of intimal cholesterol in the various forms mentioned may not be correct.

In summary, there is little evidence at present to support the hypothesis that the bulk of the cholesterol in the lesions of atherosclerosis is produced from local synthesis. The arterial wall appears to be able to hydrolyze cholesterol esters and to be able to esterify free cholesterol; the substrates for these reactions appear, however, to originate in the plasma.

There is ample evidence to prove that LDL protein enters the arterial wall and, it is assumed although it has not been conclusively demonstrated, that the molecules of LDL would transport a certain amount of cholesterol with them. It has not been determined if the arterial endothelium is permeable to LDL at all times. Immuno-reactive LDL protein was not detected, for example, in the walls of the arteries of young children although the method may have been insufficiently sensitive. A small amount only may have been present because the arterial endothelium normally is impermeable to LDL or because of the existence of an equilibrium in which the steady-state amount was small. In the light of the relation implied by the results of Smith and Slater that associates the intimal levels of LDL with those in the plasma, low levels of plasma LDL that are found in children would be expected to result in low levels in the intima.

Intact LDL protein was detected in fibrous plaques but not in lesions containing large, fat-filled cells, although Smith and Slater were able to extract some immuno-reactive LDL from fatty streaks. It is apparent, therefore, in the light of the evidence described above that there are three major pools of cholesterol that are important in the pathogenesis of atherosclerosis. These are shown diagrammatically below:



The factors that regulate the equilibrium between pools (a) and (b) most probably include those factors which influence the permeability of the endothelium. From the work of Smith and Slater (69), it may be anticipated that an increase in the size of pool (a) would produce an increase in the size of pool (b). The onset of atherosclerosis occurs, however, when pool (c) increases to a size that begins to cause disruption in the architecture of the vascular wall. Some of the factors that are thought to cause an overloading of pool (c) shall be considered in following sections of this thesis.

I. D. The Glycosaminoglycans in Atherosclerosis

1. Structure and Function

The glycosaminoglycans (GAGs), sometimes referred to as the acidic mucopolysaccharides (AMPs), are unbranched polymers of heteropolysaccharides consisting of repeating disaccharide units. Each unit comprises a hexosamine and a hexuronic acid that are linked by a glycosidic bond. The GAGs combine with proteins by covalent linkage to form proteoglycans. These complexes are predominantly carbohydrate in composition, in contrast with the glycoproteins that are predominantly protein and have a more heterogeneous sugar content. The study of other types of polymers in biology, specifically the proteins and the nucleic acids, has revealed close relations between composition, configuration and function; no such relations have yet been discovered for the polysaccharides and it is not obvious at present what purpose is served by the glycosaminoglycans in the arterial wall. The GAGs are large, flexible, chain-like molecules, often highly charged; and because of their random configuration in solution, they occupy large domains of solvent (70).

The GAGs that are listed in Table 1 are components of the ground substance of the vascular wall, a viscous gel comprising carbohydrates associated with proteins, water, and electrolytes. The ground substance, in combination with cells and fibre, forms the mesenchyme that is present in all organs and that is the site in the arteries at which the lesions of

TABLE 1 The Glycosaminoglycans (GAG)

Old Names	New Names
Acid mucopolysaccharides	Glycosaminoglycans
	Glycosaminoglycuronoglycan (Keratan sulfate is thus excluded)
Chondroitin	Chondroitin
Chondroitin sulfate A	Chondroitin-4-sulfate
Chondroitin sulfate C	Chondroitin-6-sulfate
Chondroitin sulfate B β-heparin	Dermatan sulfate
Heparitin sulfate Heparin monosulfate	Heparan sulfate
Corneal keratosulfate	Keratan sulfate I
Skeletal keratosulfate	Keratan sulfate II
Chondromucoprotein Protein-polysaccharide complex	Proteoglycan

Reference: 71

atherosclerosis first appear. Mesenchymal tissue, as envisaged presently, is a coarse reticulum of protein-GAG complexes that is mechanically stable and of high viscosity (72).

2. Changes in Arterial Contents of GAG with Atherosclerosis

The hypothesis that early changes in atherosclerosis include an alteration and a loosening of the intimal ground substance was proposed over 100 years ago by Virchow (72). It is probable that changes in the ground substance involve changes in the GAGs and studies have produced results to support this idea. Increases in the amounts of GAGs were reported in areas of human aortas showing elastic fragmentation but no advanced lesions (73); a higher ratio of chondroitin sulphates to hyaluronic acid was found in the arteries most prone to atherosclerosis (74); the ratio of chondroitin sulphates to heparin sulphate was higher in the internal iliac artery in which atherosclerosis is frequent than in the external iliac artery in which atherosclerosis is uncommon (75); and higher total amounts of GAGs were reported to be found in the aortas of experimental animals which are most prone to atherosclerosis (76-78). Böttcher and Klynstra and associates were among the first to examine changes in the GAGs that occurred specifically in the intima and reported elevated levels of sulphated and total GAGs in fatty streaks (79). Increases in chondroitin sulphate C, decreases in hyaluronic acid, and reduced levels of total GAG were measured

in more advanced lesions (80). Kumar et al. (81) and Murata and Oshima also found reduced levels of total GAGs in advanced lesions but the latter group measured a decrease in fatty streaks (82). Stevens et al. found decreases in total GAG in the intima as the extent of atherosclerotic involvement increased. Amounts of chondroitin sulphates B and C increased and decreased respectively and the amount of hyaluronic acid was unchanged (83). Wagh et al. also found total GAG to be lower in fatty streaks (84). Others, however, reported no changes in GAG at any stage in atherosclerosis (85).

The variety of results is explained possibly by the variety of techniques used , but once explained, it is no less difficult to reconcile the seemingly opposite conclusions that are drawn. Possibly the most frequent finding is that the amounts of the sulphated GAGs, in particular the chondroitin sulphates increase in fatty streaks and increase also in comparison with amounts of hyaluronic acid (79-81, 83, 84). An increased metabolism of sulphated GAG was found in experimental animals which were fed on a high fat, high cholesterol diet that produced lipid deposition in the arterial walls of some species. Several reports have indicated that this diet produces an increased uptake of ^{35}S by the vascular wall and an elevated rate of turnover of sulphated GAG (86-88). It has been found, for example, that a species resistant to atherosclerosis, the rat, when fed cholesterol, accumulated little lipid in the aortic wall and exhibited no changes in the

rate of synthesis of aortic sulphated-GAG. In contrast, a marked accumulation of lipid in the aortic walls of rabbits fed the high cholesterol diet was accompanied by an increased incorporation of ^{35}S into GAG (88). On the basis of more recent evidence, Telner and Kalant have suggested, however, that ^{35}S incorporation may not be a reliable index of GAG synthesis (89). It is not known at present whether or not lipid deposition and changes in the GAG content of arterial walls are related causally. For the reasons given below, however, it is thought that GAGs exert some control over the movement of plasma lipoproteins in the vascular wall and that any changes in GAGs which could further restrict this movement might result in lipid deposition.

3. <u>The Role of GAGs in Lipid Deposition in the Arterial Wall</u>

a. <u>The Effects of GAGs on Transport and Solubility of Lipoproteins in Solution</u>

Polysaccharides form a molecular chain network that acts as an effective sieve when other macromolecules are forced through it. This effect has been demonstrated in the ultracentrifuge and it has been shown that the sieving effect on plasma proteins is enhanced when polysaccharides which are highly charged, are unbranched, and are of high molecular weight (90). The sieving effect of hyaluronic acid on human plasma lipoproteins was examined by Iverius (91). The effect

was increased by raising the concentration of hyaluronic acid and was greater with lipoproteins of increasing size. Thus, the sedimentation rates of high density lipoproteins (HDL) were less affected by the presence of hyaluronic acid than those of low density and very low density lipoproteins (LDL and VLDL).

Iverius described another effect of GAGs in solution with plasma lipoproteins: that of steric exclusion (91). Since molecules have finite size and since their centres cannot approach closer than the contact distance, they will deny each other space in solution (70). The activity coefficients of plasma lipoproteins were measured in 1% solutions of dextran sulphate of increasing molecular weight and were shown to increase as the molecular weight of the dextran sulphate increased (91).

The effects of molecular sieving and of steric exclusion were both demonstrated <u>in vitro</u> and it is not known how important these effects are on lipoprotein transport and solubility in the arterial wall <u>in vivo</u>. It can be shown on theoretical grounds that a solute that is transported through a network at a reduced rate resides within the network at an increased concentration (91). There is evidence that the major vehicle of plasma cholesterol, LDL, can enter the normal intima (Section I.C.5.). Once inside the arterial wall and under hydrostatic pressure, LDL would presumably be subjected to the sieving effect of the GAG and this sieving would result

in an increased concentration of LDL in the intima. In addition, the GAGs would be increasing the activity coefficient of LDL by steric exclusion. These two effects could then combine to reduce the solubility of LDL and to increase the probability of its precipitation. The precipitation of significant amounts of LDL might then lead to accumulations of cholesterol in the extracellular space.

b. Lipoprotein-GAG Complexes

Another effect of the GAGs on lipoproteins (excluding HDL) that has been demonstrated in vitro is the formation of complexes, both soluble and insoluble. The original observation that plasma β-lipoproteins (LDL) may form a stable complex with sulphated polysaccharides was made by Bernfeld et al. (92). It was then shown directly by several investigators that sulphated GAG from aortic tissue could also form complexes with plasma LDL in vitro (93-96). The exact natures of the complexes formed have not been resolved. Iverius found that a sulphated GAG would form a complex with either LDL or VLDL (but not HDL) at pH 7.4 if a critical electrolyte concentration was adhered to (97). He proposed a linkage between NH_4^+ groups on apolipoprotein B and sulphate anions on the GAG. In support of this, the acetylation of LDL (to block the NH_4^+ groups) or the replacement of LDL by HDL (which contains no apo-B) or the replacement of sulphated GAG by hyaluronic acid (which contains no sulphate groups) prevented the formation of complexes.

Srinivasan et al. envisaged, however, a calcium bridge that connected sulphate anions in the GAG to phosphate anions in the lipoprotein phospholipids (96). There is, however, good evidence that this type of linkage is unlikely: HDL did not form complexes although this type of linkage would predict that it should (96); and the removal of up to 58% of LDL phospholipids did not impair the formation of complexes of LDL with dextran sulphate (98). It is unlikely also that the concentrations of calcium (12-20 mg/ml) used by Srinivasan et al. in their studies would be comparable to the calcium concentrations in the interstitial fluid of the arterial intima.

Several attempts have been made to extract intact GAG-LDL complexes from arterial tissue. Tracy et al. studied the electrophoretic mobility of components of a salt extract of aorta and from the results inferred that intact GAG-LDL complexes were present in the extract (99). Srinivasan et al. incubated tissue from areas of aorta containing fatty streaks (100) and fibrous plaques (101) in isotonic saline at 4°C with gentle shaking. The extracts were examined for uronic acid in association with plasma lipoproteins and the results from several methods of analysis indicated that GAG-LDL and GAG-VLDL complexes were present in the extracts. The mildness of the extraction procedure indicates that the complexes are not firmly anchored in the lesion tissue. Camejo et al. have also isolated a factor from intima-medial sections of human aorta which formed insoluble complexes with plasma lipoproteins

in vitro. This "lipoprotein-complexing factor" contained very little uronic acid, however, and thus cannot be a glycosaminoglycan. A partial characterization suggests, however, that it is a glycoprotein (102, 103).

It is not known if changes in GAGs precede or follow the deposition of lipid in the extracellular space. There is much evidence to suggest that GAGs can actually cause the precipitation of plasma LDL and thus promote the accumulation of insoluble cholesterol but except in the case of the GAG-LDL complexes that Srinivasan et al claim to be able to extract from atherosclerotic lesions, this evidence has come from studies of interactions of GAGs and lipoproteins in vitro.

In summary, there is much suggestive evidence that the GAGs are involved in atherogenesis. Changes appear to occur in the proportions of individual GAGs in the atherosclerotic artery but there is presently no consensus as to what these changes are. Investigators have frequently found, however, an increase in the content of chondroitin sulphates relative to hyaluronic acid in the atherosclerotic arterial wall. The complexes formed between GAG and LDL and the GAG's effect on the solubility of LDL support a case for changes in GAG composition as initiating factors in atherogenesis. Much of this information is derived, however, from studies in vitro and its relevance to atherosclerosis in vivo remains to be established.

I. E. The Use of Cell Culture in the Study of Atherosclerosis

1. Introduction

The task of identifying the factors that initiate and aggravate the lesions of atherosclerosis is a complex one that has required the use of a variety of techniques. Among these techniques, cell culture offers an effective means of analyzing cell responses to environments that may be manipulated in a highly controlled manner.

This part of the literature survey shall deal with those aspects of atherosclerosis which have been studied in cell cultures. Four main topics shall be discussed: the effects of serum components, particularly the lipoproteins, on the proliferation of cells; the accumulation of cholesterol by cells in culture; the metabolism of LDL by cells in culture; and the production of constituents of the arterial connective tissue matrix, especially the GAG, by cells in culture.

2. The Effects of Serum Components on the Proliferation of Cells in Culture

It is accepted widely that smooth muscle cell proliferation is an early event in atherogenesis. In consequence, but perhaps more because of the general interest in biology and medicine in the control of cell proliferation, the effort to reveal "growth factors" has been great (104). Some mammalian cell mitogens that are of importance in the study of atherosclerosis are listed in Table 2. It is seen in this table that there is considerable evidence for a role for exogenous lipid in

TABLE 2 Factors Possibly Involved in the Proliferation of Aortic Smooth Muscle Cells in Culture

FACTOR	SPECIES	REFERENCE	REMARKS
Hyper-lipidemic serum	Rabbit	105	Qualitative methods; increased cell proliferation & degradation
	Rabbit	106	Increases in cell counts, cell size & cell death
	Rabbit	107	No increases in cell numbers; (control was fetal bovine serum)
	Rat	108	Increase in area of cultures
VLDL, LDL from hyper- and normo-lipidemic sera	Monkey	109,110	Increase in area of cultures; effect of hyperlipidemic LDL greater than normolipidemic LDL
VLDL	Miniature pig	111	Increase in cell numbers; no differences between lipoproteins from hyper- & normolipidemic sera
LDL	Monkey	15	Increase in cell numbers when LDL was added to a non-cholesterol containing serum
	Miniature pig	111	As for VLDL in miniature pig
Insulin	Monkey	112	Increases in cell numbers small compared with whole serum
	Rabbit	113	No increases in culture areas
Growth hormone	Rabbit	113	Small increases in culture area at concentrations greater than 1 ng/ml
Platelet factors	Monkey	114,115	Increases in cell numbers; major mitogen in serum?
Fatty acids and prostaglandins	Guinea pig	116	Inhibition of cloning of cultured cells by some fatty acids and their prostaglandin derivatives

the proliferation of arterial SMC *in vitro* and that this is true particularly when the lipid is within the low density lipoproteins (VLDL and LDL). The literature on this subject goes back to 1965 when Myasnikov and Block reported elevations in the rates of migration, proliferation, and degeneration of rabbit arterial cells (of mixed type) when normal rabbit serum was replaced in the medium by hyperlipidemic serum from rabbits fed with cholesterol (105). A similar effect of serum from swine fed with cholesterol on the mitotic activity of cells (again of mixed type) in pieces of aortic tissue was reported by Daoud *et al*. Following ultrafiltration of this serum, the "growth factor" was found to remain in the residue although the ultrafiltrate, while producing no effect by itself on mitosis, amplified the effect of the high molecular weight factor (117).

Daoud *et al*. observed the presence of several distinct types of cells in these explants which they described in an earlier report (20). Among these types the smooth muscle cell was present in both its usual form and a "modified" version which was characterized by fewer myofilaments, a large dilated granular endoplasmic reticulum, a smaller Golgi apparatus, and few mainly perinuclear mitochondria.

The difficulties of interpretation of results from cultures of mixed cell types were avoided by Ross who, by removing only the intima and inner media from animal aortas, obtained cultures of cells that maintained the phenotype of

differentiated smooth muscle for up to ten weeks (26). Simian SMC proliferated in medium containing serum from which lipoproteins were removed but the rate of proliferation was enhanced by adding back LDL. This effect was due neither to the amount of cholesterol in LDL nor to a general property of lipoproteins as was demonstrated by the failure of an equal amount of cholesterol in HDL to produce the same degree of stimulation (15).

Later reports have tended to confirm this observation although the methods used have not always been comparable. Thus, Fischer-Dzoga and colleagues found an effect of LDL on the proliferation of simian SMC but believed it was produced by LDL isolated only from hyperlipidemic serum. The ability of LDL to stimulate proliferation of cells in an explant of simian aortic media was assessed by measuring the area of the culture (109, 110). This method has been adopted by others (108) but it is susceptible to certain errors to which cell-counting is not. Both cell migration and the tendency of SMC in culture to form superimposed layers conflict with the assumption that cell number and area of culture increase in parallel. Brown et al. have in fact found no difference in the effect of LDL on porcine SMC counts when the lipoprotein is isolated from either normo- or hyperlipidemic serum (111). Nevertheless, while the effect of LDL from the blood of hyperlipidemic monkeys may not be on cell proliferation, it is clear that a difference exists in its action on cells compared with the action of LDL

from normolipidemic monkeys. It would be of great interest to know both the effects, and their exact causes, of hyperlipidemic serum lipoproteins on cells in culture.

Hyperlipidemic serum has been shown to produce three major effects on SMC in culture: increased mitotic activity, increased cell death, and increased cell size (106). The constituents of serum which caused these changes were not identified.

While it is clear that exogenous lipid, particularly in low density lipoproteins, promotes the proliferation of arterial SMC in culture, the effects of some of the other "growth factors" in Table 2 on mitosis is less certain. Insulin produced increases in cell numbers when added to a medium containing 1% whole serum but the effects of 0.1 mU/ml and 10 mU/ml were not in general substantially different and in neither case were cell numbers (in three of five cultures) close to those in 5% whole serum medium (112). The claim that growth hormone promotes the proliferation of aortic smooth muscle is even more questionable since increases in cell numbers of the order of 5% were reported. While this difference was significant statistically, the significance of such a small effect in vivo is debatable. The most striking mitogenic activity of all the serum components listed in Table 2 resides in the platelet factors. One or more of these factors stimulate proliferation in cultures of 3T3 cells (118, 119), simian skin fibroblasts and aortic SMC (115), human glial cells (120), bovine aortic endothelial cells (121), and human aortic SMC (122).

In contrast, some fatty acids and their prostaglandin derivatives have been shown to inhibit the proliferation of guinea pig aortic SMC. It was suggested that the availability of fatty acids for prostaglandin biosynthesis represents a control mechanism for cell division (116).

3. The Accumulation of Cholesterol by Cells in Culture

The accumulation of cholesterol within cells and in the extracellular matrix of the intima is a characteristic feature of the lesions in atherosclerosis. The use of cells in culture has been of particular value in the study of the regulation of cellular cholesterol content. Rothblat and Kritchevsky, in a review of the literature before 1968, came to the following conclusion (39):

(a) the mean ratio of esterified : free cholesterol in cells in culture is about 0.5. All but one of the cell types listed were established lines that are heteroploid. In the one euploid strain, WI38, derived from human embryonic lung, a ratio of 0.3 was found;

(b) cells in culture can synthesize cholesterol but are unable to degrade it;

(c) cholesterol biosynthesis is inhibited by cholesterol in the extracellular medium;

(d) cells in culture can both esterify cholesterol and hydrolyze cholesterol esters;

(e) free cholesterol, but not esterified cholesterol, is excreted from the cell;

(f) intracellular cholesterol levels are determined by the form in which cholesterol is presented to the cells. Thus,

(i) mouse lymphoblasts were found to accumulate more cholesterol from medium containing rabbit serum than from medium containing an equal amount of cholesterol in human serum;

(ii) mouse lymphoblasts maintained a constant intracellular cholesterol level even though the extracellular level was varied from 160 ug/ml to 800 ug/ml;

(iii) non-lipoprotein-bound cholesterol was accumulated with a greater avidity than lipoprotein-bound cholesterol;

(iv) phospholipids appear to reduce the accumulation of cholesterol by cells in culture.

The accumulation of cholesterol by cells in culture continues to be of great interest particularly now that techniques are available to produce from mesenchymal tissue homogeneous cultures of cells that remain diploid and appear to preserve their differentiated state in vitro.

Cells that are cultured in medium to which serum has been added contain more free cholesterol than esterified cholesterol (39, 123). When the whole serum is replaced by lipoprotein-free serum, the total cholesterol content falls and the free : esterified cholesterol ratio rises. The addition of LDL to the medium increases the total cholesterol content but reduces the

free : esterified cholesterol ratio. The amount of cellular free cholesterol remains, however, higher than the amount of esterified cholesterol. These changes have been observed in cultures of cells from a variety of species: mouse fibroblasts (124), human fibroblasts (123, 125), porcine SMC (126), and canine SMC (126). Comparable results were reported from experiments in which fetal calf serum was replaced by hyperlipidemic serum in the medium of cultures of rabbit SMC (127) and simian SMC (45).

A role of HDL in regulating the cholesterol content of cells by accepting free cholesterol at the plasma membrane was postulated originally by Glomset (128). This hypothesis is supported by the results of studies in which it was shown that delipidated HDL that was recombined with lecithin or sphingomyelin removed cholesterol from Landschütz Ascites cells (129) and that HDL removed endogenous sterol from mouse fibroblasts (130). The removal of cholesterol from cells by HDL is not, however, the only mechanism by which HDL influences the cell cholesterol content: HDL appears also to inhibit LDL binding to cells, thus preventing the deposition of cholesterol from this lipoprotein (131, 132).

4. <u>The Metabolism of LDL by Cells in Culture</u>

It is believed generally that most of the LDL circulating <u>in vivo</u> is degraded ultimately in the liver. The experimental evidence for this theory includes the observations that following the injection of ^{125}I-LDL into the rat (133) and into humans (62, 63), ^{125}I-activity accumulated most rapidly in

the liver. In the light of more recent work it is apparent, however, that the liver is one of many potential sites of LDL degradation. In the pig, following hepatectomy the fractional catabolic rate of ^{125}I-LDL was shown to increase rather than decrease as the theory above would predict (134). This result suggests not only the existence of non-hepatic sites of LDL degradation, but it implies also that a function of the liver is to stabilize LDL.

It is now evident from a variety of studies that skin fibroblasts and arterial SMC, among other non-hepatic somatic cells in a variety of species are able to degrade LDL. Furthermore, the degradation of LDL is part of a plasma membrane receptor-mediated mechanism that regulates the metabolism of cholesterol within the cell. In essence, the so-called "LDL pathway" comprises the following steps (135, 136).

(a) LDL is bound at the plasma membrane at a high-affinity site which exhibits saturation kinetics at low lipoprotein concentrations. It was thought originally that the binding site was specific for apolipoprotein B and thus could bind only VLDL and LDL (137). It is now known, however, that a cholesterol-rich lipoprotein, HDL_c, that contains mainly apo E, apo AI, and apo C but no apo B, and is found in the plasma of swine and dogs fed with cholesterol, possesses a binding activity at the same site that is 10- to 100-fold greater than

that of LDL (138). It has also been demonstrated that HDL at a molar ratio higher than 5 : 1 with LDL, inhibits the binding of LDL although the mechanism for this inhibition is not known at present (131, 132).

(b) The high-affinity receptor bound-LDL is internalized by a process that requires metabolic energy.

(c) The internalized LDL is degraded by lysosomes. Apo B is hydrolyzed rapidly and completely and lipoprotein cholesterol esters are hydrolyzed to free cholesterol. The receptor-mediated process of internalization and hydrolysis is saturated in human skin fibroblasts at LDL concentrations of about 50 ug/ml medium. Chloroquine, an inhibitor of lysosomal degradative processes, inhibits LDL degradation and causes an accumulation of intact LDL particles within the cell (125, 139).

(d) The release of free cholesterol from LDL produces reciprocal effects on the activities of two enzymes involved in cellular cholesterol metabolism. The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), a microsomal enzyme that catalyzes the rate-limiting step in the biosynthesis of cholesterol, is depressed while that of acyl-CoA : cholesterol acyltransferase (ACAT), an enzyme that esterifies free cholesterol, is

increased. Non-lipoprotein-bound free cholesterol when added to the culture medium of human skin fibroblasts has similar but smaller effects on these enzymes. The release of free cholesterol from LDL produces also an effect of suppressing the synthesis of LDL receptors. Non-lipoprotein cholesterol again has a similar action.

At concentrations of extracellular LDL above the receptor-saturating level, LDL is internalized by bulk-phase pinocytosis and degraded at a rate proportional to its concentration in the medium (137). The results of binding studies (137) and studies of cholesterol accumulation (123) suggest that cholesterol is not accumulated by cells from LDL internalized by the low-affinity process. After 24 hours, only a small difference in cholesterol contents was found between cells incubated with 150 ug or 450 ug of LDL cholesterol per ml of medium and it was inferred that cholesterol taken up by the cells was balanced by that removed (123). In an earlier section (I. E. 3.) it was seen that there is evidence that HDL promotes the efflux of cholesterol from cells. The culture medium in the experiments just referred to contained no HDL and thus, since cholesterol is insoluble in an aqueous medium, some other protein that is not a lipoprotein in the accepted sense, was removing cholesterol. This point was omitted, however, in the authors' discussion (123).

LDL, internalized by bulk-phase endocytosis, does not regulate the activities of HMG CoA reductase, of ACAT, or of LDL-receptor synthesis. This low-affinity binding is, however, inhibited by HDL (132).

Aspects of the LDL pathway have been demonstrated in human skin fibroblasts, arterial SMC, and leucocytes (135, 140), in rat aortic SMC (141, 142, 143), in swine aortic SMC (144, 145), in monkey aortic SMC (45), and in the lung and kidney of the rat (146) as well as in some established cell lines (135). It appears, therefore, that the "LDL pathway" is a general mechanism in somatic cells for controlling cholesterol contents in environments in which the cholesterol levels may fluctuate extensively. If the LDL pathway operates in somatic cells in vivo, as its demonstration in freshly-isolated lymphocytes has indicated (140), then it may be inferred that arterial smooth muscle cells have at some stage in atherogenesis lost their ability to control their cholesterol content. Although the reasons for this loss of control are unknown, a similar inability to control LDL-cholesterol accumulation has been demonstrated experimentally by incubating skin fibroblasts and aortic SMC with LDL modified chemically so as to carry a net positive charge. Cholesterol was deposited in both types of cell to an extent that produced lipid-laden cells, morphologically similar to the characteristic "foam cell" of atherosclerosis (147, 148). The significance of the modification of LDL in atherogenesis in vivo is, however, unknown. As was referred to previously (Section I.E.3.f.iii), non-lipoprotein-bound

cholesterol in alcoholic emulsions is accumulated by cells in culture more readily than LDL-cholesterol. In human arterial cells (of mixed type) this effect has been demonstrated qualitatively by Rutstein et al. (149), and it has been confirmed in human skin fibroblasts by Brown et al. (123). There remains, in consequence, no doubt that the intact LDL structure is of importance in the regulation of cholesterol accumulation. It is possible, therefore, that changes in this structure in vivo are involved in the conversion of intimal cells into "foam cells".

5. The Production of Glycosaminoglycans (GAG) by Cells in Culture

The role of changes in the intimal GAG in atherogenesis was discussed previously (Section I.D.).

There is evidence that arterial smooth muscle cells produce the GAG and other connective tissue components in the arterial wall. Thus, collagen and elastic fibres were shown to be secreted from aortic SMC in rats in vivo (18), and GAG were found to be in close association with intimal SMC membranes, collagen fibrils, and elastic fibres in monkeys in vivo (19). Explants of aortic tissue from swine (20) and monkey (150) yield a variety of cell types which include endothelial cells, fibroblasts, and smooth muscle cells. Explants from the aortas of cholesterol-fed rabbits revealed lipid-laden macrophages also (151). Ross obtained cultures of cells from explants of aortic intima and inner media that maintained the phenotype of SMC for

ten weeks. In these cultures, he demonstrated the production of microfibrils that resembled, both in morphology and in their reaction with histochemical stains, the elastin seen in ligaments and blood vessels (26). Aortic SMC have since been shown by incorporation of radiolabelled amino acids to secrete collagen in cultures from monkeys (22), rabbits (21), and humans (23, 24), elastin in cultures from rabbits (21), and GAG in cultures from monkeys (27).

Since there is evidence from studies in vitro that suggests that GAG may impede the mobility of LDL in the intima (Section I.F.3.), there is much interest in studying factors that control the production and secretion of GAG by intimal cells. The effect of cell proliferation, for example, on the secretion of GAG is of particular interest because proliferation of SMC is an early event in atherogenesis (Section I.B.). Cell culture is a convenient tool for this kind of study since the environment of the cells may be controlled and the accumulation of GAG in the medium may then be related to changes in the culture conditions that are known.

The synthesis of collagen, another connective tissue component, by aortic SMC appears to be part of a general increase in protein synthesis that occurs with an increase in DNA synthesis before mitosis (22, 25). It is difficult, however, to make a concise statement about the relation of GAG synthesis and cell proliferation at present. The production of total GAG during the logarithmic phase of growth declines

in cultures of chick corneal fibroblasts (152) and simian aortic SMC (27). The synthesis of hyaluronic acid and the synthesis of DNA occur in parallel in chick embryo fibroblasts (153, 154) and chondrocytes (155), but sulphated-GAG production falls in proliferating chick embryo fibroblasts (156). Cells that are confluent and thus are in a stationary phase of growth secrete GAG into the medium (152, 157, 158, 159). It was reported that the addition of fresh serum to confluent cultures of rat fibroblasts stimulated hyaluronic acid synthesis within four hours, long before DNA synthesis and mitosis occurred at 15-22 hours (160). The mitogenic activity was removed by ultrafiltration but the residue remained able to stimulate hyaluronic acid synthesis (160). The results of Koyama et al. suggested also the existence of separate factors since c-AMP and dibutyryl-cAMP increased hyaluronate secretion while they inhibited mitosis. Conversely, bromodeoxyuridine did not affect cell proliferation but decreased markedly the accumulation of hyaluronic acid in the medium (161). The independence of cell proliferation and GAG production has been reported also in cultures of human skin fibroblasts (158).

Differences have been reported in the types of GAG secreted by simian skin fibroblasts and aortic SMC. These dermal fibroblasts secrete primarily hyaluronic acid (50-60% of total) with lesser amounts of dermatan sulphate (10-20% of total) and chondroitin sulphates. The simian aortic SMC secrete very little hyaluronic acid (0-5% of total)

but much more dermatan sulphate (60-80% of total), proportions which appear to be reflected in the aorta of this species *in vivo* (27).

Some other factors that influence GAG production have been reported. Thus, Kim and Conrad found that D-glucose was limiting for GAG synthesis (162) and Nevo and Dorfman found that sulphated-GAG synthesis was enhanced by the presence of sulphated-GAG in the medium (163). Hyaluronic acid synthesis however, was inhibited by hyaluronic acid in the medium (164). In addition, it has been reported that a platelet factor of about 9,000 daltons stimulates both hyaluronate and DNA synthesis in human synovial cells but not the synthesis of sulphated-GAG (165).

In summary, it is reasonable to accept that GAG and other connective tissue components are synthesized and secreted by SMC in the intima. There is evidence that hyaluronic acid is synthesized in parallel with DNA synthesis and mitosis but that the sulphated-GAG production is unrelated to cell proliferation.

I. F. Diabetes Mellitus and Atherosclerosis

The literature on the association of atherosclerosis with diabetes mellitus has been reviewed (166, 167, 168). The main points one may draw from this literature and from more recent reports on this subject are as follows:

(a) deaths from coronary heart disease (C.H.D.) are more frequent among diabetics than non-diabetics. Estimates of the excess mortality from C.H.D. in the diabetic population have ranged from 40 to 200% (166-171);

(b) deaths from cardiovascular diseases occur earlier in diabetics (167);

(c) an accelerated development of vascular disease is probable in diabetes of long duration. The question of whether the progress of vascular disease may be retarded by maintaining normal plasma glucose levels has not, however, been resolved (166);

(d) the greater prevalence of C.H.D. among men in the general population is absent in the diabetic population (168, 170, 171).

The evidence, therefore, is strongly in favour of the concept that diabetes mellitus is predisposing to accelerated vascular disease. This view, however, is not held unanimously. Opposition to it has been expressed because of the absence of adequate control groups in some of the studies reported, or more

simply, because of a failure to find a special association between diabetes and atherosclerosis (172). Other critics, while they have accepted the concept that the mortality attributable to cardiovascular disease is greater among diabetics, have discounted a diathesis. Havel, for example, has proposed that a greater prevalence among diabetics of hypertension, that in itself is a risk factor in atherogenesis, and the frequency of diabetic microangiopathies, which may impair the development of collateral circulation during ischemic events, could each account for a part of the increase in the number of deaths from ischemic disease (167). Although others have subscribed to the former proposal (166), the accounting for all other major risk factors for C.H.D.* could not remove completely the excess mortality among diabetics in the 16-year follow-up study conducted in Framingham (169). While the explanation for a unique role of diabetes in vascular disease remains elusive, there is evidence which implicates glucose intolerance as a risk factor for C.H.D. (173). Many patients, ostensibly non-diabetics, with C.H.D. have an impaired tolerance to glucose (167, 168, 172). Moreover, insulin-treated diabetics, who, in the absence of good control would be especially vulnerable to an effect of glucose intolerance

* The risk factors accounted for were hypertension, hypercholesterolemia, obesity, cigarette smoking, and electrocardiographic abnormalities.

on C.H.D., have been shown, in two widely separated populations (Framingham, Massachusetts and Warsaw, Poland), to be particularly prone to fatal vascular disease (169, 171).

It is accepted widely that hyperlipidemia, an independent risk factor in C.H.D. (35, 173), is prevalent among diabetics. Elevations in the plasma lipids of diabetics have been reported often (166, 174, 175, 176). These elevations appear to precede the detection of diabetes by several years (173), and are manifested most frequently as an elevated plasma content of very low density lipoproteins (VLDL) (169). Adequate control groups have not, however, always been included in these studies (eg., 175). Mean serum cholesterol levels found in diabetic populations are, moreover, often comparable with those in the general population (166, 169) and some studies have revealed in fact, no abnormalities in the plasma lipoproteins of diabetics (167, 177).

While many studies have focussed on the levels of lipids and lipoproteins in diabetics, few have given attention to the possibility of abnormalities in composition. The major reason for this is that knowledge of the composition of plasma lipoproteins has been acquired only recently (178-181). Some of the more important properties of the lipoproteins are shown in Table 3. From the few studies in which the composition of plasma lipoproteins in diabetics was analyzed, one may infer that the only differences detected were ones that are accounted for by the changes accompanying hyperlipidemias (174, 177, 183).

TABLE 3 Properties of Plasma Lipoproteins

Lipoprotein	Hydrated density (gm/ml)	S_f*	Electrophoretic mobility (on paper)	Apoproteins**	Lipid (% of total lipoprotein dry weight) Triglyceride	Free Cholesterol	Esterified Cholesterol
Chylomicrons	0.95	> 400	origin	A B C E	80-95	1-3	2-4
VLDL	0.95-1.006	20-400	pre-β	A B C D E	50-70	10	5
LDL	1.019-1.063	0-12	β	B D E	10	8	37
HDL	1.063-1.210	—	α	A C D E	3	15	22

* Lipoprotein flotation rate in Svedberg units (10^{-13} cm/sec/dyne/gm) in a sodium chloride solution of density 1.063 gm/ml at 26°C.

** Apo A comprises two distinct apoproteins, AI and AII
Apo C comprises three distinct apoproteins, CI, CII, and CIII. Apo CIII reveals two bands following polyacrylamide-gel electrophoresis, $CIII_i$ and $CIII_{ii}$. Apo $CIII_i$ and Apo $CIII_{ii}$ differ only in their content of sialic acid .
Apo D is known also as the " thin-line " protein.
Apo E is known also as the " arginine-rich " protein .

References : 178-182.

Nonetheless, Schonfeld et al. reported small increases in LDL- and HDL-triglycerides among normolipidemic diabetics (183), and they and Lopes-Virella et al. found lower levels of HDL-cholesterol in the normolipidemic diabetics (183, 184). Calvert et al. observed a decrease of HDL-cholesterol only in diabetics treated with sulphonylurea (185). HDL-cholesterol has recently acquired a cynosural position in atherosclerosis because of an association of subnormal levels with C.H.D. (34, 35, 186) and because of the smaller accumulations of cholesterol found in cells cultured in the presence of HDL (Section I.E.3.). It is noteworthy, therefore, that Reckless et al. failed to find an association between HDL-cholesterol levels and vascular disease in a group of diabetics although they did find a relation between LDL-cholesterol and vascular disease (187).

In summary, and as Keen has remarked, there is little evidence for a characteristic abnormality in the lipoproteins of diabetics (172).

The suggestion has been made that the accelerated vascular disease in diabetics arises from an inherited defect that causes the degeneration and replacement of somatic cells to occur more frequently than in non-diabetics (188). The familial nature of maturity-onset diabetes is established (189). In addition, studies of dermal fibroblasts in culture have revealed several abnormalities in cells obtained from diabetics. Dermal fibroblasts possess a limited capacity for

replication *in vitro* and appear to lose the ability to divide after 40-60 mean population doublings (190). The number of population doublings a culture may undergo *in vitro* is related inversely to the age of the donor when the biopsy was performed (191). The implication, therefore, is that somatic cells *in vivo* possess a finite life-span similar to that of the cells *in vitro*. When compared with cells from age-matched controls, a lower number of pre-diabetic cells* formed colonies (192), and the number of population doublings in culture before the onset of cellular senescence was smaller in diabetic cultures (188, 191). Skin fibroblasts from diabetics tend, therefore, to possess characteristics in culture that are found normally in donors of a more advanced age. While it is true that "older" cells *in vivo* may arise from the dying and replacement of cells at a faster pace than is normal, direct evidence for this process as a mechanism of accelerated vascular disease is scarce at present.

In conclusion, while it may be presumed that deaths from vascular diseases are more frequent among diabetics, the causes of this increase are not known. There are conflicting views on the prevalence of hyperlipidemias among diabetics, and the existence of abnormalities in lipoprotein composition has

* The authors of this report defined a pre-diabetic as an individual with repeatedly normal glucose tolerance test results but who is at risk, genetically, of developing diabetes in later life. The off-spring of conjugal diabetics and the non-diabetic monozygotic twin of a diabetic are thus pre-diabetics.

little support from analytical studies. The number of these studies has been small, however, and it may be premature to discount a defect in diabetic lipoproteins. While it is accepted generally that hypertension is found in many diabetics, the Framingham study has ruled this out as the cause of the excess mortality from C.H.D. in the diabetic population. In the midst of this uncertainty, the prevailing speculation that an inherited defect in diabetic cells may be involved in a predilection for vascular disease offers a welcome alternative domain in which to search for causes of premature atherosclerosis and perhaps even of diabetes mellitus itself.

II. INTRODUCTION

Evidence has accumulated to verify a fundamental role for the arterial smooth muscle cell in atherosclerosis and to support the hypothesis that abnormalities in rates of proliferation and in metabolism, particularly in cholesterol metabolism, of these cells are key, and possibly, initiating factors, in the disease. Of great interest at present are the effects of blood components on the initiation and progression of atherosclerosis and the recent literature contains many reports of the effects of lipoproteins and platelet-derived factors on the proliferation and metabolism of mammalian and avian cells in culture. These effects may be crucial factors in atherogenesis in vivo.

This investigation examined aspects of human cellular responses to serum LDL in the presence and absence of platelet factors. Fibroblasts from human deltoid and genital skin were used in most of the studies although an attempt was made to determine if similar effects of LDL could be shown in cultures of porcine aortic smooth muscle cells. It is known that fibroblasts and arterial smooth muscle cells differ in morphology and in characteristics of growth in culture. The metabolism of LDL may however, be similar in these two cell types since both have specific membrane receptors for LDL, internalize LDL, degrade the LDL protein, hydrolyze LDL-cholesterol esters, re-esterify free cholesterol from this hydrolysis, and suppress cellular synthesis of cholesterol in response to one or more of these processes. LDL has been shown to enhance the rate of replication of mammalian arterial smooth muscle cells in culture

but this effect has not been demonstrated in cultures of human skin fibroblasts.

It is recognized that elevated plasma lipoproteins are associated with atherosclerosis. It is recognized also that diabetics often are hyperlipidemic and develop atherosclerosis earlier and more severely than the general population. The evidence available suggests however, that other risk factors in atherosclerosis such as hyperlipidemia and hypertension cannot account for all of the excess mortality from coronary heart disease in the diabetic population and in consequence it has been suggested that diabetes mellitus itself is a risk factor. The present investigation attempted to examine the relation between diabetes mellitus and atherosclerosis in more detail by measuring the responses of diabetic cells in culture to serum LDL to determine if abnormalities in these responses exist, and by analysing the composition of lipoproteins from diabetics to determine if an abnormality could be found that was characteristic of diabetes. Both a cellular defect in response to LDL and abnormal compositions of lipoproteins could provide the basis of the predisposition to atherosclerosis that is characteristic of diabetics.

III. EXPERIMENTAL

III. A. General Methods

1. Isolation of Serum Low-Density Lipoprotein (1.019-1.063 gm/ml)

Low-density lipoprotein was isolated according to standard methods (193).

Blood was obtained from healthy volunteers after a fast of at least 12 hours. The blood was collected into 30 ml glass tubes and allowed to stand for 2 hours at 24°C. The serum was separated by centrifugation (International PR-6, 3000 rpm, 4°C., 15 minutes x 2). EDTA (Na_2) was added (0.3 mM) and 18.51 mg potassium bromide/ml were added to increase the non-protein solvent density to 1.019 gm/ml.

Cellulose nitrate tubes[1] were filled with the density-adjusted serum and centrifuged in a 30.2 rotor at maximum speed (30,000 rpm) in a Spinco Model L Ultracentrifuge for 16-24 hours at 15°C. Two fractions separated by a clear space were seen in each tube; the infranatant fraction was collected by tube-slicing and the remainder was discarded. The infranatant fractions from all tubes were pooled, adjusted to the original total volume of the serum with a potassium bromide / sodium chloride / EDTA (Na_2) solution of density 1.019 gm/ml, and adjusted to a density of 1.063 gm/ml by addition of 64.40 mg potassium bromide/ml.

1. Beckman Instruments ($3\frac{1}{2}$" x $\frac{1}{2}$")

Ultracentrifugation was repeated to yield two fractions separated by a clear layer; the supernatants were orange, were pooled and replaced into the ultracentrifuge for 16-24 hours.

The "washed", concentrated LDL was removed by tube-slicing and dialyzed (NaCl 0.15 M, EDTA (Na_2) 0.3 mM, pH 8.6, 200 volumes x 5, 4°C.) over a period of 24 hours. An aliquot was assayed for inorganic bromide (194) and if this was less than 1 mg/dl, the LDL was passed through a sterile 0.22 um cellulose acetate filter[1] into a sterile bottle (3 ml) and was stored at 4°C. An aliquot was assayed for protein (195). It has been reported that LDL retains specific binding activity to fibroblasts up to 3 months after preparation (137); it was observed, however, that increasing turbidity and precipitation occur after 1 month, and at this time, fresh LDL was prepared.

2. Preparation of Platelet-Poor, Lipoprotein-Free Serum (PPLFS)

Platelet-poor serum was prepared according to the method of Ross et al. (114).

Freshly-drawn whole blood, collected into acid citrate - dextrose (USP Formula A) was obtained from the Diagnostic Unit of the Jewish General Hospital, Montreal. The donors were out-patients undergoing therapeutic phlebotomies.

The blood was centrifuged in 250 ml plastic bottles (International PR-6, 3000 rpm, 4°C., 20 minutes x 2). The

1. Millipore Corporation

plasma supernatants were centrifuged in 30 ml stainless steel tubes (Sorvall SS-2, 20,000 G, 4°C.,30 minutes). Careful removal of the plasma revealed a "button" of platelets at the bottom of the tube. The platelet-poor plasma was dialyzed (Ringers solution, 8 volumes, 4°C.) overnight and the coagulated contents of the bag were emptied into a beaker. Serum was forced from the clot with a glass rod and passed through sterile gauze. A second dialysis under the same conditions was performed.

The platelet-poor serum was made lipoprotein-free by adjusting the non-protein solvent density to 1.250 gm/ml with the addition of 398.37 mg potassium bromide/ml serum and by centrifuging as in the isolation of LDL above.

The turbid lipoprotein supernatants were removed by tube-slicing and the lipoprotein-free fractions were collected and dialyzed (NaCl 0.15 M, 4°C., 200 volumes x 6).

The serum was heated at 56°C. for 30 minutes, filtered through paper (Whatman #1), and subsequently passed through a sterile filter (0.22 um) into sterile bottles.

Aliquots were taken for estimation of protein (195) using bovine serum albumin, fraction V[1] as a standard, of total cholesterol (196), and of inorganic bromide (194). A preliminary study indicated that no differences in residual cholesterol levels were detectable in sera centrifuged at

1. Sigma Chemical Company

densities of 1.210 and 1.250 gm/ml for 24 and 48 hours in each case. All preparations were centrifuged for 24 hours at the higher density, however, to ensure the maximum removal of cholesterol.

No batch of PPLFS was used if the cholesterol concentration was higher than 8 mg/dl, if inorganic bromide exceeded 1 mg/dl, or if the serum failed to maintain cultures of human skin fibroblasts for at least one month.

An amount of PPLFS was added to Dulbecco's modification of Eagle's minimum essential medium (DME) to yield a final protein concentration of 6 mg/ml, which is about 10% of a normal protein concentration in human serum.

In one experiment of 6 days' duration, 10% PPLFS reduced the number of labial skin fibroblasts to 69% of the final count (average of 4 dishes) of the same cells cultured in 10% whole human serum. This may be compared with a 21% reduction in cell numbers of monkey dermal fibroblasts grown in 5% human platelet poor serum relative to whole human serum at the same concentration (115). The experiments are not directly comparable, however, since the sera in the simian cell cultures were at lower concentrations and contained lipoproteins.

3. Cell Culture Methods

Mass culture of cells was carried out in plastic flasks (Corning). The surface areas were 75 and 150 cm^2 and these flasks shall henceforth be referred to as T-75 and T-150 respectively. The culture medium was DME (Flow Labs Inc.) and

was supplemented with fetal calf serum (10% v/v, Reheis Chemical Company and Microbiological Associates), non-essential amino acids (1 mM, Gibco), sodium pyruvate (1 mM), streptomycin (60 ml/l), penicillin G (10^5 units/l), and sodium bicarbonate (2.25 gm/l) according to the formulation used by Ross (26). This medium shall be referred to as 10% FCS. Medium was sterilized by passage through a sterile 0.22 um filter, stored at 24°C. for three days to confirm sterility, and stored at 4°C. thereafter.

Medium in culture vessels was changed three times every week (T-75 : 20 ml; T-150 : 40 ml) until the cells had reached confluency (i.e., had formed a single layer of cells which covered the entire surface of the culture vessel). The cells were released with trypsin (Difco 1 : 250, 2.5 gm/dl) and EDTA (Na_4), (60 mM) according to standard techniques. The cell suspension was added to 10% FCS to inhibit further proteolysis and was centrifuged (150 x g, 4 minutes). The cell pellet was resuspended in a volume of 10% FCS to yield approximately 0.5×10^6 cells.ml^{-1}, and the cells were counted. A volume containing 1.5×10^6 cells was pipetted into a new T-75 or a volume containing 3.0×10^6 cells was pipetted into a new T-150 flask. Each flask contained medium that had been previously "gassed" with air : CO_2 (19 : 1) and incubated at 37°C. If the cells were to be used for an experiment, 2 ml of 10% FCS were added to each of the appropriate number of plastic petri dishes (P-35, 35 mm diameter, Corning) and the dishes

were incubated at 37°C. in a humidified atmosphere of air : CO_2 (19 : 1). After counting the cells, the suspension was diluted with 10% FCS to yield the desired number of cells to be plated in a volume of 0.2 ml. A 1 ml graduated pipette was used to pipette the cell suspension into the dishes. The coefficient of variation of plating was found to be less than 5%.

4. Cell Counting

Cell counts were performed with one of two instruments: the Coulter Model F (threshold 2.5, attenuation 2, aperture current 512) or the Cytograf 6300 A (Biophysics Systems Inc., threshold 14, medium gain).

Preliminary studies on each instrument showed that the counts were linear with increasing cell density, were precise (coefficient of variation 2-6%) and were in close agreement with simultaneous counts using a hemacytometer. The accuracy of counting electronically is superior to that with a hemacytometer because of the much larger number of cells actually counted. These instruments do not allow, however, the operator to distinguish between cells and debris as does the hemacytometer. The problem of debris was reduced to a minimum by filtering all solutions before washing, releasing, and suspending the cells for counting (Whatman #1 paper). The counting of cells during routine culture has been described. The counting of cells during experiments required a procedure that would allow estimation of cholesterol and protein in the same sample. The medium was removed by aspiration and the

cells were washed five times with 1 ml warm phosphate-buffered saline (calcium- and magnesium-free, PBS). Incubation for 5-10 minutes with trypsin (1ml) and EDTA (Na_4) (1.5 ml) was sufficient to release the cells. The suspension was carefully transferred to a clean, siliconized (Siliclad) centrifuge tube (12 ml). The suspension was centrifuged (100 x g, 4 minutes) and the pellet washed twice with 1 ml PBS. The pellet was suspended in 6 ml PBS and aliquots were taken for counting each sample in duplicate. Four ml of the cell suspension were transferred volumetrically into a clean glass centrifuge tube (12 ml); the cells were pelleted by centrifugation as before, and prepared for extraction as follows.

5. Estimation of Total Cholesterol in Cell Pellets

Cholesterol was extracted from the cells into a mixture of chloroform and methanol (2 : 1, v/v) and the extract was washed (197). Both solvents were of reagent quality and were redistilled prior to use. Warming the cell pellet in 1.5 ml of extraction mixture by suspending the tube in a boiling-water bath, or allowing the pellet to stand overnight at 24°C in the same volume of extraction mixture yielded identical recoveries of cell cholesterol.

The mixture was centrifuged to pellet the disrupted cells (3000 rpm, International PR-6, 20°C) and the residue was washed with 1 ml of the extraction mixture which was removed after centrifugation and combined with the first extract. Distilled and deionized water was added (0.6 ml) and the mixtures were

shaken. Two phases were separated by centrifugation: the upper phase was discarded and the surface of the lower phase was carefully washed with "pure upper solvents" (197). The lower phase was dried under nitrogen. Cholesterol was estimated in the dried residue by a fluorometric method (196). A fluorometer equipped with filters of 528 nm for activation and 565 nm for emission was used (G. K. Turner Associates). The fluorescence yields of cholesterol, cholesteryl palmitate, cholesteryl oleate, and cholesteryl linolenate are identical with this method. Coprostanol and lanosterol produce fluorescent yields lower by a factor of ten.

6. Estimation of Total Protein in Cell Pellets

The lipid-extracted pellet, dried under nitrogen, was dissolved in 0.5 ml NaOH (1 N) during incubation for 1 hour. A solution of bovine serum albumin (0.75 mg/ml, 0.15 M NaCl, 1 N NaOH) was incubated simultaneously and used to prepare standards for the assay. Aliquots (0.1 ml) were analyzed in duplicate from each sample using the method for insoluble material of Lowry et al. (195).

III. B. Effects of LDL on the Proliferation, Cholesterol Content, and Protein Content of Human Skin Fibroblasts

1. Introduction

The study of the effects of LDL on cells *in vitro* has an advantage in enabling the investigator to select conditions such that any effect observed may be attributed specifically to LDL. When studying the effect of a specific agent on cell proliferation, it is essential obviously to ensure that no other factor becomes the limiting one for proliferation. As long as knowledge of factors that control cell replication is incomplete, this condition cannot be satified with absolute confidence. The medium used in the following experiments was chosen to ensure that no known essential nutrient was likely to be exhausted during the course of the experiments. DME is enriched substantially in amino acids and vitamins. Glucose is present at 22 mM as supplied and the medium was supplemented further with pyruvate and non-essential amino acids. To ensure that an approach to physiological protein metabolism by these cells was possible, human serum, free of lipoproteins, was added. Furthermore, since platelet factors possess potent mitogenic effects on cultured cells, it was desirable to remove these as much as possible in order that any effect of LDL on proliferation was not obscured by a much larger effect of platelet factors.

This investigation included an analysis of cholesterol accumulation in LDL-treated cells and of the effect of LDL on protein accumulation. Although little information exists

about the latter effect in atherogenesis, there is an abundance of evidence to show that cholesterol accumulation, both within arterial cells and in the extracellular space, is an integral part of the development of atherosclerotic disease.

2. Materials and Methods

The effects of LDL on cell proliferation, total cholesterol content per cell, and total protein content per cell were examined at two levels of LDL in strains of deltoid skin fibroblasts from diabetic and non-diabetic subjects. The study of these effects was continued with cells from genital skin of young subjects using the higher level of LDL. Control cells were incubated in medium containing PPLFS alone. LDL, PPLFS, and culture medium were prepared by methods described in the General Methods section.

Human skin fibroblasts were obtained from cultures initiated and maintained in the laboratories of Dr. R. G. Germinario and Dr. L. Pinsky at the Lady Davis Institute for Medical Research of the Jewish General Hospital, Montreal. Only those cultures which had been passaged less than 12 times were used. Methods of culturing and counting cells are described in the General Methods section (III.A.).

Cell strains are identified in the Results section, Table 4. Methods of extracting cholesterol from the cells and its estimation, and the method of estimating the total protein content of the cells are described in the General Methods section (III.A.).

The statistical analysis of the results was performed as follows. The data consisted of the total number of cells in each of the triplicate plates (three replications) for each treatment group (three treatment groups for cells from mature

TABLE 4 Details of Cell Strains Used

Experiment	Strain	Biopsy Site	Passages	Health Status	Age	Sex	Number of Cells Plated	Levels of LDL (ug protein/ml Low	High
1	AJD	d	6	D	54	M	30,000	19	190
2	LIC	d	6	N	55	F	33,000	19	190
3	FAD	d	4	N	40	F	40,000	24	192
4	JRD	d	4	D	43	M	37,000	24	192
5	FAD	d	6	N	40	F	40,000	22	177
6	JRD	d	6	D	43	M	42,000	22	177
7	LIC	d	4	N	55	F	33,000	20	203
8	AJD	d	4	D	54	M	32,000	20	203
9	AJD	d	4	D	54	M	32,000	20	136
10	CID	d	3	D	50	F	33,000	20	136
11	LIC	d	4	N	55	F	38,000	20	136
12	YXF	f	11	N	10	M	45,000	-	130
13	FRE	f	4	N	14	M	41,000	-	143
14	RMF	f	5	N	26	M	49,000	-	143
15	PAL	l	8	N	20	F	46,000	-	143

Sites of Biopsy:
- d - deltoid
- f - foreskin
- l - labia majora

Health Status:
- D - diabetes mellitus of the maturity-onset type
- N - normal, non-diabetic

Cell Strains from Diabetics:
- AJD: very obese, treated for 9 yrs. with insulin, family history of diabetes.
- CID: treated with insulin, no family history of diabetes.
- JRD: treated with an oral agent + diet, mother and a brother also diabetic.

donors; two treatment groups for cells from young donors) for each of the counts performed every two days (0-8 days), after adding the experimental media on day 0. The data were left unchanged in the case of total cell cholesterol but the cell counts and the total protein per cell were transformed by computing the percentage of the corresponding average value of three dishes on day 0. This was done to eliminate any variability attributable to differences in 0 day values among experiments.

The sets of data were each subjected to a three-way analysis of variance with replications using a computer program (Balanova 5) which accepts designs with missing values as long as there is at least one value in each cell. Missing values are indicated in the tables of Means of Triplicates in the top right-hand corner of each cell. Where this number is 3, this indicates that all three values were missing and an estimate has been made (198). Linear regression by the method of least squares was carried out on a Texas Instruments TI-55 calculator which yielded the slope and intercept of the regression line and the associated correlation coefficient.

3. Experimental Protocol

Cells ($3\text{-}5 \times 10^4$) were pipetted into 40 P-35 dishes. Dishes were assigned to appropriate groups by selecting consecutive numbers from a table of random numbers (199).

The cultures were incubated for 2-3 days and three dishes were counted for cells. If the counts were close to the number

pipetted, the experiment was started; if not, the 10% FCS medium was changed and counts were made at later times until the original number was reached. At this time (day 0) the medium was removed from the dishes and the cells were washed twice with serum-free DME (1 ml). DME containing platelet-poor, lipoprotein-free human serum (PPLFS, 10% v/v) was added to each dish (2 ml), and LDL at the level(s) indicated in Table 4 was added to the experimental dishes. The lower level of LDL ranged from 19 to 24 ug protein per ml medium and the higher level ranged from 130 to 203 ug per ml medium. The latter range was determined by the amount of LDL obtained from each of the donors.

Medium and LDL were changed at 3 days and at 6 days.

Three dishes in each group were taken at 2, 4, 6, and 8 days and the cells were released for counting, extraction, and estimation of cholesterol, and for estimation of total protein. These procedures are described in detail in the General Methods section (III.A.).

An attempt at matching diabetic and non-diabetic strains for age and sex was made, but this was unsuccessful because of the limited number of strains available. Close matches for age and passage number were possible; thus, experiments 1 and 2 were run at the same time, as were experiments 3 and 4, 5 and 6, 7 and 8, and 9, 10, and 11. In the young donor group, experiment 12 was run alone, and experiments 13, 14, and 15 were run together.

4. Results

a. Effects of LDL on Cell Proliferation

Changes in cell numbers in all experiments with cells from mature donors are shown in Table 5. No differences in the proliferation rates of diabetic cells compared with normal cells in the presence and absence of LDL were found (Table 6). Diabetic and normal strains were subsequently treated as one group. The results from all experiments were subjected to an analysis of variance, the major results of which are presented in Table 7. Differences in the effects of one or more of the three treatments on cell numbers were found to be highly significant. The effect of time was also significant. The variability of results among experiments was large and it is clear from the interaction terms that the main effects are not independent of each other.

Several sources of variation are unavoidable and to be expected in experiments of this type since the factors and events which result in cell proliferation are complex and largely unknown. It was thus anticipated that the magnitude of any response to LDL by different cell strains would vary, and variability in cell counts was found even among experiments with the same strain. It was anticipated also that LDL would not produce the same increment in cell number during each interval in each experiment as progressively less LDL became available to an increasing number of cells. The magnitudes of the interaction terms in the analysis of variance

TABLE 5 Strains from Mature Donors: Effect of LDL on Cell Proliferation

Treatments (ug LDL protein/ml medium):	% of Cell Count on Day 0											
	0				21				176			
Days:	2	4	6	8	2	4	6	8	2	4	6	8
Experiments												
1 (D)	115[3]	131	188	198	116[3]	133	234	253	112[3]	124	303	302
2 (N)	135[3]	175	172	224	154[3]	208	248	270	116[3]	230	244	317
3 (N)	158	170	213	243	174	198	260	294	176	210	233	249
4 (D)	259	263	202	372	247	279	285	548	353	459[1]	440	496
5 (N)	139	201	266	348	133	203	296	418[1]	153	245	247	300[1]
6 (D)	105	165	246	248	128	160	267	269[1]	141	227	291	238[1]
7 (N)	258	273	365	418	253	327	429	497	295	339	417	413
8 (D)	121	180	218	253	120	182	242	270[1]	149	189	243	303[1]
9 (D)	178	187	188	201	138	191	214	246	153	223	239	297
10 (D)	395	514	599	529	459	577	643	743	505	565	555	762
11 (N)	307	420	415	478	282[1]	372	423	496	306	475	542	652
Mean:	197	244	279	319	200	257	322	391	224	299	341	393
Standard Error:	18	20	22	20	20	22	23	30	23	24	22	32
Number of Culture Dishes:	27	33	33	33	26	33	33	30	27	32	33	30

Table 6 Non-Diabetic compared with Diabetic Cell Strains

LDL Treatment Level :	ZERO		LOW		HIGH	
	N*	D**	N	D	N	D
Cell Count (% of that on day 0)						
n***	5	6	5	6	5	6
2 days	200(20)****	196(28)	199(19)	201(33)	210(21)	235(38)
4 "	248(25)	240(32)	262(20)	254(37)	300(27)	298(40)
6 "	286(25)	274(36)	331(22)	314(39)	336(33)	345(29)
8 "	342(27)	300(29)	395(28)	388(52)	386(42)	400(49)
Total Cholesterol (ug / 100,000 cells)						
n	2	3	2	3	2	3
2 days	-	-	-	-	-	-
4 "	2.1(.2)	2.4(.4)	2.7(.1)	3.2(.3)	4.2(.6)	3.6(.4)
6 "	3.1(.3)	2.1(.1)	3.6(.1)	3.1(.2)	4.3(.5)	4.3(.6)
8 "	2.9(.4)	2.8(.4)	3.3(.3)	3.0(.2)	4.6(.5)	5.1(.9)
Total Protein (%of that on day 0)						
n	3	3	3	3	3	3
2 days	106(7)	102(8)	105(8)	93(9)	126(6)	110(8)
4 "	166(7)	156(32)	189(14)	158(26)	247(13)	214(26)
6 "	213(20)	220(36)	201(24)	201(33)	279(7)	300(38)
8 "	242(24)	246(45)	214(19)	235(50)	294(15)	334(69)

* Non-Diabetic cell strains

** Diabetic cell strains

*** Number of experiments (Triplicate dishes, Table 5)

**** Standard Error of the Mean

Table 7 Analysis of Variance : Cell Count (% Of that on day 0)

Source of Variation	Degrees of Freedom : Numerator	Sum of Squares	Mean Square	F Ratio	Level of Significance
(a) Mature Donors					
A. Levels of LDL	2	176468	88235	94.37	< 0.001
B. Days	3	1118526	372842	398.77	< 0.001
C. Experiments	10	4905690	490569	524.68	< 0.001
Interactions :					
A x B	6	83953	13992	14.96	< 0.001
A x C	20	166563	8328	8.91	< 0.001
B x C	30	250604	8353	8.93	< 0.001
A x B x C	60	230546	3842	4.11	< 0.001
Replications (26 missing values)	243	227200	935		
(b) Young Donors					
A. Levels of LDL	1	154587	154587	245.31	< 0.001
B. Days	3	231165	77055	122.28	< 0.001
C. Experiments	3	322951	107650	170.82	< 0.001
Interactions :					
A x B	3	17095	5698	9.04	< 0.001
A x C	3	5364	1788	2.84	< 0.05
B x C	9	23943	2660	4.22	< 0.001
A x B x C	9	11592	1288	2.04	< 0.05
Replications (6 missing values)	60	37811	630		

confirm that these factors have considerable influence on the results of this type of experiment.

The effect of each level of LDL is seen more clearly when the means of all experiments in each treatment group were plotted against time (Fig.1). The greatest increase in cell number occurred between days 0 and 2 in all three treatment groups. The rate of increase in cell number diminished with time in a similar fashion in all treatment groups except that the low level of LDL, in contrast with the high level, maintained a greater rate than controls after 4 days . Cell numbers were always greater in the high LDL group than in the control group. The low level of LDL exerted a small effect on cell numbers in the early part of the experiment, but by 8 days it had equalled the effect produced by the high level of LDL.

The results from four experiments with cells from young donors are presented in Table 8 and the results from the analysis of variance appear in Table 7. The effect of LDL and the effect of time on cell numbers in these experiments were essentially the same as in the experiments with cells from mature donors and the interpretation of the analysis of variance is similar to that given above.

The means of all experiments in each treatment group were plotted against time in Fig.1 . The greatest increase in cell numbers occurs between days 0 and 2 in control medium and with the addition of LDL. After day 2, the rate of increase diminishes in both cases. LDL exerted an effect which was

Fig. 1 - Increase in cell number of skin fibroblasts without (●) and with the addition of LDL to a medium containing PPLFS. (o) 20 ug LDL protein/ml medium; (Δ) 160 ug LDL protein/ml medium.

A. Cells from mature donors.

B. Cells from young donors.

——— Cell count.

– – – – Percentage increase in cell count over preceding count.

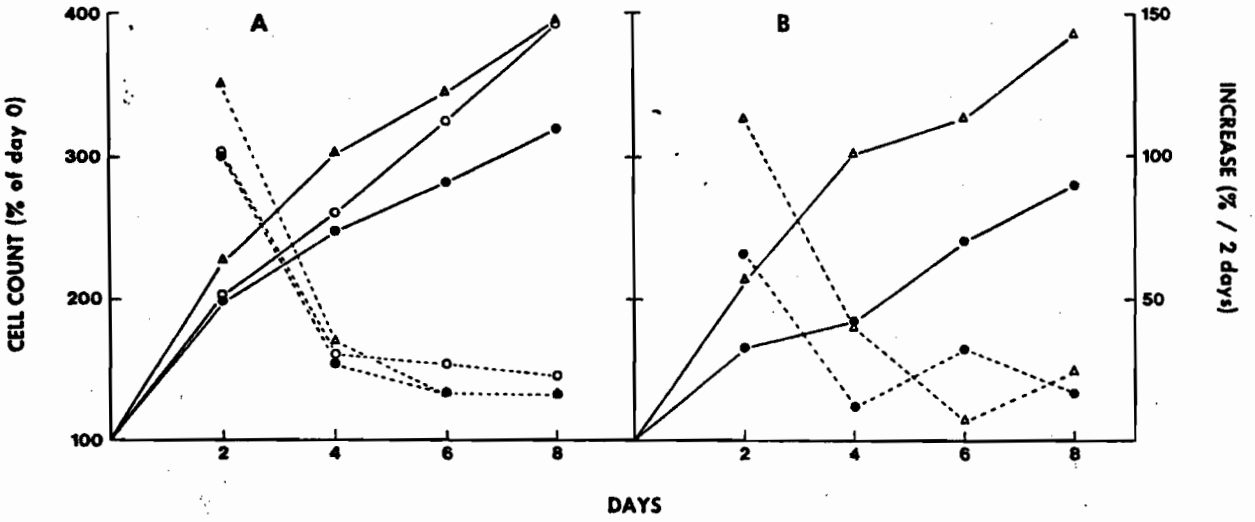

A
B
CELL COUNT (% of day 0)
INCREASE (% / 2 days)
DAYS
400
300
200
100
150
100
50
2
4
6
8

Table 8 Cell Strains from Young Donors

LDL Treatment Level	ZERO				HIGH			
Days	2	4	6	8	2	4	6	8
Cell Count (% of that on day 0)								
Expt. 12	217	237	280	366[3]	263	360	337	475[3]
" 13	116	167	241	250	181	271	280	323
" 14	211	203	301	353	266	391	388	469
" 15	122	133	144	156	148	192	234	282
Mean	166	185	242	281	214	303	310	387
n*	12	12	12	9	12	12	12	9
Standard Error	15	13	20	28	17	24	20	29
Total Cholesterol (ug / 100,000 cells)								
Expt. 12	0.7	0.9	0.7[1]	0.7[3]	0.8	1.3[1]	1.0	1.2[3]
" 13	2.0	1.9	1.5	1.4	1.8	2.8	2.8	2.8
" 14	0.4	0.3[1]	0.9	0.8	0.8	1.6	1.3	1.6
" 15	1.3	1.3	1.4	1.6	2.2	2.8	2.4	2.4
Mean	1.1	1.2	1.2	1.3	1.4	2.2	1.9	2.2
n	12	11	11	9	12	11	12	9
Standard Error	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.2
Total Protein Per Cell (% of that on day 0)								
Expt. 12	112	196	208[1]	96[3]	265[1]	356	272	367[3]
" 13	178	102	84	152	228	119[1]	220	310
" 14	197	214	318	172	340	396	570	518
" 15	190	132	172	171	362	320	350	461
Mean	169	161	195	148	299	298	353	414
n	12	12	11	9	11	11	12	9
Standard Error	16	15	31	9	30	39	44	45

* Number of culture dishes

measurable at the earliest time of counting cells and maintained this effect throughout the experiment. After day 4, however, the difference between cell numbers in medium with and without LDL did not grow larger.

The qualitative effect of control medium, alone or with the addition of LDL, was similar in cultures of cells from mature and young donors. The mean percentage increases in cell numbers for both groups are presented in Table 13 where it is seen that the effect of adding the high level of LDL is identical. The control medium alone produced an effect which was about 30-40% greater on cells from mature donors although this was apparent at 2 days and appeared not to change during the experiment.

b. Effects of LDL on Total Cholesterol Content of Cells

The cholesterol contents per cell in strains from mature donors are shown in Table 9 . The method for measuring cholesterol was available from the time of experiment 7. It was insufficiently sensitive to yield reliable results before day 2 in experiments in the mature cell group. Results from experiments 7-11 were from 2 experiments with 1 non-diabetic and 3 experiments with 2 diabetic strains. No difference in the cholesterol content of diabetic cells was found either in the presence or in the absence of LDL (Table 6). The diabetic strains and the normal strains were subsequently treated as one group and the combined results were subjected to an analysis of variance, the major results of which appear in

Table 9 Strains from Mature Donors: Effect of LDL on Total Cholesterol per Cell

Cholesterol (ug / 10^5 cells)

Treatments (ug LDL protein/ml medium) :	0			20			163		
Days:	4	6	8	4	6	8	4	6	8
Experiments									
7 (N)	2.1	3.8	3.9	2.8	3.8	3.9	5.5	5.2	5.6
8 (D)	2.7	2.3	4.0	3.3	3.5	3.9[1]	4.5	6.7	9.0[1]
9 (D)	3.5	2.2	2.3	4.0	3.4	2.9	4.1	3.7	4.0
10 (D)	1.0	1.6	2.0[1]	2.0[1]	2.5	2.6	2.2	2.5	3.5
11 (N)	2.1	2.4	2.0	2.7	3.4	2.7	2.9	3.4	3.7
Mean:	2.3	2.5	2.9	3.0	3.3	3.2	3.9	4.3	5.2
n*	15	15	14	14	15	14	15	15	14
Standard Error:	0.2	0.2	0.3	0.2	0.1	0.2	0.3	0.4	0.5

* Number of culture dishes

Table 10. Significant differences in cholesterol content per cell were found with treatments and with days; thus LDL and time were both producing effects. As expected from the previous analyses of variance on changes in cell numbers, the variability among experiments and the interaction among the main effects were also significant. The interpretation of these was given above. The mean values of all experiments with mature strains in each treatment group were plotted against time (Fig.2). During the 4-day period of measuring cholesterol content, increases occurred in all groups except in that which was given the low level of LDL. Early in the experiments there was a relation between the total cholesterol content per cell and the amount of cholesterol in LDL in the medium, but at 8 days the control and the low LDL groups had similar contents.

Results from the experiments with cells from young donors were essentially the same (Table 8). As seen in Fig.2, LDL increased the cholesterol content per cell while the cholesterol content per control cell was virtually unchanged.

Under the light microscope, the cells from the young donors appeared to be smaller than those from the mature donors. It was expected therefore, that the cholesterol content of the young cells would be lower than that of the older cells. This is seen to be the case in Table 13. Cells from young donors in control medium after 8 days contained 1.3 ± 0.1 pg of cholesterol; cells from mature donors contained 2.9 ± 0.3 pg.

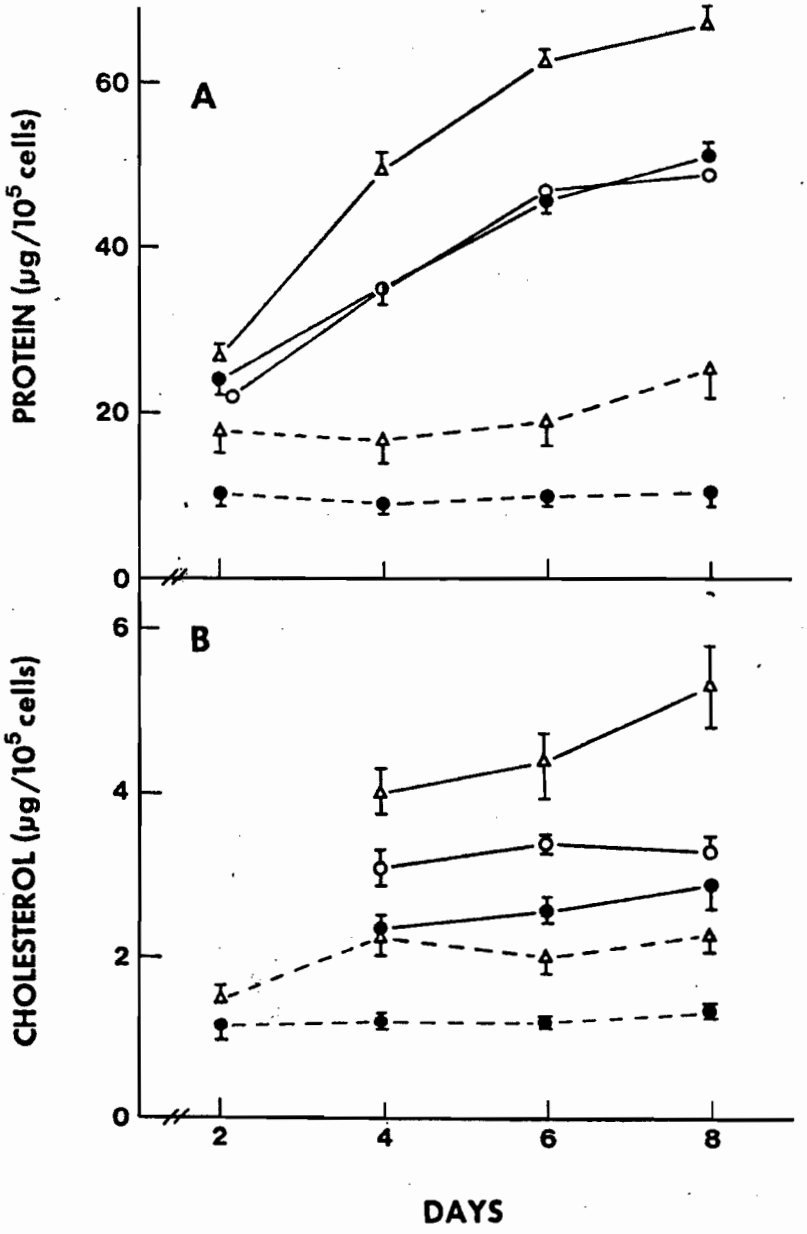
Table 10 Analysis of Variance : Total Cholesterol (ug/10^5 cells)

Source of Variation	Degrees of Freedom : Numerator	Sum of Squares	Mean Square	F Ratio	Level of Significance
(a) Mature Donors					
A. Levels of LDL	2	8191	4095	287.449	<0.001
B. Days	2	1038	519	36.443	<0.001
C. Experiments	4	8528	2132	149.648	<0.001
Interactions :					
A x B	4	536	134	9.405	<0.001
A x C	8	3317	414	29.105	<0.001
B x C	8	2343	292	20.562	<0.001
A x B x C	16	1470	91	6.450	<0.001
Replications (4 missing values)	86	1225	14		
(b) Young Donors					
A. Levels of LDL	1	1055	1055	158.816	<0.001
B. Days	3	163	54	8.214	<0.001
C. Experiments	3	2508	836	125.795	<0.001
Interactions :					
A x B	3	142	47	7.136	<0.001
A x C	3	152	50	7.657	<0.001
B x C	9	61	6	1.034	N.S.
A x B x C	9	177	19	2.966	<0.01
Replications (9 missing values)	57	378	6		

Fig. 2 - Effect of LDL on total protein per cell and on total cholesterol per cell. (●) Control; (o) 20 ug LDL protein/ml medium; (Δ) 160 ug LDL protein/ml medium.

——— Cells from mature donors.

– – – – Cells from young donors.


A
B
PROTEIN (μg/10⁵ cells)
CHOLESTEROL (μg/10⁵ cells)
0
20
40
60
0
2
4
6
2
4
6
8
DAYS

Total cholesterol contents per cell after 8 days' incubation with LDL were 69% higher in the young cells and 79% higher in the mature cells than the cholesterol contents of cells in control medium. Thus the two groups appeared to respond similarly to LDL although the level of cellular cholesterol was maximum at 4 days in the young cells, whereas it was still rising at 8 days in the mature cells.

c. Effects of LDL on Total Protein Content of Cells

The changes in the total protein per cell in cultures from mature donors are shown in Table 11. It may be seen in Table 6 that there were no differences in the changes in protein content of diabetic cells and, as before, the two groups were subsequently treated as one and the combined results subjected to an analysis of variance. The results of this analysis (Table 12) are similar to those found with cell counts and cholesterol contents: the addition of LDL produced a significant effect on protein contents per cell and there was a significant effect of time. The meanings of the variation among experiments and of the interaction terms were explained above. As indicated in Fig. 2 , the high level of LDL produced a clear effect on the protein content of cells; however, the low level did not.

A similar effect of LDL was found in experiments with cells from young donors (Table 8). The results of the analysis of variance indicated that the effect of LDL was significant but that the effect of time was not (Table 12). As seen in

TABLE 11 Strains from Mature Donors: Effect of LDL on Total Protein per Cell

Treatments (ug LDL protein/ml medium):	Total Protein per Cell (% of That on Day 0)											
	0				22				191			
Days:	2	4	6	8	2	4	6	8	2	4	6	8
Experiments												
3 (N)	114	155	174	188	107	165	145	183	131	201	254	271
4 (D)	76	56	114	123	72	60	105	96	82	101[1]	170	176
5 (N)	123	187	174	206	130	213	165	173[1]	142	288	286	270[1]
6 (D)	127	181	210[1]	195	112	180	195[1]	321[1]	129	231	298	321[1]
7 (N)	81	158	292	332	78	190	293	274	106	253	297	332
8 (D)	103	262	334	420	96	235	301	359[1]	118	273	432	582[1]
Mean:	104	167	216	244	99	174	201	234	118	224	289	325
Standard Error:	5	15	19	24	6	14	19	24	5	14	19	31
Number of Culture Dishes:	18	18	17	18	18	18	17	15	18	17	18	15

Table 12 Analysis of Variance : Total Protein per Cell (% of that on day 0)

Source of Variation	Degrees of Freedom : Numerator	Sum of Squares	Mean Square	F Ratio	Level of Significance
(a) Mature Donors					
A. Levels of LDL.	2	160625	80312	197.313	< 0.001
B. Days	3	745404	248468	610.440	< 0.001
C. Experiments	5	659255	131851	323.934	< 0.001
Interactions :					
A x B	6	36901	6150	15.110	< 0.001
A x C	10	23041	2304	5.661	< 0.001
B x C	15	315290	21019	51.641	< 0.001
A x B x C	30	68457	2282	5.606	< 0.001
Replications (9 missing values)	135	54949	407		
(b) Young Donors					
A. Levels of LDL.	1	610144	610144	111.759	< 0.001
B. Days	3	43850	14617	2.677	N.S.
C. Experiments	3	298634	99545	18.233	< 0.001
Interactions :					
A x B	3	62159	20720	3.795	< 0.02
A x C	3	58807	19602	3.590	< 0.02
B x C	9	129964	14440	2.645	< 0.02
A x B x C	9	25199	2800	0.513	N.S.
Replications (9 missing values)	57	311190	5459		

Table 13 Mature compared with Young Cell Strains

LDL Treatment Level :	ZERO				HIGH			
Days :	2	4	6	8	2	4	6	8
Cell Count (% of that on day 0)								
Mature (n=11)*	197 ±18**	244 ±20	279 ±22	319 ±20	224 ±23	299 ±24	341 ±22	393 ±32
Young (n=4)	166 ±15	185 ±13	242 ±20	281 ±28	214 ±17	303 ±24	310 ±20	387 ±29
Total Cholesterol (ug / 100,000 cells)								
Mature (n=5)	-	2.3 ±.2	2.5 ±.2	2.9 ±.3	-	3.9 ±.3	4.3 ±.4	5.2 ±.5
Young (n=4)	1.1 ±.2	1.2 ±.2	1.2 ±.1	1.3 ±.1	1.4 ±.2	2.2 ±.2	1.9 ±.2	2.2 ±.2
Total Protein (% of that on day 0)								
Mature (n=6)	104 ± 5	167 ±15	216 ±19	244 ±24	118 ± 5	224 ±14	289 ±19	325 ±31
Young (n=4)	169 ±16	161 ±15	195 ±31	148 ± 9	299 ±30	298 ±39	353 ±44	414 ±45
Total Protein (ug / 100,000 cells)								
Mature (n=6)	23.4 ±1.5	34.8 ±2.1	45.2 ±1.5	50.4 ±2.2	26.4 ±1.6	49.0 ±2.6	61.8 ±1.4	66.3 ±2.6
Young (n=4)	9.8 ±1.6	8.3 ±0.7	9.6 ±1.1	10.0 ±1.4	17.4 ±2.7	16.2 ±2.3	18.3 ±2.3	25.0 ±3.5

* Number of experiments ** Standard Error of the Mean

Fig.2, the protein content of cells remained unchanged in control medium but appeared to change with LDL in the medium. The protein content of cells in LDL-containing medium was higher than that in control medium at all times. At the higher level, a clear effect of LDL on protein content per cell is evident in cultures of both young and mature cells. Changes in protein content in each group are summarised in Table 13. A 50% increase in the young control cells is in contrast with the 150% increase in protein content in mature control cells. The effect of LDL was however, smaller in the mature cells both in the change with time in LDL medium and in the change in protein content relative to that in control cells. The absolute values of protein content per cell are shown in Table 13 and confirm the observations referred to earlier that the mature cells were larger. It is seen that although the relative effects of LDL on protein content were smaller in the mature group, the net increases in protein content per cell (compared with control cells after 8 days) were 150-160 pg in cells from both groups of donors.

d. Correlation of Cell Protein and Cell Cholesterol

Pairs of values of cholesterol and protein (expressed as ug/10^5 cells) from all experiments in which both measurements were made are presented in Table 14. Cell protein was not measured on day 0 in experiments 9-11, and thus these experiments could not be included in the results in the

Table 14 Total Protein and Cholesterol (ug / 10^5 cells)

LDL Level:	Zero		Low		High	
	Cholest.	Protein	Cholest.	Protein	Cholest.	Protein
Expt.7						
Day 2	-	14.7	-	14.2	-	19.2
" 4	2.1	28.6	2.8	34.5	5.5	46.0
" 6	3.8	53.0	3.8	53.3	5.2	54.0
" 8	3.9	60.3	3.9	49.7	5.6	57.8
Expt.8						
Day 2	-	15.1	-	14.3	-	17.5
" 4	2.7	39.0	3.3	34.9	4.5	40.5
" 6	2.3	49.6	3.5	44.8	6.7	64.2
" 8	4.0	62.4	3.9	53.4	9.0	86.7
Expt.9						
Day 2	-	32.7	-	28.6	-	30.2
" 4	3.5	44.2	4.0	39.1	4.1	46.4
" 6	2.2	35.8	3.4	36.0	3.7	43.8
" 8	2.3	36.7	2.9	35.2	4.0	49.0
Expt.10						
Day 2	-	24.0	-	24.8	-	28.0
" 4	1.0	30.2	2.0	29.3	2.2	35.8
" 6	1.6	29.4	2.5	28.2	2.5	36.7
" 8	2.0	35.4	2.6	39.9	3.5	54.2
Expt.11						
Day 2	-	25.2	-	29.7	-	26.7
" 4	2.1	31.1	2.7	34.2	2.9	36.9
" 6	2.4	30.3	3.4	28.5	3.4	36.5
" 8	2.0	39.1	2.7	39.9	3.7	44.9
Expt.12						
Day 2	0.7	5.2			0.8	12.3
" 4	0.9	9.1			1.3	16.5
" 6	0.7	9.6			1.0	12.6
" 8	0.7	-			1.2	-
Expt.13						
Day 2	2.0	13.1			1.8	16.8
" 4	1.9	7.5			2.8	8.8
" 6	1.5	6.2			2.8	16.2
" 8	1.4	11.2			2.8	22.9
Expt.14						
Day 2	0.4	5.4			0.8	9.1
" 4	0.3	5.9			1.6	10.9
" 6	0.9	8.7			1.3	15.7
" 8	0.8	4.7			1.6	14.2
Expt.15						
Day 2	1.3	15.6			1.4	29.7
" 4	1.3	10.9			2.2	26.3
" 6	1.4	14.1			1.9	28.8
" 8	1.6	14.1				

preceding section (III. B. 4. c.).

The results are presented on a graph of Total Protein versus Total Cholesterol (Fig.3) from which it is evident that a good correlation exists between protein and cholesterol content in these cells. The equations for the regression lines and the associated correlation coefficients were calculated for the pairs of values in each treatment group in each of the mature and young donor cell groups, for all pairs of values in each of the two donor groups, and for all pairs of values pooled from all groups. These are presented in Table 15.

Only the correlation of cholesterol and protein of the young donor cells treated with the high level of LDL was not significant at the $p < 0.05$ level. When these results were pooled with those from the cultures treated with control medium alone, the correlation was significant at the $p < 0.01$ level. The correlation of cholesterol and protein was highly significant for the mature donor cells in all treatment groups. When all the results were pooled, the correlation was significant at the $p < 0.001$ level, indicating a definite association between the levels of cholesterol and protein in human skin fibroblasts that was independent of the age of the donor and the availability of LDL cholesterol in the medium.

Fig. 3 - The relation between total protein and total cholesterol per cell in cultures of fibroblasts in the presence and absence of LDL.

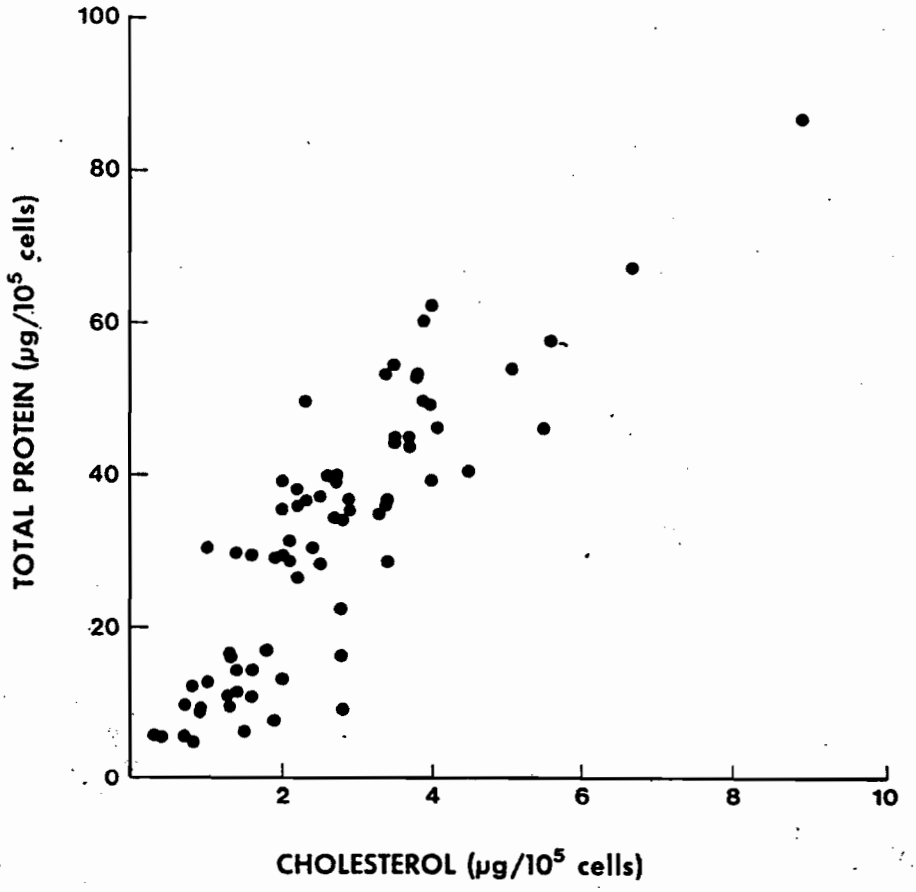

100
80
60
40
20
0
2
4
6
8
10
TOTAL PROTEIN (μg/10⁵ cells)
CHOLESTEROL (μg/10⁵ cells)

Table 15 Regression of Cholesterol per Cell on Protein per Cell

Donor Group	Level of LDL	Regression Equation	Correlation Coefficient	Level of Significance
Mature (n*=15)	0	y=10.816x + 13.011	0.85613	< 0.001
Mature (n=15)	Low	y= 9.321x + 9.272	0.68265	< 0.01
Mature (n=15)	High	y= 7.005x + 17.840	0.91608	< 0.001
Young (n=15)	0	y= 4.082x + 4.767	0.57969	< 0.05
Young (n=15)	High	y= 3.742x + 12.019	0.29984	N.S.
Mature (n=45)	All	y= 6.969x + 19.144	0.83288	< 0.001
Young (n=30)	Both	y= 6.227x + 4.991	0.52580	< 0.01
Both (n=75)	All	y=10.063x + 4.975	0.86367	< 0.001

* Number of pairs of values (Each pair is the average of triplicate culture dishes).

5. Discussion

Major events in the development of atherosclerosis include the accumulation of cholesterol within arterial smooth muscle cells, the death of these cells, and the deposition of cholesterol in the extracellular space in the arterial wall (Sections I.A. and I.B.). While it is accepted generally that diabetes mellitus is a risk factor in atherosclerosis, the reasons for this are presently unknown. Among the many possible causes of a greater susceptibilty to arterial disease is the intracellular accumulation of cholesterol at a rate higher than normal.

The accumulation of cholesterol by diabetic cells in culture was examined. As the results in Table 6 show, no difference in the cholesterol content of diabetic and normal cells could be found. An abnormal number of necrotic cells was not observed in the diabetic cultures on routine inspection during the experimental periods; as the results in Table 6 indicate, the numbers of cells were in fact, virtually the same in the diabetic and normal groups at all times during the experiments. It is concluded, therefore, that the diabetic cells do not accumulate an abnormal amount of cholesterol and that there was no observable increase in the rate of cell death in diabetic cultures that may have resulted from an excessive accumulation of cholesterol within these cells.

The metabolism of LDL by skin fibroblasts and by arterial smooth muscle cells is similar (Section I.E.4.). It is reasonable to suggest therefore, that it is unlikely

that diabetic aortic smooth muscle cells would respond to LDL differently from normal cells. This, of course, remains to be verified. There is, however, no proof at present that the effects of LDL on cells in vitro are representative of the effects of LDL in vivo and the present results can only suggest that an abnormality in the cellular response to LDL is not a cause of premature atherosclerosis in diabetics.

LDL increased the proliferation of cells from both mature and young donors. Both levels of LDL used in the experiments with cells from the mature donors produced this effect although increased proliferation was measurable at an earlier time with the higher level; after 8 days, however, cell numbers with the two levels of LDL were identical. This demonstration of an effect of serum LDL on cell proliferation agrees with the results of Ross and Glomset with monkey smooth muscle cells (15) and with those of Brown, Mahley, and Assmann (111) with swine aortic smooth muscle cells. The present work does not support the claim of Fischer-Dzoga and Wissler that LDL enhances cell proliferation only when prepared from hyperlipidemic serum (109,110) since LDL in these experiments with skin fibroblasts was prepared from normolipidemic subjects. Fischer-Dzoga and Wissler used monkey aortic smooth muscle cells; Ross and Glomset used cells from a different species of monkey and found an effect of LDL (presumed to have been prepared from normolipidemic animals); and Brown, Mahley, and Assmann found no difference in LDL-stimulation of cell

proliferation in cultures of swine aortic smooth muscle cells when the LDL was prepared from hyperlipidemic serum.

It is seen in Figure 1 that cell proliferation is most rapid during the first two days of the experiments. This is evident in both control and LDL-treated cultures, and the slower proliferation that follows possibly reflects an increasing insensitivity to mitosis-promoting agents present in the PPLFS as cell density increases. It is also possible that the rapid increase in cell numbers in the first two days reflects a carry-over of the effect of mitogens present in the fetal calf serum in which the cells were incubated prior to day 0. This is unlikely, however, because Ross and Glomset found the same diminishing rate of proliferation after pre-incubating their cultures in 1% whole monkey serum, which is generally considered to produce little effect on cell proliferation (15). Ross et al. found the same effect when monkey aortic smooth muscle cells were pre-incubated with 1% whole monkey serum followed by incubation with 5% whole monkey serum which should have produced a maximum effect on cell proliferation (114). The maximum effect on proliferation of young and mature cells of the LDL relative to the controls appeared to have been established at four days.

The effect of the higher level of LDL after eight days was to have increased the cell number to 393% of that on day 0 in the mature cell cultures and to 387% of that on day 0 in the young cell cultures. The control medium had a greater effect

on proliferation of the mature cells than on the young cells but the difference appears to have been established during the first two days and remained fairly constant throughout the experiment. It is generally believed that younger cells proliferate more rapidly in culture; thus it was unexpected that the mature cells proliferated more rapidly.

Although LDL has been shown to stimulate proliferation in human cells in vitro, it is not known if this result is applicable to aortic smooth muscle cells in vivo, and if it is, whether this in itself can cause atherogenesis. Smooth muscle cell proliferation probably occurs in normal arteries following endothelial injury without necessarily initiating atherosclerosis (13). It is possible, however, that if proliferation is accompanied by a process that favours cholesterol accumulation, then formation of an atherosclerotic lesion is the result.

Cells from mature donors contained more cholesterol than cells from young donors (Table 13). The increased content in the older cells was probably due to a larger cell size which is the result of aging in diploid fibroblasts (200), although an alternative, or possibly additional, cause is an inherent difference in the cholesterol content of the tissues of origin of the two groups of cells (mature cells were from deltoid skin and young cells were from genital skin).

LDL elevated the cholesterol levels in both mature and

young cells and in general, the cholesterol content per cell reflected the amount of LDL-cholesterol in the medium. These results are in agreement with the results of previous studies that have shown that serum lipoproteins in the medium are the major source of cholesterol in cultured cells (39,123,126 144). An inverse relation is apparent between the rate of increase in cell numbers and the amount of cholesterol per cell in the cultures from mature donors. As seen in Fig.1 the effect of LDL on proliferation is greatest during the first two days after which it rapidly declines; the effect of LDL on cholesterol content per cell is maintained, however, and cholesterol levels were still rising when the experiments were terminated at eight days. In contrast, a declining effect of LDL on the proliferation of cells from young donors was accompanied by a disappearance after two days of the LDL effect on cholesterol content although the overall increase in cholesterol per cell was similar in the two groups: relative to controls, a 79% increase was observed in cultures of mature cells and a 69% increase was measured in cultures of young cells.

Intracellular cholesterol synthesis operates only in the absence of serum lipoproteins in the medium (201). The addition of LDL to the medium results in a series of events initiated by LDL binding to a high-affinity, saturable, plasma membrane receptor that includes: the lysosomal hydrolysis of internalized LDL, the release of cholesterol from LDL by this

hydrolysis, a suppression of the synthesis of the rate-limiting enzyme in cholesterol biosynthesis (3-hydroxy-3-methylglutaryl coenzyme-A reductase) as a result of cholesterol release, and the suppression of the synthesis of the plasma membrane LDL receptor which then causes a reduction in the amount of LDL internalized by the high-affinity mechanism (Section I.E.4.).

The low-affinity mechanism appears to be under no control since the amount of LDL bound to cells, internalized and degraded, is proportional to the extracellular LDL concentration (137). The formation of "foam cells" in culture was not observed when levels of up to 400 ug LDL-cholesterol per ml of medium were used for periods of 24 hours (Section I.E.4.). In the present work, levels of LDL equivalent to up to 260 ug LDL-cholesterol per ml of medium were used and the cells from mature donors continued to accumulate cholesterol up to eight days when the experiments were terminated. It is not known if foam cells would have been produced in experiments of longer duration. In the cultures of cells from young donors, cell cholesterol levels reached a plateau at two days and remained virtually unchanged until the experiments were terminated on day 8. This difference in the pattern of cholesterol accumulation may be attributable to a relative loss of control of cellular cholesterol content with aging but it may also be due to the different tissues of origin of the two groups of cells.

The data in Table 13 indicate that considerably larger amounts of protein are associated with the mature cells compared with the young cells. As mentioned previously, cell size increases in aging diploid fibroblasts, and one would expect that this would require additional structural protein.

An effect of LDL on increasing the protein of cells from mature and young donors was demonstrated. This effect, however, is dependent on the amount of LDL to which the cells are exposed: the lower level of LDL did not produce any change in cell protein content different from that produced by the control medium.

The young cells in control medium alone, increased their protein contents during the first two days but maintained it at this level for the remaining six days resulting in an overall increase of 75% over the eight-day period. The mature cells increased their protein per cell by about 120% after eight days in control medium but this increase was a steady one after two days, before which, in contrast to the young cells, virtually no protein increase per cell was observed. Relative to controls, at eight days the effect of LDL was an 81% increase in protein per cell in the mature group and a 266% increase in the young group. While this is a large difference between the two groups, it is interesting that the absolute amounts of protein these figures represent are similar; LDL produced an effect of increasing the protein of each cell by 150-160 pg (Table 13). Since all of this increase could be accounted for

by the uptake of LDL-protein from the medium, experiments were designed to examine the source of this protein, initially with the purpose of determining if it was, in fact, LDL-protein accumulating in the cells. Results of this work are reported in a later section (Section III.C.).

Compared with the numbers of studies in the literature dealing with proliferation and cholesterol metabolism of cultured cells, relatively few have focussed on variations in protein levels and their causes. The estimation of replication rates in cultured cells by measuring the total protein of the culture has quite recently been recommended in a text on tissue culture methodology (202). It is evident from the few reports dealing with cellular protein levels in proliferating cultures, however, that only under certain conditions would this method be free of significant error. The general conclusions from studies on protein contents of HeLa (203), Chinese Hamster (204), and mouse L cells (205) in proliferating cultures are that protein content is maximum during lag phase, declines during the exponential phase of proliferation, and varies considerably from cell to cell even at mitosis. These studies, in addition to the previously mentioned changes in cell size in aging diploid fibroblasts (200), do not support an argument for a unique value for cellular protein content even within presumably homogeneous cultures such as the established lines (HeLa cells, mouse L cells, etc.)

The data from experiments with human skin fibroblasts

indicate no consistent relation between the rate of increase in cell number and the protein content of these cells: a) the high level of LDL increased both the rate of proliferation and the protein content of mature cells compared with those in control medium alone, while the low level increased the rate of proliferation but not the protein content; all three treatments produced their greatest effects on cell proliferation during the first two days when virtually no effect on protein per cell was observed; b) the increases in cell numbers and protein contents were parallel in the first two days in the young cell cultures with both treatments and maintained this pattern in the LDL-treated cultures until the end of the experiments; but while the numbers of cells were increasing in the control medium, the protein contents remained fairly constant until the end of the experiments. This lack of a relation between proliferation and cell protein is in agreement with the results of Salzman from cultures of HeLa cells (203) and Kimball et al. from cultures of Chinese hamster cells (204). The loss of protein during the latter stage of exponential proliferation found by these workers and by Tsuboi et al. (205) could not be verified with human skin fibroblast cultures because a logarithmic phase of proliferation was never observed. In contrast, however, an increasing cell density in the fibroblast cultures in the present study was generally accompanied by an increase in protein content per cell rather than by the decrease found with the established lines. Whether

this represents an inherent difference between cell lines (with an apparently infinite capacity for replication) and cell strains (with a limited capacity for replication), or whether it is a characteristic of all cell cultures after a logarithmic increase in numbers remains to be determined.

As discussed previously, the high level of LDL produced an increase in cholesterol in human skin fibroblasts. The relation between the effects of LDL on cholesterol and protein contents was examined. A correlation was found between protein and cholesterol contents when all treatments were combined which suggests that LDL-cholesterol is incorporated into the cell structures (probably the membranes). If a relation had not been found and the cholesterol : protein ratios were higher in the cells treated with LDL, this would have implied that the cells were storing cholesterol in a non-structural form such as within lysosomes or vacuoles.

Linear regression analysis was performed on the combined data from all treatments in the young and mature cell experiments separately and then on the pooled data from both groups. The results are presented in Table 15 . Each of the analyses of results from mature and young cells yields a highly significant correlation of cholesterol and protein content per cell. The slopes of the lines of regression are similar (6.969 for the mature cells; 6.227 for the young cells) and as expected from the higher absolute amount of mature cell protein that was reported earlier, the regression line intercept on the ordinate is

higher (19.144) than that for the young cells (4.991). The difference between the two conceivably represents an amount of cell protein that is not associated with cholesterol and accumulates with aging *in vivo*. When the data from these two groups were combined, the intercept was essentially the same as that for the young cells alone (4.975), the slope had increased to 10.063, and the correlation between cholesterol and protein was significant at the $p < 0.001$ level. This result suggests that although LDL increases the cellular content of cholesterol, it also produces an effect of increasing the protein content by an amount that maintains a constant ratio of cholesterol : protein (of 0.1) after subtracting an amount of protein which may represent functional rather than structural protein. This observation may be relevant to the failure to find a report in the literature describing the production of lipid-filled cells following incubation with serum LDL. Furthermore, it adds to the current information on the regulation of cholesterol accumulation in mammalian cells in culture by suggesting that a mechanism exists to incorporate the LDL-derived cholesterol into the structure of the cell rather than into lipid-filled droplets or lysosomes in the cytoplasm. These droplets accumulate readily when cultured cells are incubated with non-lipoprotein bound cholesterol (123,149) or with LDL modified to alter the net charge on the molecule to a positive one (147,148).

III. C. Source of the Increased Protein Content per Cell

It was shown in the previous section that LDL elevated the protein content per cell. The increase amounted to 150 pg per cell after eight days when the high level of LDL was added to the medium. The increase relative to controls was 81% in the mature cells and 266% in the young cells, although the additional protein in the medium resulting from the addition of LDL was only 3%. This effect of LDL has not been reported in the literature. Thus an examination of the possible sources of the additional protein was carried out to determine the exact effect of LDL on cellular protein metabolism. Several processes were examined: synthesis, degradation, internalization of extracellular proteins, and export of proteins to the extracellular space.

1. Accumulation of ^{125}I-LDL Protein by Human Skin Fibroblasts in Culture

a. Introduction

The maximum number of cells counted after eight days of incubation with LDL in any of the previously described experiments was approximately 3×10^5. The maximum amount of LDL added was 203 ug protein per ml which was added three times during the eight-day period. The volume of the medium was 2 ml at each time and thus the total amount of LDL to which the cells were exposed was 1.2 mg. The minimum amount of LDL available to each cell was, therefore, 4×10^3 pg, which is

more then 26 times the absolute protein increase of 150 pg. It was possible then, that the increase was due to undegraded LDL protein taken into the cells.

Skin fibroblasts and aortic smooth muscle cells have been shown to degrade LDL protein (46,137,144). The rate of degradation of LDL protein is constant after a lag period of approximately 2 hours. Since the amount of cell-associated LDL was seen to have reached a constant level at this time, it was proposed that a steady state had been established in which the rates of internalization and of removal of LDL protein were equal (144). The amount of LDL used was similar to the lower level used in the present work, a level that produced no increase in protein content per cell. A steady state has not been reported in cells exposed to high levels of LDL similar to the ones used in this work, and the possibility that cells were unable to degrade completely LDL protein at these levels was examined.

Cells were incubated for up to 6 days in medium containing ^{125}I-LDL. At 3 and 6 days the cell protein was precipitated and cell lipid was extracted with chloroform/methanol. ^{125}I-activity was counted in the precipitate and the specific activity of the ^{125}I-LDL originally added was used to estimate the intact LDL protein associated with the cells. The accumulation of intact LDL protein by fibroblasts was further examined by radial immunodiffusion.

b. Materials and Methods

Methods of preparation of LDL from human serum, of cell culture, of cell counting, and of estimation of cell cholesterol and protein contents have been described in the General Methods section (III. A.).

i. Iodination of LDL with ^{125}I

LDL was iodinated using the iodine monochloride, ICl, method of McFarlane (206) as applied to LDL by Dr. D. Bilheimer to whom I am indebted for the details (207). This method was recommended by Shepherd, Bedford, and Morgan (208) who compared four methods of iodinating LDL and found that by using this technique they could prepare ^{125}I-LDL that:

- was immunologically indistinguishable from native LDL;
- eluted as a single peak from a gel filtration column;
- was removed in a mono-exponential manner from plasma after injection into the vascular compartment of the rat.

Less than 5% of the label was extracted with ethanol : ether.

Glycine buffer (0.75 ml, 1 M, pH 10.0) was added to 0.4 ml LDL solution (at least 10 mg LDL protein/ml, NaCl 0.15 M, EDTA 0.01%, pH 7.4); sodium ^{125}iodide (1.5 uCi per mg LDL protein) was added to 0.5 ml glycine buffer, which was then added to the above solution; ICl (0.033 M) was diluted with NaCl (2 M) to a concentration which would yield an average of 1 atom of iodine per molecule of LDL. (The molecular weight of apo-LDL is uncertain: Dr. Bilheimer's method assumed a molecular weight of 10^5 and this was used here.) Diluted

ICl (0.4 ml) was rapidly mixed with the LDL solution and incubated on ice for 5 minutes. The mixture was dialyzed for 24 hours (NaCl 0.15 M, EDTA (Na_2) 0.01%, pH 7.4, 500 ml x 6), sterilized by passage through a sterile cellulose acetate filter (0.22 um) into a sterile 3 ml bottle, and stored at 4°C. All of the above operations were performed at 4°C.

The efficacy of the ICl reagent and the method were established by iodinating BSA and subjecting the ^{125}I-BSA and native BSA to simultaneous electrophoresis on separate polyacrylamide gels. The migrations of ^{125}I-activity and Coomassie Brilliant blue-staining material corresponded, and the relative activities and densities of staining were similar for each band.

Although ^{125}I-LDL has been reported to retain characteristic immunologic and cell-binding properties for up to one month (137), it was prepared immediately before use in the following experiments. The specific activities ranged from 5742 to 8071 cpm/ug LDL protein (average: 7075).

More than 98% of ^{125}I-activity was precipitated by TCA (10%) and less than 10% of the ^{125}I-activity was extracted into a mixture of chloroform/methanol (2 : 1). ^{125}I-LDL co-migrated with native LDL during electrophoresis in polyacrylamide gels (10%) (209).

The liberation of free ^{125}iodide from ^{125}I-LDL by FCS has been reported (142). ^{125}I-LDL was added to PPLFS and incubated for 3 days at 37°C. More than 95% of the original ^{125}I-activity

was recovered from the TCA precipitate. It was thus concluded that no active de-iodinase was present in the PPLFS that could cause an underestimation of LDL protein in the cells.

ii. Counting of ^{125}I-activity

All samples were counted in a gamma counter (Nuclear Chicago) with an efficiency of approximately 50%. All tubes were pre-counted for background and were rejected if more than 60 cpm were obtained. The average value of background counts was subtracted from the total counts of each sample prior to calculating the final count to be used to estimate the amount of ^{125}I-LDL protein present in the samples. In all experiments, except for #5, at least 10,000 counts per sample were obtained, and in this experiment, at least five times the background number were counted.

iii. Preparation of gels for immunodiffusion

The procedure for radial immunodiffusion was originally developed by Ouchterlony (210) and has been applied to the study of serum lipoproteins (193). Gels were prepared from agarose instead of the more frequently-used agar because of the latter's interaction with LDL (193).

Agarose (Sea-Kem, 2.5 gm) was added to 250 ml barbital buffer (pH 8.2) in a boiling-water bath. The suspension was filtered while hot and was poured into glass petri-dishes, the surfaces of which had been previously smeared with the agarose solution and allowed to dry in air. Wells were cut with the fire-polished tip of a pasteur pipette connected to a

vacuum aspirating flask. Gels were stored in a humidified container at 4°C until required. The capacity of each well was approximately 4 ul.

iv. Anti-human-LDL antiserum

The antiserum was obtained from Miles-Yeda Ltd. It had been raised in goats and was monospecific by immuno-electrophoresis with human serum. It did not precipitate human HDL. Seven mg of antibody per ml were present and a preliminary experiment showed reaction at full strength with 60 ug LDL protein per ml but not with 6 ug per ml. Reactions were observed at a four-fold dilution of antiserum but not at a sixteen-fold dilution.

c. Experimental Protocol

i. Measurement of cell-associated ^{125}I-LDL

Cells (4.5-7.0 x 10^4) from each of two strains of human skin fibroblasts were plated into each of 20 petri-dishes (3.5 cm diameter) containing 2 ml FCS medium. After incubation for three days at 37°C in a humidified atmosphere of CO_2 : air (1 : 19), three dishes were counted for cells. If the number was below that plated, the medium was changed and the cells were counted at a later date. If it was similar to the number plated, the medium was removed, the cells were washed twice with serum-free DME (1 ml), 2 ml of PPLFS were added to all dishes, and ^{125}I-LDL (156 ug LDL protein/ml medium, range: 130-170 ug/ml) was added to the experimental dishes.

Three dishes in each of the two groups (± LDL) were

taken at 3 and at 6 days; medium was removed, the cells were washed 5 times with warm PBS, released with trypsin and versene, and pelleted by centrifugation (General Methods, Section III. A. 4.). The trypsin-versene supernatant was removed, and was pipetted into an equal volume of TCA (20% w/v). This was allowed to stand at 4°C for 1 hour and was then centrifuged (International PR-6, 3,000 rpm, 4°C, 15 minutes). The supernatant was discarded, the pellet was washed twice with cold TCA (10% w/v) and was dissolved in 1 ml NaOH (1 N). An aliquot was counted for ^{125}I-activity to yield an estimate of cell-bound, but not internalized, LDL (143). The cell pellet was washed twice with PBS and was suspended in 6 ml PBS for cell counting, extraction and estimation of cholesterol, and estimation of protein as described in the General Methods section (III. A. 4., 5., and 6.). An aliquot of the dissolved, lipid-free residue (after incubation at 37°C with NaOH) was counted for ^{125}I-activity to yield an estimate of internalized, intact LDL protein.

ii. Measurement of cell-associated LDL by radial immunodiffusion

In one of the above experiments, native LDL was added (193 ug/ml medium) to three additional dishes of cells and three more dishes were included as controls. All six dishes contained 2 ml of PPLFS. This was changed after three days; the experimental dishes also received the original amount of LDL. At 6 days, medium was removed from all dishes. Cells

were released with trypsin and EDTA (Na_2) and were centrifuged. The cell pellets were washed with PBS (1 ml x 2). The final volume of the cell pellet was estimated to be 50 ul. An equal volume of sodium deoxycholate (2%) was added and the pellets were incubated overnight at 37°C (137). A small amount of material remained insoluble after adding a further 50 ul of sodium deoxycholate and was sedimented by centrifugation. Aliquots of the supernatants from the three LDL-treated samples and of the supernatants from the control samples were placed in the appropriate wells of an immunodiffusion gel. An amount of native LDL equivalent to the increase in protein content of cells incubated with LDL was added to portions of the supernatants from control cells and was placed in wells of the immunodiffusion gel. The gel was allowed to stand at 22°C overnight in a humidified chamber.

d. Results

Five experiments were performed and as the results in Table 16 show, the effects of ^{125}I-LDL on cell proliferation, cholesterol content per cell, and protein content per cell were similar to those found previously with native LDL.

Trypsin-releasable and trypsin-resistant, protein-associated ^{125}I-activities are shown in Table 17. The specific activities of ^{125}I-LDL in each experiment are indicated and were used to calculate the ^{125}I-LDL protein associated with the ^{125}I-activities.

Although the protein content per cell relative to

Table 16 Effects of ^{125}I-LDL

Expt.	Time (Days)	LDL-Treated Cell Number (% of control)	LDL-Treated Cholesterol Content per Cell (% of control)	LDL-Treated Protein Content per Cell (% of control)
1	3	183 (177-191)*	85 (66-101)	142 (136-152)
	6	157 (142-173)	111 (78-167)	124 (115-133)
2	3	143 (125-154)	125 (107-138)	109 (103-112)
	6	150 (145-154)	166 (164-172)	129 (120-136)
3	3	146 (126-139)	191 (170-220)	136 (126-147)
	6	130 (118-137)	150 (138-163)	148 (145-153)
4	3	151 (140-168)	141 (124-150)	108 (108-109)
	6	150 (142-158)	162 (156-167)	126 (116-131)
5	3	130 (125-134)	128 (124-133)	109 (106-114)
	6	214 (148-330)	93 (73-108)	122 (121-124)

* The range of values from 3 culture dishes.

Table 17 Trypsin-Resistant and -Releasable ^{125}I-activity

Expt. #	Time (Days)	Protein Increase in LDL-treated Cells (ug/10^5 cells)	Specific Activity of ^{125}I-LDL cpm/ng protein	Trypsin-Resistant ^{125}I-Activity cpm/10^5 cells	Trypsin-Resistant ^{125}I-LDL Protein ng/10^5 cells	Trypsin-Releasable ^{125}I-Activity cpm/10^5 cells	Trypsin-Releasable ^{125}I-LDL Protein ng/10^5 cells
1	3	12.0 (10.1-14.9)*	5.7	200 (191-210)	35	562 (394-719)	99
	6	6.8 (4.4- 9.3)		245 (213-267)	43	900 (664-1137)	158
2	3	3.3 (1.2- 4.3)	6.7	669 (624-730)	100	1054 (708-1265)	157
	6	10.2 (7.2-13.2)		836 (738-957)	125	1222 (1147-1334)	182
3	3	11.2 (8.1-14.5)	6.7	865 (746-1025)	129	1360 (1211-1585)	203
	6	15.8 (14.7-17.4)		1276 (1243-1323)	190	1377 (1240-1600)	205
4	3	2.1 (1.9-2.3)	8.1	699 (649-774)	86	1652 (1397-1904)	204
	6	9.7 (5.9-11.8)		1204 (1176-1259)	149	1825 (1702-1905)	225
5	3	3.2 (2.1- 5.1)	8.1	258 (244-267)	32	2196 (1564-2828)	271
	6	10.4 (9.7-11.4)		712 (541-984)	88	11835 (5329-15746)	1461

* The range of values from three culture dishes.

controls was higher in the LDL-treated cultures by 63.6 (21.0-120.0) pg in three days and by 105.8 (68.0-158.0) pg in six days, no more than 1.9 pg of this could be attributed to LDL protein at any time. An average increase of 68% occurred between days 3 and 6 in the amount of ^{125}I-LDL protein that was resistant to trypsin indicating that undegraded LDL protein was indeed accumulating in these cells although its contribution to the total cellular protein was insignificant.

Trypsin-releasable ^{125}I-LDL increased on average by a smaller amount between days 3 and 6 (22%). The atypically large increase in experiment #5 was excluded from the calculation. The explanation for this unusually large increase is not obvious; all other results in this experiment were consistent with those from the other four experiments. It is possible that a small number of injured cells were present in the cultures since injured cells are known to bind a disproportionately large amount of protein (211).

The results of the immunodiffusion experiment were as follows. In one quadrant of the gel, solubilized cells from each of the three dishes to which LDL had been added were allowed to diffuse against the antiserum at full strength; in another quadrant, one sample of solubilized, LDL-treated cells was allowed to diffuse against each of three concentrations of antiserum (100%, 50%, and 25% v/v in NaCl (0.15 M)); in a third quadrant, solubilized cells to which

native LDL had been added were allowed to diffuse against antiserum (100%); and in the fourth quadrant, the antiserum at three concentrations was allowed to diffuse against native LDL (6 mg protein/ml). LDL-treated cells did not react with the antiserum to LDL at any dilutions of cell solution or antiserum used. The antiserum did form lines of precipitation with native LDL and with solubilized cells to which native LDL was added. The additional protein content per cell in the LDL-treated cultures was 97 pg (Experiment #4). Cells (0.5×10^6) were dissolved in 150 ul of which 4 ul were placed in each well of the agarose gel. Thus if all of this additional protein were LDL protein, each well would contain 1.29 ug of LDL protein. A comparable amount (1.60 ug LDL protein) was added to 4 ul of the solubilized control cells, which reacted with the antiserum, indicating that the antiserum was effective in precipitating LDL protein at these concentrations and under these conditions. The gel was washed overnight in NaCl (0.15 M), stained with Coomassie Brilliant Blue G 250 in acetic acid : isopropanol : water (2 : 5 : 13), and de-stained in acetic acid (10%). The background staining, however, was too intense to permit a photograph.

e. Discussion

The purpose of these experiments was to determine if an amount of LDL protein comparable with the increase in total protein per cell previously demonstrated, could be detected in human skin fibroblasts exposed to LDL for up to

6 days. Except for two cholesterol results (at 3 days in Experiment #1 and at 6 days in Experiment #5), the effects of LDL on cell proliferation, cholesterol content per cell, and protein content per cell were similar to those found previously. This shows that ^{125}I-LDL was producing similar effects to those of native LDL.

The first set of experiments showed that although increases in cell-associated ^{125}I-activity were measureable from 3 to 6 days, the absolute amount of ^{125}I-LDL protein that these represented was insignificant when compared with the absolute increase in total protein per cell. This result was confirmed by the second type of experiment which did not reveal any immunologically detectable LDL-protein in these cells after 6 days when the total protein per cell had increased by 50%. An amount of native LDL protein comparable to the net increase in cell protein was added to the solubilized control cells and was shown to form a precipitate with the antiserum, indicating that the sensitivity of the method was adequate for the purpose.

The inference of these results is that accumulation of undegraded LDL protein in human skin fibroblasts cannot account for more than a small fraction of the increased total protein content per cell which follows the addition of a sufficiently high level of LDL to the culture medium.

2. Accumulation of ^{125}I-Human Serum Albumin by Human Skin Fibroblasts in Culture

a. Introduction

LDL protein does not accumulate in cultured fibroblasts to an amount which approaches the net increase in cell protein content produced by a high level of LDL in the medium. This increase might be produced, however, by an effect of LDL on increasing the internalization of serum proteins which might then be followed by an inability of the cells to degrade completely these proteins. In fact, some evidence that high levels of LDL in the medium may promote a greater rate of endocytosis has appeared: the volume of medium estimated by counting cell-associated ^{125}I-activity which was taken up by human skin fibroblasts in 3.5 hours was 4.1 ul/mg cell protein when ^{125}I-BSA (250 ug protein/ml) was added to Eagle's MEM, but was 9.4 ul/mg cell protein when the same amount of ^{125}I-LDL protein was added (137). This result could be explained by a specificity of protein internalization which favours LDL over BSA but may also be explained by a stimulation of non-specific endocytosis of medium proteins by LDL.

The purpose of the following experiments was to determine if the addition of a high level of LDL produced an increased amount of cell-associated ^{125}I-Human Serum Albumin (HSA). Albumin is the major protein in human serum. Because of the greater specific activity that would result, albumin, rather than all serum proteins, was iodinated. The duration of the

experiments was 10 hours, which was considered adequate for the expression of an effect of LDL on stimulating endocytosis. In fact, differences in the stimulation of cellular uptake of BSA by basic polymers have been measured after 2 hours (211).

b. Materials and Methods

Human Serum Albumin (Factor V, Essentially fatty acid-free) was purchased from the Sigma Chemical Co. The iodination of HSA was carried out with the same method used for iodinating LDL with appropriate changes in the amounts of ICl to ensure an average of 1 atom of iodine per molecule of HSA. The molecular weight of HSA was taken as 60,000 and a labelling efficiency of 60% was assumed (206). The specific activities of ^{125}I-HSA preparations are shown in Table 18. The specific activities of ^{125}I-HSA in the culture medium were lower because of the albumin in PPLFS. More than 99% of the ^{125}I-activity was precipitated by TCA (10%). All other methods have been described in preceding sections.

c. Experimental Protocol

Cells (4.8-10.1 x 10^4) were plated into each of 40 petri dishes (3.5 cm diameter) containing 2 ml of FCS medium. Three cell strains were used (two from foreskin and one from labia majora). The medium was changed after 3 days and was removed after a further 3 days. The cells were washed with serum-free medium and 2 ml of PPLFS were added to each dish. After 24 hours LDL was added to 15 dishes and an equal volume of control solution was added to another 15 dishes. The control

solution was a sterile preparation of the solution in which LDL was dialyzed. ^{125}I-HSA was added to each dish after 1 hour. (The amount added is shown in Table 18.) At the indicated time periods (Table 18), medium was removed from 3 dishes in each group, the cells were washed 5 times with PBS, and cold TCA (10% w/v, 1.5 ml) was added to each dish. The dishes were allowed to stand at 4°C for 1 hour before scraping off the cells which were then transferred to a 12 ml centrifuge tube. The dishes were each rinsed with 1 ml TCA twice and inspected under the light microscope for complete removal of cells. The TCA rinsing solutions were combined with the original 1.5 ml and centrifuged to dissolve completely the pellets. Aliquots were taken for estimation of protein (0.1 ml x 2) and for counting of ^{125}I-activity (0.2 ml). The total, cell-associated, TCA-insoluble ^{125}I-activity was calculated and divided by the protein estimate for each sample to correct for different recoveries of cell protein.

d. Results

The results given in Table 18 show no increase in the amount of cell-associated ^{125}I-HSA up to 10 hours after adding the LDL. The ^{125}I-HSA associated with the cells at 5 minutes probably represents simple adsorption of the HSA to the cell surface (212). In 2 of the 3 experiments only slightly greater amounts were seen at 7 or at 10 hours. It cannot be determined from these experiments alone whether these small increases indicate a slow rate of uptake of HSA or a steady state in

Table 18 Accumulation of ^{125}I-HSA by Human Skin Fibroblasts

	cpm / mg cell protein						
Time:	5 min	1 hr	3 hr	4 hr	5 hr	7 hr	10 hr
Expt.							
1							
+LDL	214 * (188-255)	161 (138-184)	179 (164-190)	-	250 (198-300)	236 (211-280)	-
-LDL	144 (119-163)	212 (172-243)	197 (148-250)	-	210 (180-239)	184 (174-203)	-
2							
+LDL	2564 (1893-3236)	1932 (1728-2137)	-	3947 (2861-5342)	-	2456 (2111-3077)	2042 (1890-2195)
-LDL	1923 (1378-2569)	2222 (2137-2380)	-	4290 (3948-4631)	-	3412 (2488-4649)	2864 (2500-3381)
3							
+LDL	2964 (1430-4213)	3461 (2678-4111)	-	4356 (3500-5215)	-	5104 (4210-6257)	-
-LDL	2517 (1794-3367)	4094 (3684-4431)	-	5348 (4137-6821)	-	6954 (4644-9263)	-

* The range of results from triplicate dishes.

Specific activities of ^{125}I-HSA:
Expt.#1: 3 cpm/ng HSA (0.9×10^6 cpm added in total)
Expt.#2 & #3: 27 cpm/ng HSA (8.1×10^6 cpm added in total)

which the rate of internalization is equal to the rate of degradation, or both. In Experiment #3 where the cell-associated ^{125}I-HSA did increase in 7 hours, the amounts were similar in the presence and absence of LDL.

e. Discussion

These results do not support the hypothesis that LDL enhances the rate of endocytosis of serum proteins. In Experiments #2 and #3 the amounts of ^{125}I-HSA that were associated with cells after the first 5 minutes were slightly lower in LDL-containing medium than in the control medium. Ryser has shown that negatively-charged polypeptides reduce the amount of albumin taken up by tumour cells in 2 hours (211). Since LDL carries a net negative charge, Ryser's results might possibly be extended to diploid fibroblasts. No attempts were made, however, to examine this in more detail. The purpose of these experiments was to determine the cause of the LDL effect on increasing the cell protein content; these results indicate that LDL does not promote the accumulation of serum proteins within the cell.

3. Effect of LDL on Cellular Protein Synthesis

a. Introduction

The previous experiments showed that neither a specific accumulation of LDL protein nor a non-specific accumulation of other serum proteins could account for the increased protein content per cell after exposure to LDL.

The following experiments were designed to determine if LDL increased cellular protein synthesis. Cellular protein synthesis is conveniently measured by the incorporation of a radio-labelled amino acid into macromolecules precipitable by TCA (213). An amino acid commonly used is L-leucine which is distributed widely in cellular protein.

b. Materials and Methods

Methods of preparation of LDL from human serum, of cell culture, of cell counting, and of estimation of protein have been described (Section III. A.).

L-leucine-1-C^{14} (28.7 mCi/mmole) and L(-4,5-^{3}H(N))-leucine (5 Ci/mmole) were purchased from New England Nuclear. Immediately prior to use, the labelled leucine was diluted to 0.25 uCi/ml with Eagle's MEM (leucine-free, Gibco). The radioactivity in each cell sample was counted in 10 ml of a solution comprising: 2,5-diphenyloxazole (PPO, scintillation grade, 4 gm/l); 1,4-di-(2-(5-phenoxyazoylyl)) benzene (POPOP, scintillation grade, 0.2 gm/l); napthalene (60 gm/l); ethylene glycol (B. Pt.: 196-198°C, 20 ml/l); methanol (redistilled, 100 ml/l); and 1,4-dioxane (Spectra grade,

to complete the volume).

Each counting vial was pre-counted for background with 10 ml of a solution containing PPO (4 gm/l); POPOP (0.2 gm/l) in toluene (A. R. grade) to a final volume of 1 litre.

The samples were counted in the single-channel mode of a Unilux II-A or Intertechnique SL30 scintillation spectrometer. At least 10,000 counts were obtained from each sample and disintegrations per minute (dpm) were calculated using the appropriate set of standards and the external standards in the spectrometers. Dpm per 10^5 cells for each sample was then calculated from the cell number counted in a culture that was treated identically. Five experiments were performed with four strains of fibroblasts from genital skin.

c. Experimental Protocol

Cells (4-7 x 10^4) were pipetted into each of 60 petri dishes (3.5 cm diameter) and were incubated at 37°C in 10% FCS for 3 days. The medium was removed and the cells from 3 dishes were counted. If the average count equalled the number that was plated, the medium was removed from the other dishes and the cells were washed twice with serum-free medium. PPLFS (2 ml) was added to each dish and LDL (144 ug protein/ml, range 129-160 ug) was added to each of the experimental dishes.

At 3 days, cells from 3 dishes in each group were counted and the protein content per cell was estimated to determine if LDL had produced an increase. If not, the medium was

removed from all dishes and fresh medium and LDL were added where appropriate. An effect of LDL was then looked for on day 5 and if found, the medium was removed and 2 ml of Eagle's MEM containing radio-labelled leucine was added to 6 dishes in each group which were then replaced in the incubator. The medium was aspirated from duplicate dishes in each group at 30, 60, and 90 minutes. The cells were washed 5 times with cold PBS, were allowed to stand at $4^{\circ}C$ for 1 hour with 1 ml of TCA (10% w/v), and were scraped into 12 ml centrifuge tubes. The dish-surfaces were rinsed with TCA (2 x 1 ml) and the washings were added to the centrifuge tubes. The precipitated material was sedimented by centrifugation, the pellets were washed again with TCA (2 x 1 ml), and were then dissolved in 0.5 ml of NaOH (1 N) at $37^{\circ}C$. Aliquots were taken for estimation of protein (0.1 x 2) and for counting of ^{14}C- or ^{3}H-activity (0.2 ml). A preliminary experiment was performed to determine the linearity of incorporation of radio-labelled leucine with time.

d. Results

As shown in Fig. 4, the incorporation of leucine into TCA-precipitable material was linear during a 2-hour period.

Cell numbers and protein content at the indicated times after addition of LDL are shown in Table 19. An average increase in protein content per cell of 34% (4-57%) was produced by LDL. In contrast with previous experiments minor increases or actual decreases in cell numbers with LDL were

Fig. 4 - Incorporation of radiolabelled-leucine into TCA-insoluble material with time.
Each point represents the mean result of triplicate dishes.

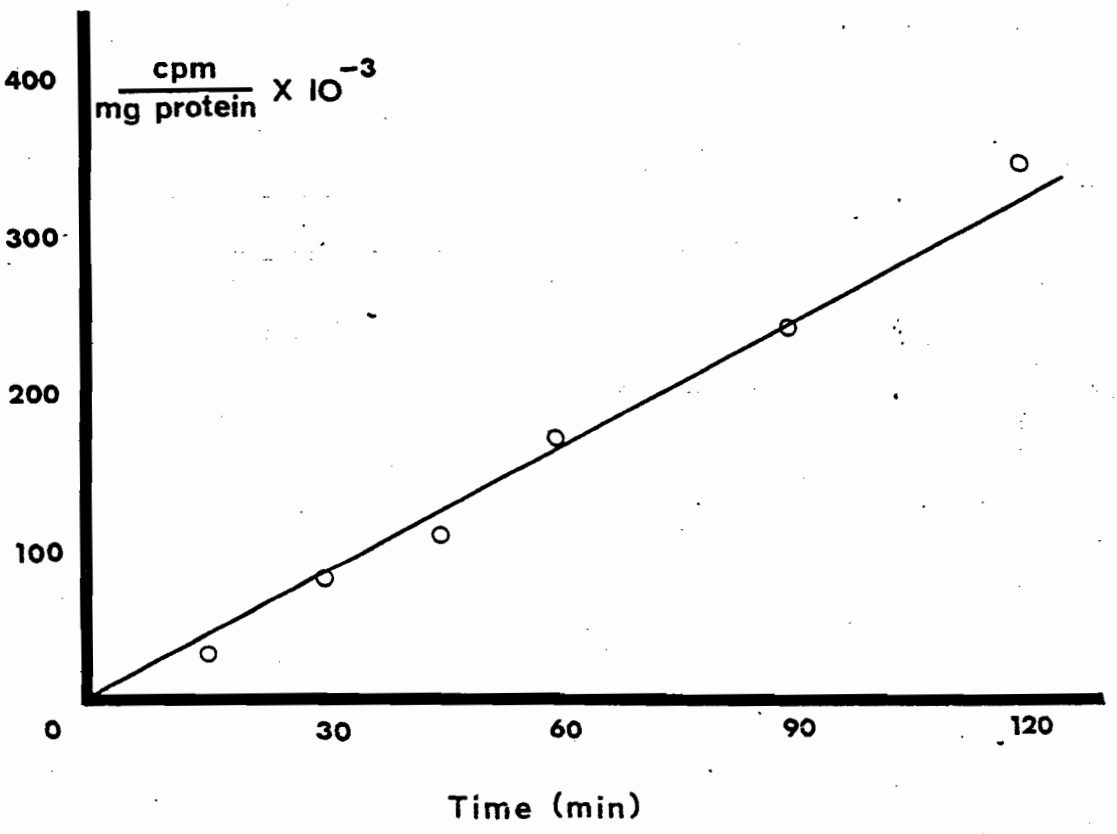

400
300
200
100
0
cpm / mg protein X 10^{-3}
30
60
90
120
Time (min)

Table 19 LDL Effect on Cell Protein Synthesis: Cell Numbers and Protein Content per Cell

Expt. #	Treatment Group	Cell Number ($\times 10^{-3}$)	Cell Protein ($ug/10^5$ cells)	Time after Addition of LDL (hrs)
1	+LDL	189*	47.2*	72
	-LDL	209	38.5	
2	+LDL	182	45.1	72
	-LDL	177	43.3	
3	+LDL	364	75.5	120
	-LDL	528	48.2	
4	+LDL	326	52.6	120
	-LDL	469	37.3	
5	+LDL	305	44.1	72
	-LDL	563	30.7	

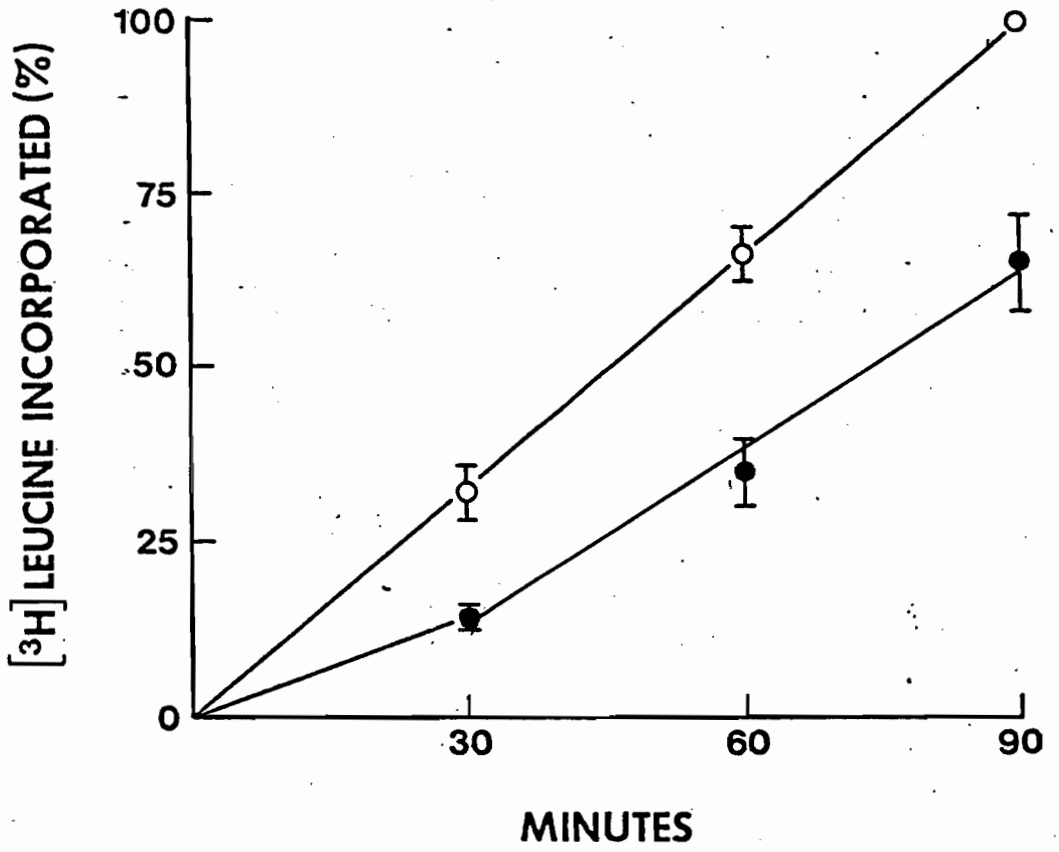
* Mean result from triplicate dishes

seen. Observations of the cultures under the light microscope revealed no obvious signs of a toxic effect of the LDL. The incorporation of radio-labelled leucine into TCA-insoluble material was linear with time in the control cultures. Addition of LDL to the control medium produced a rate of incorporation that was lower than that in the controls initially but that approached the control rate after the first 30-minute period of leucine incorporation (Fig. 5). This effect of the LDL was seen in all experiments and there was no evidence of an increase in the rate of protein synthesis in LDL-treated cells.

e. Discussion

The rates of protein synthesis in the presence and absence of LDL were examined at a time when the protein content per cell in LDL-treated cultures had increased relative to that in the controls. The rate of synthesis was not increased by LDL. Cells previously exposed to LDL incorporated less radio-labelled leucine into TCA-insoluble material, particularly during the first 30 minutes of the labelling period. This effect of LDL could be explained by a larger intracellular pool of leucine in LDL-treated cells that would produce a greater dilution of the isotope than in the controls and result in less incorporation of label. The absence of "cold" leucine in the labelling medium may then have caused a rapid depletion of the intracellular pool so that the rate of incorporation would then have risen to the level of the controls.

Fig. 5 - Incorporation of radiolabelled-leucine into cell protein in the presence (●) and absence (o) of LDL (160 ug protein/ml medium).
The figure shows the mean DPM/cell (±s.e.m.) of five experiments. DPM/cell was determined in each experiment in duplicate dishes in each group at each time point.
The means of results from 5 experiments are expressed as a percentage of the radiolabelled-leucine incorporated into protein in the control cells at 90 min.


100
75
50
25
0
30
60
90
[3H] LEUCINE INCORPORATED (%)
MINUTES

The numbers of cells in cultures exposed to LDL were in general lower than in control cultures. An obvious toxic effect of LDL was not observed and the reasons for these lower counts are not known. The effects of LDL on cell proliferation and on protein content per cell have been shown, however, to be independent (III. B. 4. c.). The results of the present work indicate that the effect of LDL on cell proliferation is also independent of the rate of protein synthesis. A comparison of results in Experiments #2 and #3 for example, shows different effects of LDL on cell proliferation but similar effects, relative to controls, on incorporation of leucine.

It is concluded, therefore, that the increased protein content per cell in cultures exposed to LDL is not due to an increase in the rate of protein synthesis.

4. Effect of LDL on Intracellular Protein Degradation

a. Introduction

The previous experiments demonstrated that the effect of LDL on protein content per cell could not be accounted for by accumulation of protein from the medium or by an increased rate of intracellular protein synthesis.

Of the possible sources of cellular protein listed in the introduction to this section, only a decreased rate of protein degradation and/or a decreased rate of export of intracellular proteins remain to account for the LDL effect. The purpose of the following experiments was to determine whether LDL was affecting either or both of these.

Cellular protein was labelled for several days by incubation in medium containing radiolabelled leucine. The medium was removed and the cells washed several times to remove radioactivity unincorporated into cellular protein. The release of free radiolabelled leucine from the degradation of labelled protein was then monitored at various times by removing the culture medium and counting the supernatants after precipitation of proteins with TCA and centrifugation, and by counting the TCA-insoluble radioactivity remaining in cellular protein. Using a similar method, Poole and Wibo have labelled two classes of proteins in rat fibroblasts in culture: one class with a rapid turnover and another class with a slow turnover (214). The present work eliminated the contribution of rapidly turning-over proteins from the final estimate of protein degradation. Previous results showed

that LDL was producing an effect on structural proteins which would be expected to be more stable and have longer half-lives than the rapidly turning-over proteins that release radio-labelled leucine into the medium during the first hour after removing the medium containing the radioactive leucine. The rapidly turning-over proteins would conceivably contribute a significant fraction of free leucine to the medium and possibly mask any effect of the LDL.

The method used contains theoretical sources of error which arise from the oxidation and from the reincorporation of labelled leucine released by protein degradation, each of which might result in an underestimation of the degradation rate. Glucose, pyruvate, and other amino acids in DME have been shown however, to eliminate the oxidation of leucine to CO_2 by mouse fibroblasts that occurs when these cells are incubated in Krebs-Ringer buffer alone (215). The reincorporation of leucine has been shown to be insignificant in cultures of WI-38 (216), 3T3 (217), and HeLa (218) cells provided that the extracellular concentration of leucine during the "chase" period was several orders of magnitude greater than the intracellular concentration and that the flux between the two pools was rapid. In consequence, only a small fraction of the intracellular leucine was found to be labelled. The work with HeLa cells indicated also that transfer-RNA was aminoacylated with leucine from the extracellular pool with little dilution from the intracellular pool (218). The medium present during the "chase" period in the following experiments contained a large

excess of unlabelled leucine relative to the intracellular labelled-leucine and since these cells were human fibroblasts (as are the WI-38 cells referred to above), it was assumed that the flux between the two pools would be rapid enough to prevent the accumulation of a significant fraction of isotopic leucine available for reincorporation. Losses of radio-labelled leucine by oxidation and the reincorporation of label into protein were assumed, therefore, to be insignificant.

b. Materials and Methods

Methods of preparation of LDL, of cell culture, of cell counting, and of estimation of protein have been described (III. A.). The method of counting radioactivity has been described also (III. C. 3. b.).

L-leucine-1-C^{14} (28.7 mCi/mmole) and L(-4,5-$^{3}H(N)$)-leucine (5 Ci/mmole) were purchased from New England Nuclear.

Four experiments were performed using one strain of fibroblasts.

c. Experimental Protocol

Cells (7-10 x 10^{4}) were pipetted into each of 60 petri dishes (3.5 cm diameter) containing 2 ml of 10% FCS and were incubated at 37°C for 2 days.

In the first experiment only, the medium was changed to fresh 10% FCS containing L-leucine-1-^{14}C (0.1 uCi/ml) and the cells were labelled with this for four days. The medium was removed and the cells were washed with serum-free medium (2 ml) containing 0.8 mM leucine. PPLFS (2 ml) was added to each

dish for 60 minutes. The purpose of this was to "wash out" labelled leucine from degraded proteins with a very rapid turn-over period. The medium was removed, the cells were washed with serum-free medium (2 ml) containing 0.8 mM leucine and PPLFS (2 ml) was added to each dish. LDL (144 ug protein) was added to dishes in the experimental group. At 0.5, 1, 3, 5, 7, 24, and 48 hours the medium and cells were analyzed for ^{14}C-activity as described below.

In the subsequent 3 experiments, cell proteins were labelled by incubation for 2 days in 10% FCS containing L(-4,5-$^{3}H(N)$)-leucine (1 uCi/ml). The labelling medium was removed, the cells were washed as before, and PPLFS (2 ml) was added for the 1 hour "wash out" period. This was removed, the cells were washed, and PPLFS was added. LDL (145 ug protein, range: 138-157 ug) was added to the experimental dishes. Medium and cells were analyzed for ^{3}H-activity at 24, 48, and 72 hours.

Triplicate cultures in each group in each experiment were treated identically except that labelled-leucine was omitted; cells were counted and their total protein content was estimated at 0, 24, 48, and 72 hours. Thus, three dishes were used in each of the 2 groups for counting cells and protein estimation, and 2 dishes were used in each group for analysis of radioactivity.

For the analysis of radioactivity, medium was removed completely, added to 0.5 ml of TCA (50% w/v) with mixing,

and allowed to stand at 4°C overnight. After centrifugation radioactivity was counted in 0.2 ml of the supernatant. The precipitate was washed twice with TCA (10% w/v) and dissolved in NaOH (1 N, 0.5 ml). In all but the first experiment, radioactivity was counted in these solubilized precipitates (0.2 ml of the NaOH solution was used).

The cells were washed 4 times with cold PBS (1 ml), were allowed to stand at 4°C overnight in TCA (1 ml, 10% w/v), and were scraped from the dishes with 2 washings into 12 ml centrifuge tubes. The disrupted cells were sedimented by centrifugation, were washed twice with TCA (10% w/v), and were dissolved in 0.5 ml NaOH (1 N) of which aliquots were taken for estimation of protein (2 x 0.1 ml) and for counting of radioactivity (0.2 ml). In the first experiment only, the TCA-soluble fraction of the cells was counted for ^{14}C-activity and was found to contain less than 1% of the total counts in cells and medium. This agreed with the results of Poole and Wibo (214) and was disregarded in the subsequent experiments.

d. Results

Table 20 shows a general increase in cell numbers in cultures to which LDL was added although the increases in Experiments #3 and #4 are small and probably insignificant. LDL increased the protein content per cell in all experiments except that the initial increase in Experiment #2 during the first 24 hours was followed by a relative decrease. The explanation for this is not obvious and although the LDL

Table 20 Protein Degradation in LDL-Treated Cultures. Cell Numbers, and Protein Content per Cell and per Culture.

Expt. #	Time after Addition of LDL (hrs)	Cell Number (% of Control)	Protein Content (% of Control)	
			per Cell	per Culture
1	24	98*	102*	102*
	48	112	107	119
	72	140	110	153
2	24	117	114	133
	48	159	99	157
	72	198	81	160
3	24	70	91	64
	48	102	118	122
	72	105	115	121
4	24	98	106	104
	48	105	104	109
	72	102	117	119

* Mean result from triplicate dishes

effect on cell numbers was greater than average in this experiment, it was shown previously that the effects on cell numbers and on protein content were independent (Section III.B.4.c.). The protein content of the LDL-treated cultures increased relative to controls in all 4 experiments.

LDL caused a consistently higher proportion of radioactivity to be retained in cellular protein (Fig.6) and a consistently lower proportion to be released as free leucine into the medium (Table 21). These results demonstrate that LDL reduced the rate of protein degradation in these cells. Radioactivity in the TCA-insoluble fraction of the medium was always less than 5% of the total and was similar in the LDL and control cultures. Since this represents exported protein (and possibly dead cells) there was no indication that LDL increased the protein content per cell by inhibiting the export of proteins from the cells.

e. Discussion

After 72 hours about 25% more radioactivity remained in the protein and the protein content was 39% higher in the LDL-treated cultures than in the controls. Although an enhanced rate of protein synthesis could have accounted for both the additional radioactivity (by diluting the labelled protein) and for the additional protein, it was shown previously that LDL produced no increase in the rate of protein synthesis (Section III.C.3.). The results presented in this section support therefore, the conclusion that LDL reduces the rate of protein degradation.

Fig. 6 - Radiolabelled-protein remaining in cells in the presence (●) and absence (o) of LDL (145 ug protein/ml medium).

The figure shows the mean (± s.e.m.) percentage of the total radioactivity in the cultures in four experiments that was found in TCA-insoluble cellular material. Each experiment contained triplicate cultures in each group at each time.

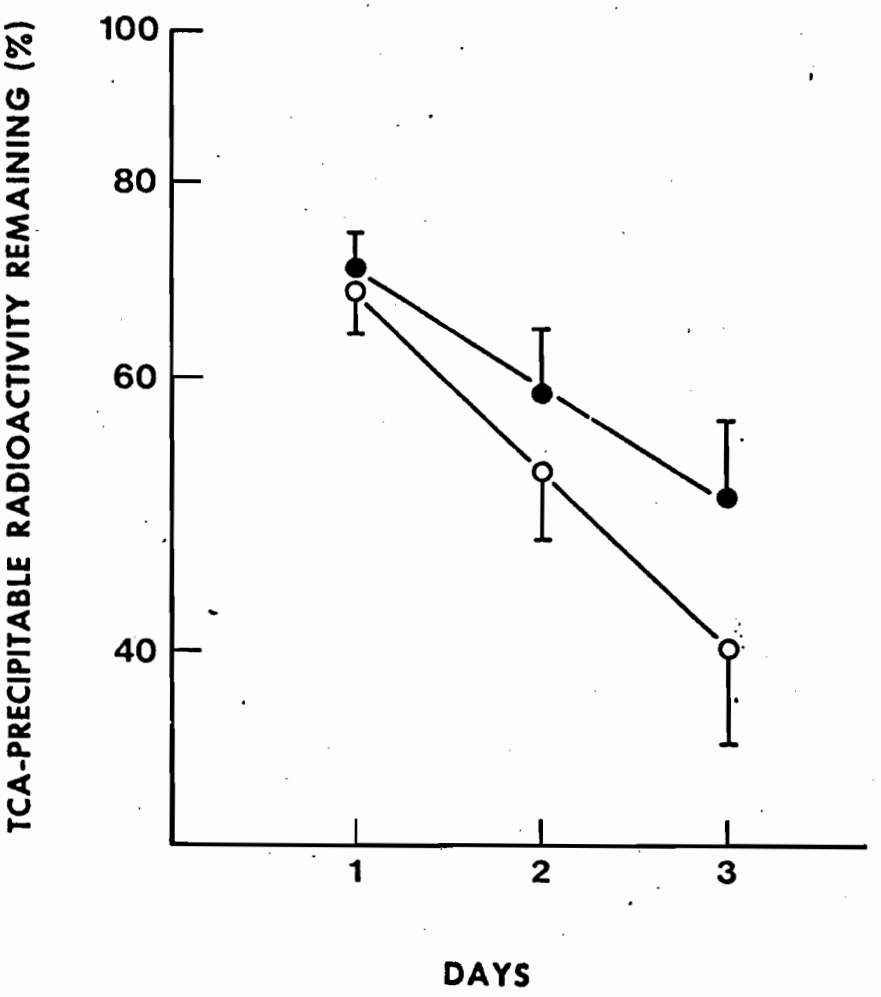

TCA-PRECIPITABLE RADIOACTIVITY REMAINING (%)
100
80
60
40
1
2
3
DAYS

Table 21 Protein Degradation in LDL-Treated Cultures: TCA-Soluble Radioactivity in the Medium

TCA-soluble activity in medium as a percentage of the total activity in cells and medium

Time(hrs):	7 (n=1)*	24 (n=4)	48 (n=4)	72 (n=3)
+LDL	10	28±4	40±6	48±8
-LDL	11	30±4	47±6	56±6

It was unlikely that reincorporation of radio-labelled leucine was occuring in these experiments (Section III. C. 4. a.). Unlabelled leucine was present at levels 10^5 times greater in the first experiment and 2×10^6 times greater in the other three experiments than the initial amounts of labelled leucine used to label cell proteins. (The difference is due to the use of ^{14}C-leucine in the first experiments and to ^{3}H-leucine in the other three experiments.) Furthermore, in the absence of an effect of LDL on protein synthesis (Section III. C. 3.), it is unlikely that the higher amount of labelled protein in LDL-treated cells could be explained by a greater degree of reincorporation of labelled leucine. Rates of protein catabolism in cultured cells have been shown to be sensitive to the composition of the medium. Addition of serum lowers the rates of proteolysis in 3T3 cells (213, 217) and in human fibroblasts (219, 220). Insulin alone has a similar effect in 3T3 cells (213) and in a variety of tissues in vivo and in vitro, (reviewed by Kanter, 221). These results have been interpreted as part of a "pleiotypic response" to changes in the environment which promote cell proliferation (213). Lower rates of protein catabolism have been found in proliferating cultures compared with density-dependent growth-inhibited cultures of 3T3 cells (215, 217) and of human fibroblasts of the MRC-5 strain (222). Bradley has claimed that the two growth states produce no difference in rates of proteolysis in cultures of

human fibroblasts of the WI-38 strain (219). In the figures presented, however, it is evident that a study longer than one of the 25 hours reported may have revealed a difference.

Significant contributions of decreases in rates of protein catabolism to net protein gain have been reported in compensatory renal hypertrophy, regenerating liver, and isoproterenol-stimulated salivary gland (223) as have similar results in skeletal muscle hypertrophy and other tissues (reviewed by Goldberg and St. John, 224). In some of these cases, a lower rate of protein degradation during stimulated growth was shown to be accompanied by minor or no changes in the rates of synthesis. The present work showed a similar pattern of events in cultures of human fibroblasts exposed to LDL: cell growth is stimulated (as shown by increases in protein content) and this was shown to be caused by a decrease in the rate of protein degradation with no increase in the rate of synthesis.

None of these effects of LDL has been reported previously. The literature of protein degradation in mammalian cells is relatively new and is sparse compared with that of protein synthesis (225). It is generally believed that the site of protein degradation is the intracellular lysosome which has been shown to degrade a wide variety of cell constituents (225). Some evidence suggests that LDL too, is degraded in lysosomes. In cultures of human skin fibroblasts, chloroquine, a drug which inhibits intralysosomal degradation, significantly

reduces the degradation of LDL (139) and greatly increases the amount of LDL found to be associated with the cell (125). At present, it is not known whether all proteins are degraded in lysosomes or whether other sites of proteolysis exist within the cell. It is also unknown if the total capacity for protein catabolism is limited and if an acute challenge from a "preferred" substrate would reduce the rates of degradation of other proteins. A "preferred" substrate (according to Goldberg, quoted in reference 225) is large and negatively-charged; LDL is large and negatively-charged but one may only speculate that it reduces the rate of general degradation of cellular proteins by overloading the capacity of the lysosomal system. An alternative explanation, again speculative, is that the deposition of cholesterol into the cell by LDL is followed by a requirement for additional protein to incorporate the lipid into integral cell structures such as membranes, and that this protein is made available most readily by conservation of existing proteins. In a previous section it was shown that even though the protein content per cell is increased in the presence of LDL, it maintains a constant ratio with cellular cholesterol content over a wide range of values (Section III. B. 4. d.).

III. D. The Effect of LDL Added to Ultrafiltered PPLFS on Cell Proliferation, Cholesterol Content, and Protein Content per Cell

1. Introduction

The effects of LDL described above were measured against a background of platelet-poor, lipoprotein-free human serum. It was unlikely that the method of preparing PPLFS would have completely eliminated platelet factors because the centrifugation and washing procedures are thought to damage some platelets and to cause the release of factors involved in blood coagulation (226, 227).

One partially-characterized constituent of platelet lysates stimulates the proliferation of 3T3 cells (118, 119). Uncharacterized constituents of platelets promote the proliferation in culture of monkey skin fibroblasts and aortic smooth muscle cells (115), of human glial cells (120), of bovine aortic endothelial cells (121), and of human aortic smooth muscle cells (122). Platelet factors may provide most of the effect of whole serum on cell proliferation (122). It is not known if platelet factors involved in promoting cell mitosis are the same as those involved in blood coagulation, but it is likely that damage to platelets in any case releases not only coagulation factors but also mitogens. One of these mitogens is heat-stable (228) and has a molecular weight of 13,000 (118). This factor is probably retained in the serum during dialysis of PPLFS because the dialysis membranes that

were used had an exclusion limit of 11,000 daltons. In order to determine what effects the removal of this factor from PPLFS would have on proliferation and on cholesterol and protein contents of cells in the presence and absence of LDL, PPLFS was subjected to repeated ultrafiltration.

2. Materials and Methods

Platelet-poor, lipoprotein-free serum was divided into equal volumes. One volume was added to DME as before and the other was subjected to membrane ultrafiltration and then added to DME. Final protein concentrations in PPLFS and PPLFS-f (ultrafiltered) media were identical.

Ultrafiltration of PPLFS was carried out in an Amicon Model 52 cell at room temperature and under nitrogen (50 psi). The membrane (PM-30) has a retention factor for molecules of 13,000 daltons of about 50% (Amicon Publication #403). The volume of serum was reduced from 60 ml to 30 ml and the initial volume was restored with NaCl (0.15 M). This was carried out 6 times to reduce the concentration of solutes of 13,000 daltons to less than 13% of the original. PPLFS-f was sterilized by passage through a sterile filter (0.22 um) and an aliquot was taken for estimation of protein. The maximum amount of protein lost during ultrafiltration was 3%.

In two experiments the ultrafiltrate (180 ml) was collected, dialyzed against distilled water, and lyophilized. The solid residue was dissolved in 1.5 ml water, and in one experiment only, was boiled gently for 2 minutes (228).

A volume that would contain twice the amount of the factor of 13,000 daltons that was present initially in the PPLFS (assuming losses were negligible) was added to each of 3 plates to which PPLFS-f medium and LDL had also been added. This amount of platelet factor was added at each time of changing PPLFS-f and LDL.

All other methods have been described in the General Methods section (III.A.).

3. Experimental Protocol

Cells (5.8-10.0 x 10^4) were plated in each of 60 petri dishes (3.5 cm diameter) containing 2 ml of 10% FCS. The medium was changed to serum-free medium after 1 day. Cells were counted in 3 dishes after 2 more days and at a later time if the count was lower than the number pipetted into each dish. Dishes were randomly allocated to one of 4 groups, the medium was removed, and 2 ml of PPLFS of PPLFS-f were added to each dish in each of 2 groups. LDL (155 ug protein/ml, range: 128-169 ug protein/ml) was added to half the dishes in each group. In two experiments there was a fifth group in which the concentrated ultrafiltrate was added to PPLFS-f and LDL medium. At 3, 5, and 7 days, dishes in triplicate were taken from each group; cells were counted and their cholesterol and protein contents were estimated. Four different strains of genital fibroblasts were used.

4. Results

Each set of results (cell counts, cholesterol content per cell, and protein content per cell) was subjected to an analysis of variance, the results of which are presented in Table 22. In each analysis, the differences among treatments and among days were found to be significant and as in an earlier group of experiments (Section III.B.) the variability of response among experiments was reflected in the magnitude of the interaction terms. The interpretation of the interaction terms was presented in Section III.B. Clear differences among treatments are seen in Fig.7. Ultrafiltration did not affect the rates of replication, or the cell content of cholesterol or protein in cultures in PPLFS medium alone. Cell contents of cholesterol and protein were increased by the addition of LDL but the increases were similar in PPLFS and in the ultrafiltered serum. Ultrafiltration virtually abolished however, the effect of LDL on the rate of cell replication.

The addition of the concentrated ultrafiltrate to PPLFS-f containing LDL restored some but not all of the LDL effect on replication. Thus after 7 days, the mean population doublings of triplicate cultures in PPLFS-f + LDL and in PPLFS + LDL were 3.7 and 4.3 respectively; addition of the ultrafiltrate to PPLFS-f + LDL produced 3.8 population doublings. Boiling the ultrafiltrate did not eliminate this small increase in cell numbers.

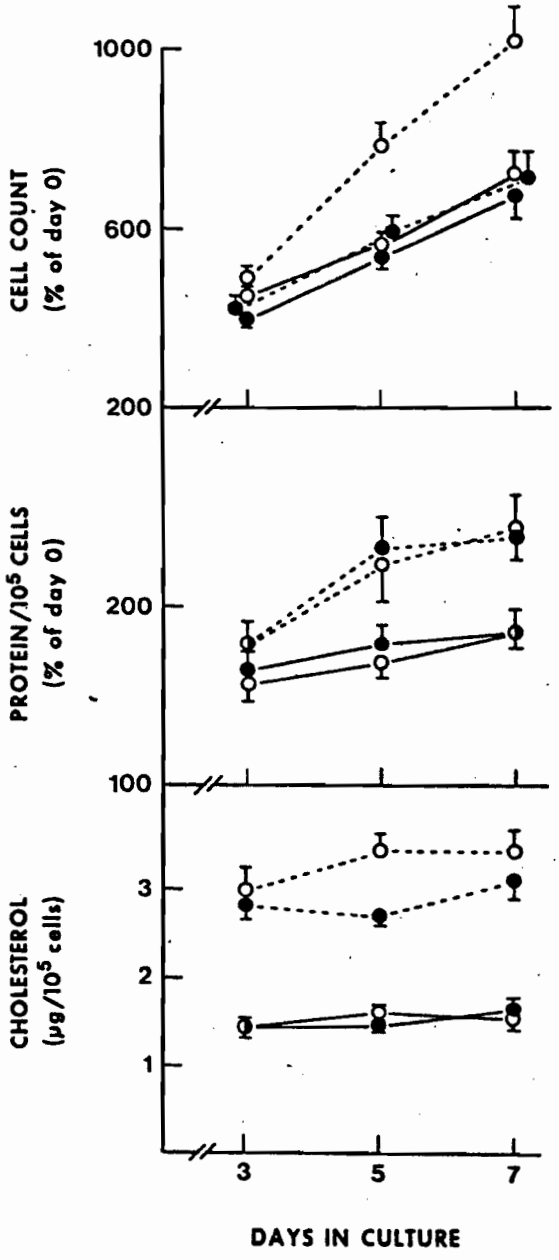
Table 22 Analysis of Variance : Effects of Ultrafiltration of PPLFS

Source of Variation	Degrees of Freedom : Numerator	Sum of Squares	Mean Square	F Ratio	Level of Significance
(a) Cell Counts (% of count on day 0)					
A.Treatments	3	2194593	731531	109.796	<0.001
B.Days	2	5566180	2783090	417.714	<0.001
C.Experiments	7	7275680	1039380	156.001	<0.001
Interactions :					
A x B	6	578126	96354	14.462	<0.001
A x C	21	3346250	159345	23.916	<0.001
B x C	14	1561890	111563	16.745	<0.001
A x B x C	42	1960640	46682	7.007	<0.001
Replications	188	1252580	6663	(4 missing values)	
(b) Cholesterol Content per Cell (ug/10^5cells)					
A.Treatments	3	10255	3418	249.151	<0.001
B.Days	2	228	114	8.342	<0.001
C.Experiments	4	1406	351	25.632	<0.001
Interactions :					
A x B	6	152	25	1.857	N.S.
A x C	12	1363	113	8.283	<0.001
B x C	8	1030	128	9.383	<0.001
A x B x C	24	602	25	1.830	<0.02
Replications	108	1481	13	(12 missing values)	
(c) Total Protein per Cell (% of that on day 0)					
A.Treatments	3	94112	31371	99.446	<0.001
B.Days	2	67270	33635	106.625	<0.001
C.Experiments	5	741991	148398	470.428	<0.001
Interactions :					
A x B	6	15587	2597	8.236	<0.001
A x C	15	83343	5556	17.614	<0.001
B x C	10	25270	2527	8.011	<0.001
A x B x C	30	23158	772	2.447	<0.001
Replications	143	45109	315	(1 missing value)	

Fig. 7 - Effects of LDL on cells in media containing PPLFS (○) or PPLFS-f (●).

——————— Medium alone.

– – – – Medium containing LDL.


1000
600
200
CELL COUNT
(% of day 0)
200
100
PROTEIN/10⁵ CELLS
(% of day 0)
3
2
1
CHOLESTEROL
(μg/10⁵ cells)
3
5
7
DAYS IN CULTURE

5. Discussion

The proliferation of several cell types in culture is stimulated by an agent released by platelets (Section I.E.2.). This agent, a cationic polypeptide of 13,000 daltons, initiates DNA synthesis in 5-10 x 10^3 confluent mouse fibroblasts when present in an amount of 1-2 ng (229). The results of Antoniades and Scher indicate that a medium containing platelet-poor human serum (10% v/v) would have about 0.7 ng of platelet factor per ml (229). Two ml of this medium would thus contain about 1.4 ng, an amount sufficient to promote DNA synthesis. Ultrafiltration would however, reduce this amount to 13% of its original value, which would leave less than 0.2 ng in 2 ml of medium, an amount insufficient apparently to promote DNA synthesis. It was anticipated therefore, that PPLFS-f medium would not support the proliferation of human skin fibroblasts. The results show however, virtually no difference in cell numbers in cultures incubated for 7 days in PPLFS and PPLFS-f. The cholesterol and protein contents per cell were also similar. Different cell types from the same species appear to vary in their dependence on platelet factors for proliferation. Thus, dermal fibroblasts from the pigtail macaque increase in number in platelet-poor serum to about 90% of the number attained by these cells in whole serum: aortic smooth muscle cells during the same period of 14 days, proliferate to only 25% of the number reached by these cells in whole serum

(115). Both the relative and the absolute effects of platelet factors on cell proliferation were greater in the smooth muscle cell cultures than in the fibroblasts. Some months after the completion of the present work, Witte et al reported that human serum prepared in essentially the same way as PPLFS produced no change in cell numbers in cultures of human smooth muscle cells in a period of 11 days (122). In comparison with the present results with skin fibroblasts, it appears that smooth muscle cells from human aortas are more dependent on platelet factors for proliferation. Although ultrafiltration produced no effect on cell numbers in PPLFS alone, it produced a marked decrease in the response of fibroblasts to the addition of LDL. As Fig.7 shows, the mitogenic effect of LDL was virtually abolished although the LDL effects on cholesterol and protein contents per cell were unaltered. The effect of LDL on proliferation was restored partially by the addition to the medium of the concentrated ultrafiltrate of PPLFS. These results support the hypothesis that LDL acts in concert with another, or other, serum factor(s) to promote proliferation. Furthermore, the results corroborate evidence presented earlier that the effect of LDL on proliferation was independent of the effects on cholesterol and protein contents. While the results of the present work are consistent with the conclusion that LDL requires the presence of a platelet-derived factor of 13,000 daltons to exert an effect on cell replication, one

cannot rule out the possibilities that the required co-factor is neither platelet derived nor mitogenic by itself. No attempts were made, however, to resolve this question. The requirement for serum factors of low molecular weight in order for the LDL effect on cell replication to be expressed is a new finding. Since several batches of serum were used in the preparation of PPLFS, it is conceivable that at least some of the variability in magnitude of the LDL effect on proliferation may be attributed to variations in the amount of the required co-factor in different sera.

III. E. The Effect of LDL on the Accumulation of Glycosaminoglycans in Culture Medium

1. Introduction

Several cell types in culture secrete glycosaminoglycans (GAG) into the medium (Section I.E.5.). Synthesis and secretion by cultured cells are stimulated in some cases by factors which are known from other work to stimulate cell division. Examples of these are: serum (153, 156, 159, 160); insulin (154, 155); and epidermal growth factor (158). In addition, a factor derived from platelets stimulates both the synthesis of hyaluronic acid and the incorporation of methyl thymidine into DNA in human synovial cells (165). It is not possible from the available data, however, to make a general statement about the relation of cell proliferation to secretion of GAG: monkey aortic smooth muscle cells (27), chick corneal fibroblasts (152), and rabbit corneal fibroblasts (157) produce GAG at maximum rates during stationary phases of growth; rat fibroblasts are reported, however, to produce more GAG during logarithmic growth (156). Changes in the GAG, as yet undefined, are thought to occur in the arterial intima during the development of atherosclerosis (Section I.D.2.). Since the proliferation of arterial smooth muscle cells is recognized to be an early event in atherosclerosis and because LDL has been shown to stimulate the proliferation of these cells in culture (15), it was of interest to examine the effect of LDL on the accumulation of GAG in the medium of cultures of human skin fibroblasts. It has been shown in the

present work that LDL produces an enhanced rate of proliferation in cultures of human skin fibroblasts (Section III. B.).

Medium was taken from dishes of cells during experiments where cells were periodically counted for other purposes. Advantage was taken of the elimination of the LDL effect on cell proliferation by ultrafiltration of PPLFS to determine if the accumulation of GAG in the medium was affected by proliferation in the presence of LDL.

2. Materials and Methods

Total GAG was estimated in pooled media from triplicate dishes in each group during experiments primarily designed to examine the effect of LDL on protein degradation and the effects of ultrafiltration of PPLFS.

Total GAG was estimated as total uronic acid by Mr. L.O. Wosu. GAG were precipitated with cetyl pyridinium chloride after pronase digestion of the culture medium (230). GAG were recovered as sodium salts and estimated as uronic acid by the modified carbazole method of Bitter and Muir (231).

All other methods are described in the General Methods section (III. A.). The results are expressed as ug of uronic acid found in 2 ml of culture medium. The average cell number over the indicated number of days is shown.

3. Results

As shown in Table 23, LDL increased the total content of GAG in the culture medium from an average value of 5.8 to 15.4 ug per 2 ml. Since no relation was found between the

Table 23 Effect of LDL on GAG Accumulation in the Medium

Expt	Time Interval (Days after addition of LDL)	Total Uronic Acid in Medium (ug)		Average Cell Number during Interval (x 10^{-3})	
		+LDL	-LDL	+LDL	-LDL
1	0-1	12.0	4.0	318	321
	0-2	8.9	5.1	390	369
	0-3	13.2	4.2	492	392
2	0-1	12.8	4.9	342	313
	0-2	19.4	3.6	549	391
	0-3	44.2	3.4	836	466
3	0-1	5.3	0*	102	118
	0-2	19.2	13.4	127	140
	0-3	26.4	15.4	179	172
4	0-1	8.9	5.1	390	369
	0-2	7.3	3.8	607	596
	0-3	7.7	7.2	594	573
	Mean :	15.4	5.8		
	Standard Error:	3.2	1.2		
	Number of Analyses :	12	12		

* None detected

GAG content of the medium and the time the medium was in contact with the cells, each pair of results was treated as being independent of any other pair and all twelve pairs were subjected to the Student t test for paired samples. The difference in accumulated GAG in the medium in control cultures and in those exposed to LDL was significant at the $p < 0.001$ level. The results in Table 23 also indicate that the effect of LDL on GAG production did not require a concomitant expression of the LDL effect on cell proliferation.

The results of experiments which examined the effect of ultrafiltration on accumulation of GAG are shown in Table 24. There was again no relation between proliferation and the accumulation of GAG in the medium, and LDL increased the accumulation of GAG. LDL produced an effect that was slightly greater in ultrafiltered serum than in control serum; the difference was significant at the $p < 0.025$ level (Student t test).

4. Discussion

The results in Tables 23 and 24 show a variable content of GAG in the medium with time. This may be attributed both to the changes in cell numbers which might influence the production of GAG and to varying rates of pinocytosis of medium GAG. As was pointed out in the introduction to this section, production of GAG appears to vary in rate with the growth phase of the culture although the relation between the two varies with cell types. Sulphated GAG are removed from the

Table 24. Effect of Ultrafiltration of PPLFS on Accumulation of GAG in the Medium

Expt	Time Interval (Days after addition of LDL)	Total Uronic Acid in Medium (ug)				Average Cell Number during Interval ($\times 10^{-3}$)			
		PPLFS		PPLFS-f		PPLFS		PPLFS-f	
		+LDL	-LDL	+LDL	-LDL	+LDL	-LDL	+LDL	-LDL
1	0-3	10.1	3.4	11.8	2.8	99	98	96	93
	5-7	7.0	2.0	9.0	2.5	300	248	218	245
2	0-3	4.8	2.0	5.1	3.1	74	77	69	72
	3-5	5.9	1.6	7.0	2.2	128	144	124	129
3	0-3	19.0	7.7	19.3	7.7	114	100	93	78
	3-5	16.2	8.6	16.2	7.6	282	168	205	132
	5-7	16.2	8.1	16.2	8.5	470	192	314	157
	Mean:	11.3	4.8	12.1	4.9				
	Standard Error :	2.2	1.2	2.0	1.1				
	Number of Analyses:	7	7	7	7				

medium by absorptive pinocytosis in cultures of human skin fibroblasts and aortic smooth muscle cells but hyaluronic acid is not (28). Thus the measurement of total GAG at different time intervals does not indicate the total amount of GAG synthesized and secreted by the cells during those intervals but rather the amount remaining after a number of metabolic processes which probably proceed at different rates for each type of GAG. It is evident, however, that LDL produces changes in one or more of these processes since at all times the total GAG accumulated in the medium is greater than in the controls. No relation was found between proliferation of cells and the accumulated GAG in the medium. The results in Table 24 show, however, that the effect of ultrafiltered PPLFS of reducing the effect of LDL on cell proliferation is accompanied by a small but statistically significant increase in the accumulation of GAG. This difference is probably too small, however, to have any physiological meaning. No differences were found between the two sera in the absence of LDL. The mechanism of the LDL effect on accumulation of medium GAG cannot be elucidated from these experiments alone. It is conceivable, however, that since LDL has been shown to form soluble complexes with sulphated GAG at neutral pH (97), sulphated GAG secreted by fibroblasts in culture may be prevented by this association with LDL from being internalized by the cells. Not only would this result in an accumulation of sulphated GAG in the medium, but it might also prevent a feedback-inhibition of new synthesis

of sulphated GAG (which after secretion into the medium would add to the increase). Although feedback regulation of sulphated GAG synthesis has not been reported, Handley and Lowther have demonstrated a decrease in incorporation of acetate into GAG which is dependent on the concentration of hyaluronic acid in the medium (164).

In summary, LDL was found to increase the accumulation of GAG in the medium during periods of up to three days. This is a novel finding and adds to the number of effects of LDL on cultured human skin fibroblasts already described in this thesis.

III. F. The Effect of LDL on Cell Size

1. Introduction

The increases in cholesterol and protein contents of cells exposed to LDL suggested that an additional effect of LDL might be to increase the cell sizes. In the experiments in which the effect of ultrafiltration of PPLFS and in those in which the effect of LDL on protein degradation were examined, the frequency distributions of cell size at various times with and without LDL were analyzed.

2. Materials and Methods

The Cytograf 6300A particle counter equipped with a 2100 distribution analyzer simultaneously counted and sized the cells (Biophysics Systems Inc.).

Cell size is measured as a function of light loss at a photo-detector caused by a cell passing through a beam of monochromatic laser light. The light loss is recorded as an electronic signal in one of a hundred channels by the distribution analyzer and the complete distribution is shown on an oscilloscope display after the cell counting has finished. The results may be stored by photographing this display or by using the printer to transfer the numbers of counts per channel to tape. The information on the tape was analyzed in the following way: the counts in the first 60 channels, which were usually greater than 90% of the total cells counted, were summed; this figure was taken to be 100% and the percentage of this in each channel was computed; this percentage was

multiplied by the channel number to yield the product for each channel; the sum of the products was then taken as a cell size index.

All other methods used were described previously in the General Methods section (III. 4.).

3. Results

The results in Table 25 show that with few exceptions LDL produced a shift in the distribution curve to a larger average cell size. The distribution curves are shown in Fig. 8 . In both the presence and absence of LDL the frequency distributions are bimodal although separation of the two peaks is greater when LDL cultures are analyzed. While the peak at low channel numbers appears in the same channel in all samples, the other peak appears in higher channel numbers in LDL cultures than in controls. There was a high correlation between the relative increase in protein content per cell and the relative increase in cell size produced by LDL: linear regression analysis yielded a correlation coefficient of 0.772 which was significant at the $p < 0.001$ level (Fig.9). No differences in the distributions of cell sizes in PPLFS and PPLFS-f were found. LDL produced the same effect on cell size in both sera.

4. Discussion

The results show a positive effect of LDL on cell size. Although Chen et al. (106) found that hyperlipidemic serum produced larger rabbit aortic smooth muscle cells, the present

Table 25 Effect of LDL on Cell Size

Expt.	Time (Days)	Protein Content per Cell (% of control) PPLFS +LDL	PPLFS-f +LDL	Cell Size Index (% of control) PPLFS +LDL	PPLFS-f +LDL
(a)	Ultrafiltration Experiments				
1	3	138	141	137	131
	5	144	136	113	115
	7	146	134	130	119
2	3	126	114	130	115
	5	131	140	141	158
	7	157	166	149	175
3	5	129	106	109	99
	7	130	114	126	119
4	3	116	98	120	108
	5	91	111	109	95
5	3	87	94	111	117
	5	84	111	95	100
	7	87	105	93	100
(b)	Protein Degradation Experiments				
1	2	107		106	
	3	110		101	
2	1	114		107	
	2	99		96	
	3	81		96	
3	2	118		106	
	3	115		112	
4	1	106		133	
	2	104		105	
	3	117		115	

Fig. 8 - Size distributions of cells in PPLFS with and without the addition of 160 ug LDL protein/ml medium. This figure was drawn from the results of a single experiment but is representative of the relative distributions obtained in other experiments.

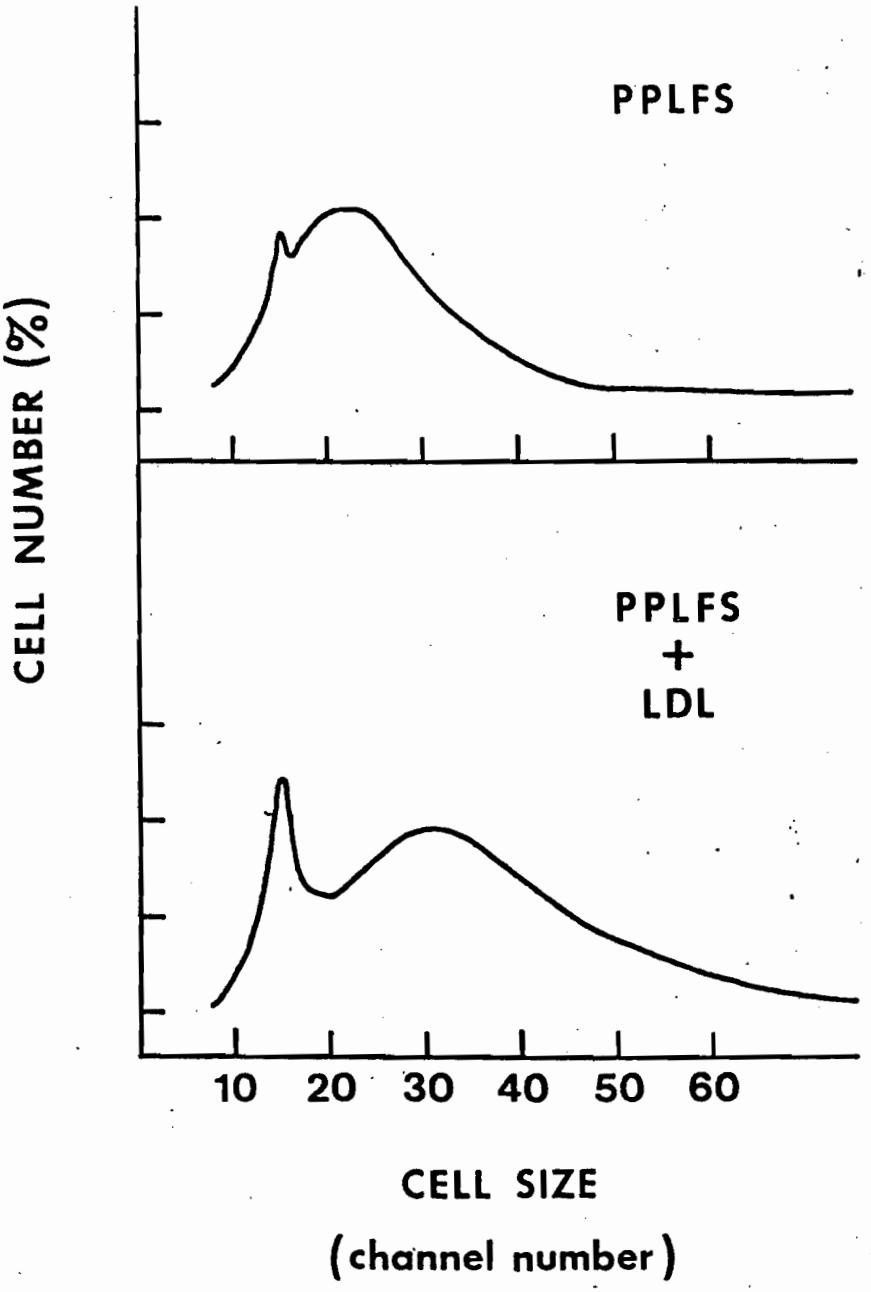
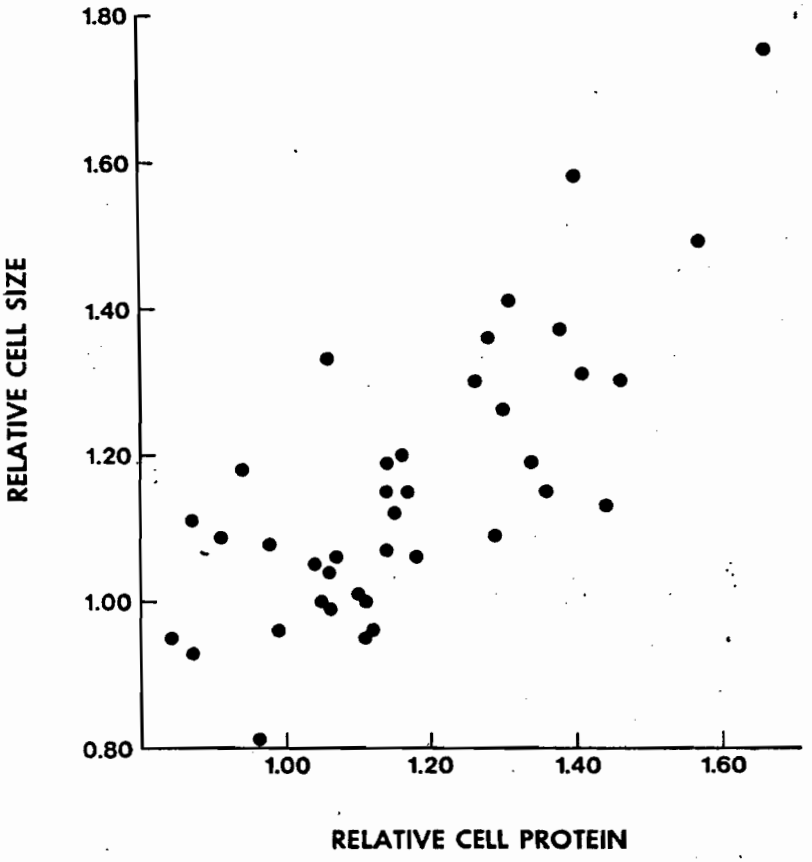

PPLFS
PPLFS
+
LDL
CELL NUMBER (%)
10
20
30
40
50
60
CELL SIZE
(channel number)

Fig. 9 - Relation between increases in cell size and total protein per cell relative to the values obtained for each of these variables on day 0 of the experiments. Each point represents the mean value from triplicate dishes.


1.80
1.60
1.40
1.20
1.00
0.80
RELATIVE CELL SIZE
1.00
1.20
1.40
1.60
RELATIVE CELL PROTEIN

results are the first to show an effect of LDL.

The frequency distributions of cell size were bimodal: the areas at lower channel numbers represent a population of relatively small cells and because of the appearence of the peaks in the same channel number with LDL and with control cultures, it is suggested that these cells are formed from recent divisions; the areas at higher channel numbers appear to be shifted in LDL cultures indicating not only a larger average size than in controls, but also the presence of a population of very large cells which does not exist in the control cultures.

As diploid fibroblasts age <u>in vitro</u>, large cells appear which seem to have limited capacities for replication. These cells bear comparison in morphology to macrophages and may represent a population of terminally-differentiated cells (232). It is possible that the very large cells seen in cultures exposed to LDL also represent a population of terminally-differentiated cells although since no attempt was made to clone them, no proof of this hypothesis is offered.

The correlation found between the increase in protein content and in cell size suggests that the accumulated protein is present in cell structures. Since cholesterol and protein contents were shown previously to be also correlated, the present finding supports the assertion made earlier, that cholesterol and protein increases in cells exposed to LDL are incorporated into structural forms.

III. G. Studies of LDL Effects in Cultures of Porcine Aortic Smooth Muscle Cells

1. Introduction

The cell type of choice for studies of atherosclerosis *in vitro* is undoubtedly the arterial smooth muscle cell. Several investigators have successfully cultured these cells from tissue obtained during corrective surgery for coarctation and atherosclerosis, and during renal transplantation (24, 233). Fetal arterial tissue has also been used (24, 148). Attempts were made during the course of the current work to culture cells from tissue obtained during aorto-coronary bypass surgery*. The average age of the patients was 58 years (range: 47-66 years). Arterial segments from ten patients were obtained and prepared for culture. Although cells migrated from tissue pieces in eight cases, proliferation was always insufficient to form a monolayer of cells which could be sub-cultured and used in experiments to examine the effects of LDL that were investigated previously in cultures of dermal fibroblasts. In comparison with fibroblasts, the arterial cells were substantially larger and had very granular cytoplasms. Rauterberg *et al*. have also experienced difficulties in maintaining cultures of arterial smooth muscle cells from tissue of older individuals (24). Both age and

* I am indebted to Drs. N. Sheiner and J. Rothschild of the Department of Surgery at the Jewish General Hospital for their constant cooperation in providing tissue.

tissue appear to determine the success of culturing rat cells: cells usually migrate from explants of liver and spleen although the time of appearance after incubation increases with the age of the donor; heart and brain cells on the other hand, migrate only from tissue of newborn rats (234). Furthermore, the highly successful culture of monkey skin fibroblasts from tissue taken at autopsy is in contrast with the failure to obtain cells from arterial tissue taken at the same time (150). A report which appeared after the completion of the present work has indicated, however, that human smooth muscle cells from aortas at autopsy proliferate in human serum (23).

Following the unsuccessful attempts with arterial segments from surgical patients, the thoracic aorta was removed from each of eight newborn farm pigs and cultures of smooth muscle cells were established in all cases. The pig is a particularly appropriate species since it develops a type of atherosclerosis which is similar to that which appears in humans (Section I.A.2.). Experiments were carried out with these cells to determine if the effects of LDL found in cultures of human skin fibroblasts were also to be found in arterial smooth muscle cells.

2. Materials and Methods

The method of obtaining explants of arterial tissue for culture of smooth muscle cells was that of Bierman et al. (142). This method was based on the original work of Ross (26).

Newborn pigs, less than 1 week old, were anesthetized with ether, shaved, surgically scrubbed, and draped. The thoracic aorta was removed from each animal and placed in medium (DME, buffered at pH 7.4 by addition of Tris, 3 mM, and containing twice the amount of antibiotics indicated in the General Methods section). Each aorta was cleaned of adherent tissue, opened longitudinally, and the endothelial surface scraped gently with a surgical blade to remove blood and endothelial cells. A well-defined cleavage plane was often present less than half-way across the thickness of the wall and this was used to strip off intimal-medial sections of the artery. These sections were cut into 1 mm squares and were placed, two to a dish, into several petri dishes (35 mm diameter) containing an amount of FCS medium (10% v/v), just sufficient to form a thin layer on the dish surface (about 1 ml). The dishes were placed into a humidified incubator in an atmosphere of air : CO_2 (19 : 1) at a temperature of 37°C. Medium was added daily in increments of 0.5 ml to avoid dislodging the adherent tissue.

Cells appeared from the tissue pieces after 2 days in a few cases but generally after 4-8 days. The surface of the dish was usually covered with cells after 3 weeks, at which time cells were collected from several petri dishes and transferred to T-75 flasks. (Removal of cells and all other techniques of cell culture are described in the General Methods section.) The initial density was $1.0\text{-}1.5 \times 10^4$ cm^{-1}; below

this density of plating, cells often became large and did not divide. The early cultures were heterogeneous: the majority of cells were irregularly-shaped (spindle-like or polygonal), and the minority were smaller and formed circular clusters. The latter type were very similar to human umbilical vein endothelial cells (235) and disappeared from the cultures after 1-2 passages. Electron-microscopy of cells at the second passage showed myofilaments, attachment plaques (dense bodies), and extracellular microfibrils were present (Fig.10). These characteristics, in addition to the pattern of growth which had the appearance of "hills and valleys", have been reported by others to be typical of cultures of smooth muscle cells (26, 126, 127, 142).

Human LDL and PPLFS were prepared as described previously in the General Methods section. Porcine LDL and lipoprotein-free serum were prepared from blood obtained at slaughter*. The blood was collected into polyethylene buckets and was immediately transferred to glass tubes (30 ml) packed in ice. After transfer to the laboratory, the tubes were allowed to stand at room temperature for 2 hours before the serum was separated by centrifugation. LDL was isolated using the method for isolating human LDL (General Methods section) and appeared turbid and white. Porcine lipoprotein-free serum was prepared

* I am indebted to Canada Packers Ltd. for allowing me to use their facilities.

Fig.10 - Thin section of porcine aortic smooth muscle cells cultured in 10% FCS medium. Cells were fixed in 1% glutaraldehyde; post-fixed in 2% osmium tetroxide; dehydrated in ethanol, dimethyl sulphoxide; and embedded in Epon. Thin section was post-stained with lead citrate and examined on a Hitachi HU 12 microscope at 75,000 Kv and at an initial magnification of 1500-20,000.

Dense bodies associated with thin filaments are visible in the top centre portion of the micrograph and the substratum shows a large quantity of extra-cellular materials, that are produced characteristically by smooth muscle and other connective tissue cells.

by ultracentrifugation of porcine serum at a density of 1.250 gm/ml using the same methods used in the preparation of lipoprotein-free serum from human platelet-poor serum (General Methods section).

All other methods have been described in the General Methods section (III.A.).

3. Experimental Protocol

Cells (6-8 x 10^4) were pipetted into plastic petri dishes (3.5 cm diameter). Each dish contained 2 ml of FCS medium and was replaced into the incubator (37°C, humidified atmosphere of air : CO_2 (19 : 1)) for 1-3 days until the cell number was similar to that pipetted (day 0). On day 0, medium was removed, the cells were washed twice with serum-free DME, and 2 ml of DME containing the concentration of various sera indicated in the Results section was added. LDL (162 ug LDL protein per ml, range: 146-172 ug/ml) from human or porcine sera was added to half the dishes. Medium and LDL in each experiment were changed at 3 and at 6 days. Three dishes were taken from each group at 2, 4, and at 6 days and cells were released for counting, estimation of cholesterol content, and estimation of protein content. Six of the eight strains of cells were used in the following experiments.

4. Results

Changes (as a percentage of cell count on day 0) in numbers of porcine aortic smooth muscle cells after 6 days were found to be related linearly to the concentration of fetal

calf serum in the medium (Fig.11). The proliferation of these cells in PPLFS or in porcine lipoprotein-free serum (PLFS) was substantially lower (Fig.12).

Little effect of concentration of serum on cell numbers was found with 1% and 5% PLFS. Higher concentrations (10% and 20%) resulted in a decrease in numbers and cells were visible floating in the medium. The deleterious effect of PLFS at high concentrations was irreversible: despite changing the medium at 4 days to 10% FCS, there was complete detachment of all cells from the dish surface.

Observations with the light microscope revealed morphological changes in cells incubated in PPLFS and PLFS relative to those in FCS. In the first two, the cells were rounded and intercellular junctions were less defined.

The PPLFS used was tested for toxicity in a culture of human skin fibroblasts and since cell numbers increased at a usual rate and no cell detachment was observed, it was concluded that the medium contained no toxic factors.

The effects of human LDL on cell proliferation, cholesterol, and protein content per cell were examined in 5 experiments using 10% PPLFS medium. The results at 6 days are presented in Fig. 12 and are representative of those at 2 and 4 days. Slightly lower numbers and protein contents were present in the LDL-treated cultures while the cholesterol contents were somewhat higher. None of these differences was significant.

Fig.11 - Changes after 6 days in numbers of porcine aortic smooth muscle cells in medium containing fetal calf serum at various concentrations.

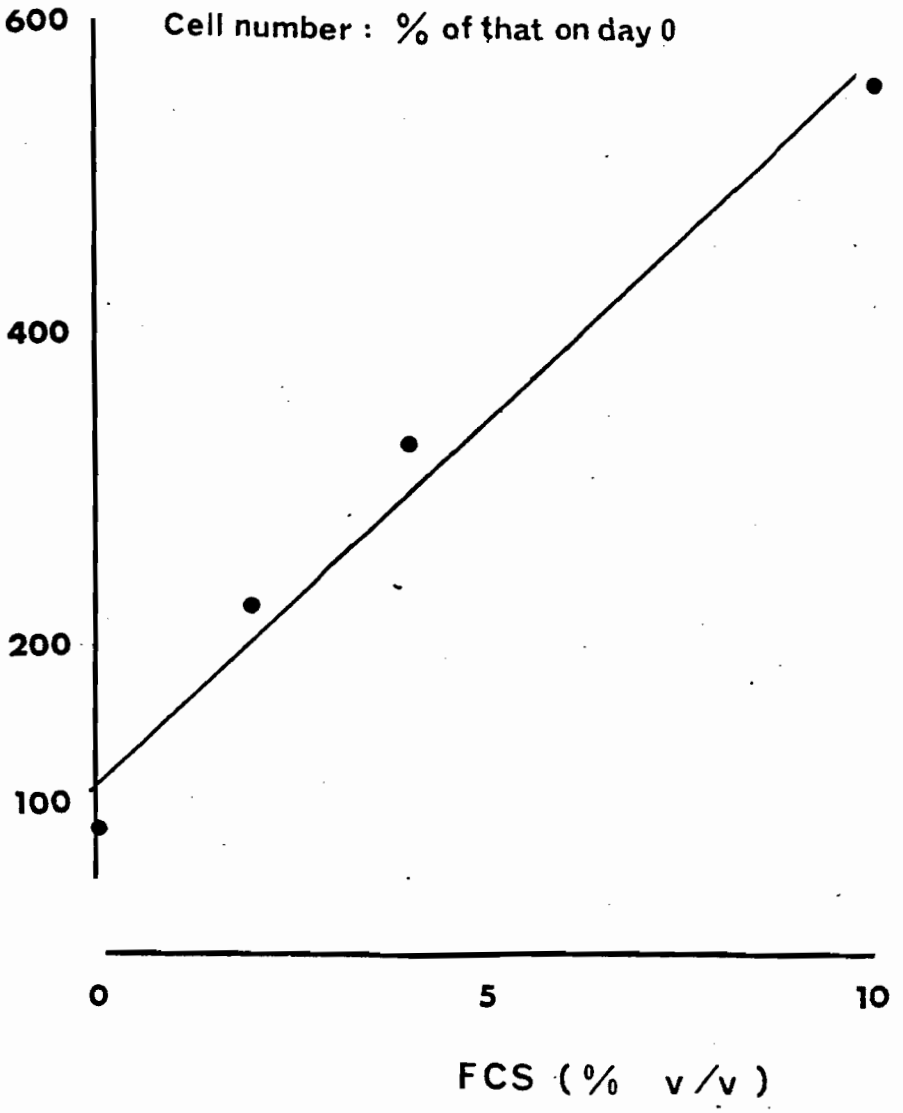
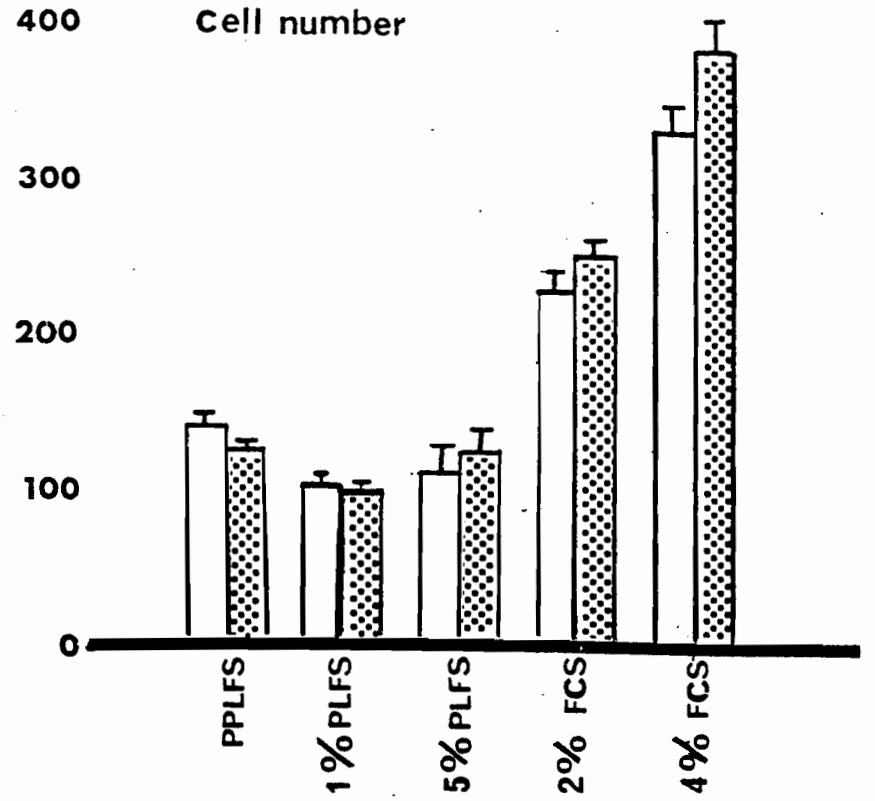
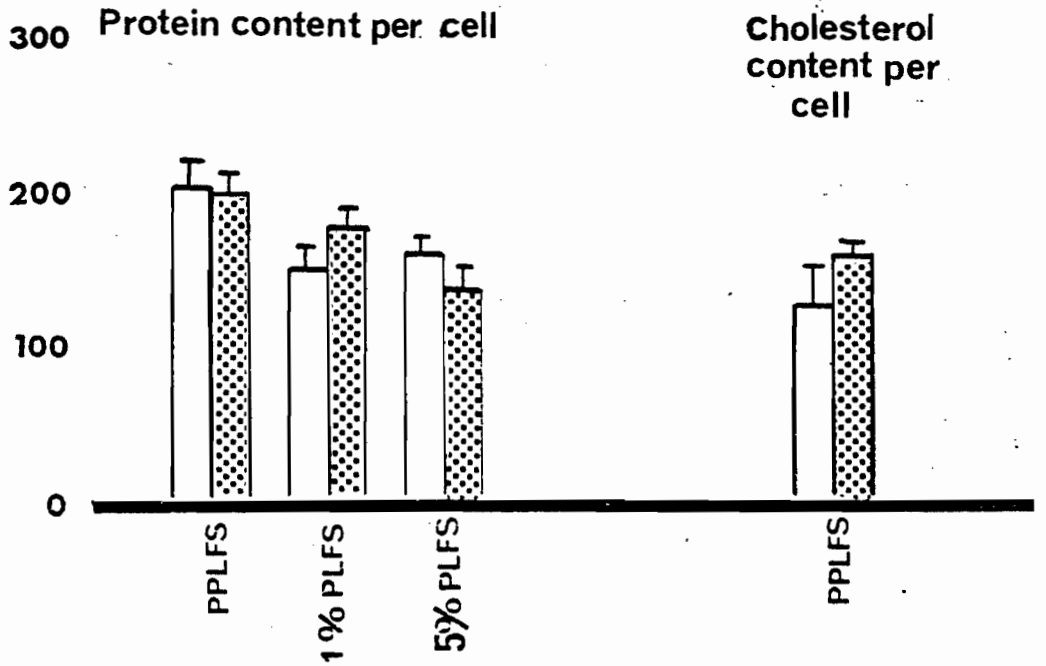

600
Cell number : % of that on day 0
400
200
100
0
5
10
FCS (% v/v)

Fig.12 - Effects of LDL on porcine aortic smooth muscle cells. LDL (▒) added to PPLFS and FCS was from humans and that added to PLFS was from swine. The ordinates show values as a percentage of those on day 0.


400
Cell number
300
200
100
0
PPLFS
1% PLFS
5% PLFS
2% FCS
4% FCS


300
Protein content per cell
Cholesterol content per cell
200
100
0
PPLFS
1% PLFS
5% PLFS
PPLFS

No significant differences were found in cell numbers and protein content per cell in cultures treated with LDL during experiments using PLFS medium (1% and 5%) and FCS medium (2% and 4%). Results of these experiments at 6 days are shown in Fig.12 and are representative of those at 2 and 4 days.

5. Discussion

Porcine aortic smooth muscle cells proliferated rapidly in medium containing fetal calf serum. A much poorer rate of proliferation was observed, however, when the serum was changed to human PPLFS and this rate was not improved by the addition of LDL. As was reported in the General Methods section, after 6 days, the number of human skin fibroblasts cultured in whole human serum (10%) was reduced by 31% by incubating the cells in medium containing PPLFS (10% v/v). The present results show that the number of porcine aortic smooth muscle cells after 6 days was about 75% lower in PPLFS than in medium containing a similar concentration of fetal calf serum. While this difference may be attributed in part to differences between human and fetal calf serum it is likely that the results also indicate a greater dependence of aortic smooth muscle cells (compared with dermal fibroblasts) on platelet factors for proliferation. Rutherford and Ross, for example, have found a substantial difference between dermal fibroblasts and aortic smooth muscle cells in the effect on cell numbers of platelet-poor serum (compared with whole serum): dermal fibroblasts in platelet-poor serum numbered 90% of those in whole serum after 14 days whereas the smooth muscle cells had

reached only 25% of the number in whole serum (115). After the completion of the present work, Witte et al. reported that human aortic smooth muscle cells remain in a quiescent state for up to 11 days in medium containing PPLFS (10% v/v), which again suggests a critical role for platelet factors in the proliferation of aortic smooth muscle cells (122). In the light of these results, the incubation of porcine aortic smooth muscle cells in medium containing lipoprotein-free swine serum from which platelet factors had not been removed, was expected to result in a degree of proliferation significantly greater than that found with PPLFS, and it was further anticipated from the previous work with human skin fibroblasts that this rate of proliferation would be augmented by LDL. Neither of these expectations was realized and at concentrations of PLFS greater than 5%, it appeared that the serum was toxic to the cells. The explanation for this result is not obvious. Brown et al. have reported an effect of swine LDL on cell proliferation when added to PLFS (111). They used the miniature pig but it is thought unlikely that differences in species of swine could account for the different results. More likely is the possibility that since the blood was obtained from animals at slaughter, when immediately before exsanguination they were in an extreme state of panic, the serum could have contained some factor(s) which inhibit(s) the proliferation of cells. Daoud et al. have in fact found a fraction of swine serum which inhibits the incorporation of

thymidine into DNA in explants of porcine aorta but they have not characterized the active agent (236).

LDL augmented neither the proliferation of cells nor the cholesterol nor protein contents per cell during incubation in PPLFS or in PLFS. In one experiment, swine LDL appeared to enhance the proliferation of smooth muscle cells already proliferating quite rapidly in fetal calf serum. It is possible, therefore, that the effect of LDL on the proliferation of aortic smooth muscle cells is to amplify the proliferation already initiated in a culture by other factors. Others have reported that LDL alone or with PPLFS is unable to promote the proliferation of human aortic smooth muscle cells (122) while the addition of LDL to monkey lipoprotein-free serum does augment the proliferation of monkey aortic smooth muscle cells (15). Fischer-Dzoga and Wissler found an effect of LDL, when added to whole serum, on increasing cell proliferation in cultures of monkey aortic smooth muscle cells but claimed that LDL had to be prepared from hyperlipidemic serum to exert this effect (109, 110).

In summary, porcine aortic SMC proliferated rapidly only in fetal calf serum . The absence of any effects of LDL (human or porcine) on proliferation , cholesterol accumulation, and protein accumulation , and the morphological changes of cells in the cultures was related perhaps to their poor adaptation to medium containing PPLFS or lipoprotein-free porcine serum .

III. H. Composition of Serum VLDL and HDL in Diabetes Mellitus

1. Introduction

A predisposition to the development of atherosclerosis is widely accepted as a part of the diabetic condition. While it is suspected that a factor in this predisposition is abnormal lipid metabolism, elevated levels of serum lipids although frequent, are not always measurably more so than in the general population (Section I.F). A characteristic feature of lipid metabolism in diabetes has yet to be found (172). Few studies have examined, however, the composition of lipoproteins in diabetic patients. The results of such a study are presented. Serum VLDL and HDL were isolated from the blood of diabetics and healthy controls by ultracentrifugation and their cholesterol and apolipoprotein contents were analyzed.

2. Materials and Methods

Thirty-seven subjects (ages: 37-88 years) were randomly selected from a group of non-obese, maturity-onset diabetic patients being treated by diet and either insulin or oral hypoglycemic agents. The control group comprised 17 healthy volunteers (ages: 30-92 years). Blood was collected after a fast (12-24 hours) and was mixed immediately with a 1.5% solution of thimerosal (pH 8.0, 0.4% v/v of blood). The blood was allowed to coagulate at room temperature for 2 hours. Serum was collected by centrifugation. Cholesterol and triglycerides were estimated by automated enzymatic methods and lipoprotein electrophoresis on thin agarose gel was

performed on an aliquot of serum*. EDTA (Na_2) was added to the remaining volume of serum (3 mM). Any chylomicrons present were removed and VLDL (density 1.006 gm/ml), IDL (1.006-1.019), LDL (1.019-1.063), and HDL (1.063-1.210) were separated by sequential ultracentrifugation using KBr for density adjustments (193). Each fraction was purified by recentrifugation at the appropriate density, dialyzed against NaCl, 0.15 M; EDTA, 0.3 mM; pH 5.6; 200 volumes x 5; and made up to a known volume. Cholesterol and protein were estimated on an aliquot, and the remainder was lyophilized. Lipids were extracted from the lipoproteins with ethanol-diethyl ether (3 : 1) following the procedure of Brown et al. (237). The protein residue was stored in an evacuated dessicator at -20°C until gel electrophoresis was performed. Samples of VLDL and HDL were dissolved in Tris buffer (0.2 M), SDS (0.1%), pH 8.2, and subjected to electrophoresis in duplicate in 10% acrylamide gels in the presence of 8 M urea (238). Urea (10 M) was deionized on columns of Rexyn I-300 immediately before use and checked for a conductivity of less than 5 umhos. Electrophoresis was carried out at 2.5 mA/gel for 4 hours. The gels were removed and placed into a solution of amido black (0.2%) in acetic acid (7%) for staining. The following day, gels were removed and destained in acetic acid (10% v/v) which

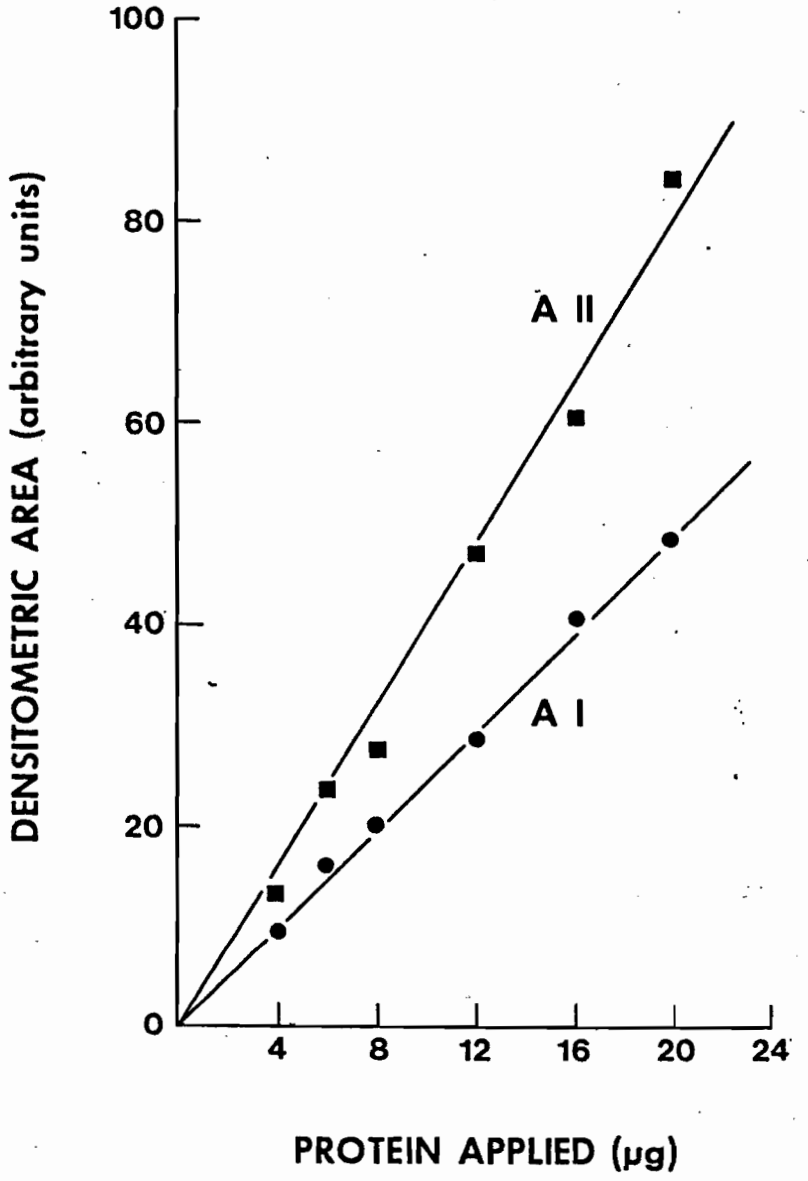
* I am indebted to Dr. R. Schucher and Mr. J. Gabor of the Biochemistry Department of the Jewish General Hospital for performing these analyses.

was changed every 4 hours.

Gels were scanned at 623 nm in a Beckman Acta V recording spectrophotometer and the scans were recorded. Areas under the peaks were measured by planimetry and expressed as a percentage of the total for the gel. No correction was made for differences in chromogenicity among the apoproteins (239). The relation between the amounts of VLDL peptides and the densitometer response was linear. Apo AI and apo AII in HDL* were found to have different chromogenicities but each produced a linear response (Fig.13). One hundred ug of VLDL protein were applied to each gel. Seven ug of HDL protein were applied to obtain relative amounts of apo AI and apo AII and 50 ug of HDL protein were applied to obtain amounts of minor components in relation to apo AII. The combined information yielded the relative amounts of all peptides. Samples of apo-LDL were dissolved by boiling for 3 minutes with SDS, 1%; EDTA, 2 mM; and dithiothreitol (DTT), 5 mM; and allowing to stand at 37°C for 18 hours. Electrophoresis was performed in polyacrylamide gels (3% stacking gel, 7½% running gel) prepared according to the procedure of Laemmli (209). A current of 0.2 mA/gel for 18 hours was used. Gels were removed and stained with Coomassie Brilliant Blue G250 (0.05%) in acetic acid : isopropanol : water (2 : 5 : 13) overnight followed by

* I am indebted to Dr. Y. Marcel for samples of purified apolipoproteins.

Fig.13 - Densitometric response (area of peak) of apoAI and apoAII. Increasing amounts of protein were applied to gels in duplicate.


100
80
60
40
20
0
4
8
12
16
20
24
A II
A I
DENSITOMETRIC AREA (arbitrary units)
PROTEIN APPLIED (µg)

diffusion destaining in acetic acid (10%). Protein and cholesterol in lipoproteins were estimated by standard methods (195, 240). Apo B in VLDL was measured as the difference between total and isopropanol-soluble protein* (241).

3. Results

a. Glucose, Lipid, and Lipoprotein Levels in Serum

Serum concentrations of glucose, cholesterol, and triglycerides are shown in Table 26. The mean value of each was higher in the diabetic group. Fifteen of the diabetics had normal lipoprotein levels, 3 had increased VLDL, 4 had increased LDL, and 3 had increases in both.

b. Distribution of Cholesterol among the Lipoproteins

The relative and absolute amounts of cholesterol distributed in each of the lipoproteins were the same in normolipidemic diabetics as in controls (Table 27). Both the relative and absolute amounts of cholesterol were increased in VLDL in those diabetics with elevated VLDL, and were increased in LDL in those diabetics with elevated LDL. Diabetics with elevated VLDL and LDL levels also had relatively lower amounts of cholesterol in the HDL fraction; only in the elevated LDL group was the absolute amount lower.

Cholesterol to protein ratios were normal in normolipidemic diabetics but were slightly higher in HDL in the diabetic group as a whole. This difference was significant (Table 28).

* Mr. F.Shareck, Ms. E. Tel, and Mr. R. White assisted at various times in the separation of lipoproteins and in the analyses of their compositions.

Table 26 Serum Glucose and Lipid Levels in Diabetic and Non-Diabetic Subjects

	Serum Concentration (mg/dl)		
	Glucose	Cholesterol	Triglycerides
Control (17)	91±2*	202±12	80±6
Diabetic (25)	152±9	244±10	169±16
Normolipidemic (15)	154±14	208± 9	102± 7
Elevated VLDL (6)**	141±10	271±17	293±23
Elevated LDL (7)**	167±25	309± 8	238±39

* Standard Error of the Mean

** 3 subjects had an increased VLDL, 4 had an increased LDL, and 3 had increases in both.

Table 27 Distribution of Serum Cholesterol among Lipoproteins

	Relative Distribution (% of total)				Absolute Distribution (mg/dl serum)		
	VLDL	IDL	LDL	HDL	VLDL	LDL	HDL
Control (17)	6±1	2.0±.3	65±2	27±2	13±2	135±9	57±5
Diabetic (25)	12±3	5.0±1	63±2	21±2**	25±4	155±10	53±5
Normolipidemic (15)	7±1	3.8±.7	63±2	26±1	14±2	131±7	58±4
Elevated VLDL (6)*	20±4**	4.0±1	56±3	20±8	48±12**	176±26	59±20
Elevated LDL (7)*	13±3	3.0±1	72±4**	14±2	41±10	221±11***	40±6**

* See Table 26

** Differs from control value, $P < 0.02$

*** Differs from control value, $P < 0.001$

Table 28 Cholesterol:Protein Ratios in Serum Lipoproteins

(w/w; mean±s.e.m.)

	VLDL	IDL	LDL	HDL
Control (17)	1.00±.02	1.21±.12	.84±.06	.29±.01
Diabetic (25)	1.09±.07	1.02±.08	.92±.06	.34±.02*
Normolipidemic (15)	1.00±.06	1.12±.12	.85±.1	.31±.09

* Differs from control value, $P < 0.05$

c. Apolipoprotein Composition of VLDL

The electrophoretic pattern of the soluble fraction of apo-VLDL shows 5 major bands, a minor but still prominent band near the origin, and several minor and less prominent bands (Fig. 14). This agrees with the results of others (239, 242). The electrophoretic patterns also indicate that relative amounts of apolipoproteins are quite variable among subjects (Fig.15). This variability was previously reported by Eisenberg et al. (243) and by Shore and Shore (242).

No major difference was found in the apoprotein compositions of VLDL of diabetics and of normals. Small but statistically significant differences were revealed, however: the minor components accounted for about 6% of total apoproteins in the normal but for less than 1% in the diabetic; and apo CII was increased slightly and the sum of apo CII, apo $CIII_1$, and apo $CIII_2$ was higher in the diabetics (Table 29).

Apo B accounted for 62 ± 8% of the apo VLDL in normals and for 65 ± 5% in diabetics.

d. Apolipoprotein Composition of HDL

The electrophoresis pattern of HDL showed 2 major bands (AI and AII) and minor bands (Fig.14). The diabetic HDL was slightly poorer in apo AI but richer in minor components (apo AIII and the apo C apoproteins). No differences existed among the diabetics sub-grouped according to serum lipoprotein levels (Table 30).

The distribution of cholesterol among the lipoproteins

Fig.14 - Gel patterns obtained with apoVLDL, apoIDL, apoHDL (50 ug), and apoHDL (7 ug). The 5 major bands in VLDL are CI, E, CII, $CIII_1$, and $CIII_2$; similar bands are seen in IDL. The two major bands in HDL are AI and AII.

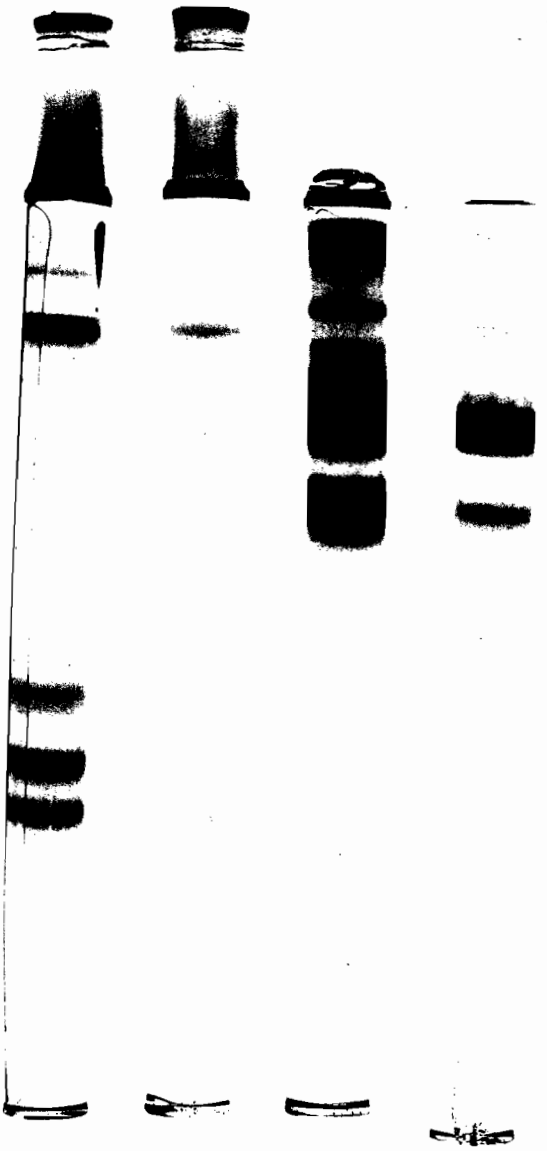


Fig.15 - Gel patterns from samples of VLDL from the serum of 2 different normolipidemic subjects. The variability in VLDL apoprotein composition among individuals is seen clearly.

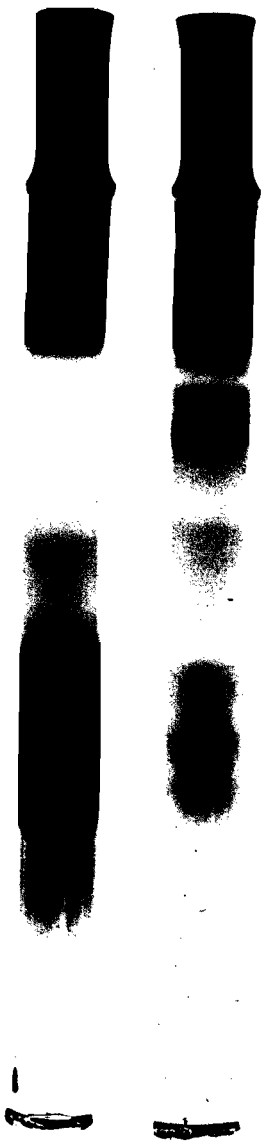


Table 29 Apoprotein Composition of Soluble VLDL Protein

	% of total soluble VLDL protein (±s.e.m)							
	(1)*	CI	E	CII	$CIII_1$	$CIII_2$	Minor** Components	CII/ $CIII_1$
Control (12)	5.2	10.6	32.4	11.4	18.2	15.8	6.1	0.63
	(±.8)	(±1.7)	(±3.1)	(±1.1)	(±1.9)	(±1.5)	(±1.8)	
Diabetic (27)	3.7	11.0	28.1	14.3***	24.4	17.9	< 1.0***	
	(±.7)	(±1.4)	(±2.1)	(±0.8)	(±1.9)	(±0.9)		
Normolipidemic (14)	3.8	13.1	28.6	13.4	21.9	17.5		0.61
	(±.9)	(±1.8)	(±2.9)	(±1.1)	(±5.8)	(±1.5)		
Elevated VLDL (6)	3.6	8.7	24.7	15.2	29.2	18.6		0.52
	(±1.0)	(±2.2)	(±3.8)	(±1.9)	(±4.5)	(±0.9)		
Elevated LDL (7)	3.6	6.5	31.5	16.5	23.4	18.4		0.70
	(±.7)	(±1.2)	(±3.8)	(±1.2)	(±2.1)	(±1.2)		

* Band 1 on the electrophoretic gel, near the origin; identity undetermined.
** Includes AI, AII and one unidentified band.
*** Differs from control value, $P < 0.025$. The sum of CII, $CIII_1$, and $CIII_2$ also differs from control value, $P < 0.025$.

Table 30 Apoprotein Composition of HDL

	% of total HDL apoprotein (±s.e.m.)								
	X	AI aggregate	CI	AI	AII	AIII	CII	$CIII_1$	$CIII_2$
Control (12)	3.2 ±.3	1.8 ±.2	0.7 ±.2	83.6 ±0.9	9.3 ±.7	0.2 ±.1	0.3 ±.1	0.4 ±.1	0.3 ±.1
Diabetic (27)	3.8 ±.4	2.0 ±.2	1.2 ±.1	79.7 ±1.0	10.1 ±.4	0.9 ±.1	1.0 ±.1	0.7 ±.1	0.6 ±.1
P			<0.025	<0.01		<0.005	<0.005	<0.005	<0.005

and the apoprotein electrophoresis were both studied in 12 control and 11 diabetic sera. A correlation was found between the absolute amount of cholesterol and of apo E in VLDL (Fig. 16). By linear regression analysis, the correlation coefficients were 0.593 in the control sera ($p < 0.05$), 0.711 in the diabetics ($p < 0.02$), and 0.697 in the combined group ($p < 0.01$).

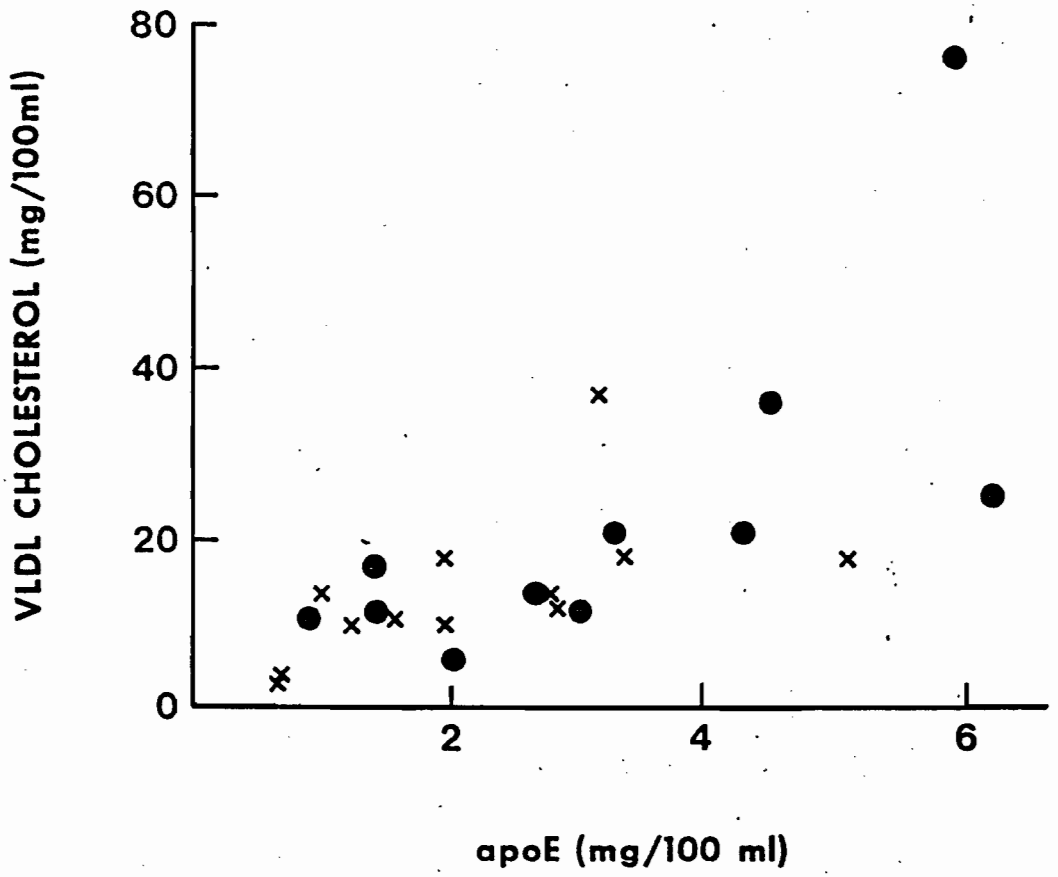
Analysis of IDL was carried out a few times but the volume of serum usually obtained was insufficient for accurate quantitation. The apoprotein pattern resembled that of VLDL (Fig 14).

Apo-LDL was completely soluble in SDS after reduction with DTT. A large number of bands was resolved by electrophoresis; no apparent differences in diabetic LDL were found.

4. Discussion

In agreement with other studies (174 , 177 , 183), major differences were not found in the composition of serum lipoproteins of diabetics. Abnormalities which were found could usually be attributed to hyperlipoproteinemia and were absent from the diabetics with normal serum lipid levels. A greater proportion of serum cholesterol, for example, was distributed in VLDL when the level of VLDL was high and likewise, in LDL when the level of LDL was high. Ballantyne et al. have reported a normal distribution of cholesterol among serum lipoproteins in diabetics (177); Schonfeld et al. (183)

Fig.16 - The relation between cholesterol and apoE in the VLDL of normal (X) and diabetic (●) subjects.


VLDL CHOLESTEROL (mg/100ml)
80
60
40
20
0
2
4
6
apoE (mg/100 ml)

and Lopes-Virella et al. (184) measured a lower amount of HDL-cholesterol in normolipidemic diabetics; and Calvert et al. found a lower HDL-cholesterol only in those diabetics treated with sulphonylurea (insulin-treated patients had normal levels) (185). HDL-cholesterol was also lower than normal in a group of diabetic women but on average the group was moderately hyperlipoproteinemic (174); the present study also showed that when the diabetics had an elevated LDL, HDL-cholesterol was significantly reduced. It is of great interest to determine whether a reduced level of HDL-cholesterol is frequent in diabetes because of the association of each of these with coronary heart disease (34, 35).

In agreement with Schonfeld et al. (183), cholesterol : protein ratios were normal in the LDL and VLDL of normolipidemic diabetics. The ratio was also normal in HDL in these patients, but was significantly higher in the diabetic group as a whole. Since the absolute amount of cholesterol in HDL was normal, these results suggest a relative deficiency in HDL apoproteins.

The analysis of VLDL apoproteins showed similar patterns in normals and diabetics. The major apoproteins reported by others were present although relative amounts differed from those reported by Kane et al. even after corrections were made for differences in chromogenicity (239). Larger proportions of apo E, apo $CIII_1$, and apo B were found in the present work. Estimates of apo B in VLDL have varied quite widely: values of 27% (183) and 40% (244) were obtained with

immunoassay methods and 37% (239), 55% (245), and 58% (246) were found with other methods. The value of 62% for normal VLDL obtained in the present study agrees best with that from a gravimetric method (246). Aside from technical differences, it is possible that variation among individuals accounts for at least part of the discrepancy in the relative proportions of VLDL apoproteins; Shore and Shore, as indicated earlier, found this source of variation to be considerable (242).

A relation between the triglyceride content and the apo C composition of VLDL has been observed by Carlson and Ballantyne: increasing triglycerides accompany a decreasing ratio of apo CII to apo $CIII_1$ (247). Triglycerides were not measured in lipoproteins in the present work but it was found that as mean serum triglyceride levels increased from the normal group to the normolipidemic diabetic group to the diabetic group with elevated VLDL, the mean ratio of apo CII to apo $CIII_1$ decreased from 0.63 to 0.61 to 0.52.

The apoprotein composition of HDL was found to be similar to that reported by Fredrickson (248). Small but statistically significant differences were found in the relative amounts of apo AI and some of the minor components in diabetics and normals. Apo AI is the major polypeptide in HDL and is associated with lower than normal amounts of HDL-cholesterol in survivors of myocardial infarction (249). The present results show that as a whole, the diabetic group has a higher ratio of HDL-cholesterol to apo AI than the control group

which suggests a greater complement of cholesterol per apo AI molecule. The mean absolute amount of HDL-cholesterol in the diabetics was, however, normal whereas that in the coronary heart disease patients studied by Albers et al. was significantly lower than normal (249).

An incidental observation of interest is the relation between the concentrations of apo E and VLDL-cholesterol. Shore and Shore suggested that an arginine-rich apoprotein (which is identical to apo E, (180)) preferentially binds cholesterol-rich particles (250). Apo E is increased in hyperlipoproteinemias of type III (180, 250) and of type V (180), and in hypothyroid patients and cholesterol-fed rabbits (250). An enhanced binding activity (compared with LDL) has been found for an apo E-containing lipoprotein in cultures of human skin fibroblasts (138). The lipoprotein, which is isolated from the serum of cholesterol-fed dogs, also enhances the rate of esterification of cholesterol in these cells, possibly because it delivers to the cells more cholesterol per mole of apoprotein compared with LDL.

The demonstration of higher concentrations of apo E accompanying increases in VLDL-cholesterol may suggest a role for VLDL in the cholesterol metabolism of cells which could have significance in atherogenesis. There is, however, no indication from the present study that a difference in this regard would exist in the diabetic.

IV. GENERAL DISCUSSION

IV. A. Introduction

Progress to a complete understanding of the development of atherosclerosis would be accelerated if it were possible to sample continuously the area in which a lesion was developing in such a way that no disturbance to that development was caused. Examination of the morphology, ultrastructure, and chemical composition of the cells and of the chemical composition of the intercellular matrix would then reveal changes during atherogenesis that could provide the framework for a complete natural history of atherosclerosis.

The techniques available at present do not permit this type of study and, therefore, alternative methods are used, each, it is clear, with its own peculiar limitations. For example, much of the current information on atherosclerosis is the result of many studies on experimental arterial disease in animals; few of these animals develop, however, a type of disease similar to human atherosclerosis (Section I.A.2.). Among the more popular techniques at present is cell culture, in which somatic cells (often from arterial tissue) are studied in conditions that are thought to be atherogenic *in vivo*, (for example, in medium containing high levels of lipid).

Cultured cells are often dedifferentiated and, therefore, may not carry out the specific functions of the differentiated cell *in vivo*. There is no organization into specialized structures that exist *in vivo* and the cultured cells are often

proliferating and experiencing cell-to-cell interactions that are changing constantly (39). In aortic SMC maintained in culture for periods of a month or less, changes have been reported in enzymic activities, centrifugal properties of organelles, and morphology (251) and in the amounts of intracellular myosin (252). These changes do not prove, however, that all functions of the cell are altered *in vitro* although it is held generally that any results obtained from cell culture experiments must be verified utimately *in vivo*.

The cell type of choice for studies of atherosclerosis *in vitro* is, of course, the arterial smooth muscle cell. The dermal fibroblast has been used frequently by others and in the present work for reasons explained in a previous section (III.G.1.). This discussion shall examine the results of the present work in the context of atherosclerosis, taking into account the results of relevant work that has been performed *in vivo* and in similar and different cell types *in vitro*.

B. The Effect of LDL on Cell Proliferation

The proliferation of arterial smooth muscle cells is an early event in atherosclerosis (Section I.A.1.). It has been demonstrated that the addition of cholesterol to the diet of swine elevates both plasma cholesterol and plasma LDL levels (253) and it is likely that the levels of cholesterol and LDL in the vicinity of intimal SMC increase also (Section I.C.5.). The addition of cholesterol to the diet of swine has been shown to cause increased mitotic counts in the aorta within

three days (16) and cholesterol was shown, moreover, to cause a higher frequency of cell death in the aorta (17).

The results of observations of the effects of LDL or hypercholesterolemic serum on the proliferation of arterial SMC in culture have been consistent with the results of the experiments in vivo. Thus, not only did LDL and hypercholesterolemic serum enhance the proliferation of animal SMC in culture, but it was observed that hypercholesterolemic serum increased the rate of cell death (Section I.E.2.).

The results from the present work indicate that human skin fibroblasts also proliferate more when human LDL is added to medium containing lipoprotein-free serum prepared from platelet-poor human plasma (Section III.B.4.a.). This is the first time that LDL-stimulated mitosis has been demonstrated in a system in which the components were derived entirely from humans. Similar experiments were to have been performed using human arterial smooth muscle cell but were precluded because of the small numbers of cells obtained from arterial explants (Section III.G.1.). The results obtained with swine SMC indicated that LDL was producing little effect on cell proliferation although it appeared that these cells adapted poorly to the experimental media (Section III.G.5.). It was found, however, that LDL augmented slightly the proliferation of swine SMC already dividing quite rapidly in low concentrations of FCS and this result suggested a requirement for other serum factors in order for LDL to exert an effect (Section III.G.4.).

In general, observations *in vivo* are consistent with the hypothesis that platelets are involved intimately with the processes of tissue injury and repair that are thought to precede the development of atherosclerosis (13,254). In addition, the disturbed flow of blood around vessel orifices, bifurcations, and points of stenosis is thought to promote the conditions favourable to local accumulation of platelets, aggregation of platelets, and release of constituents, and it is at these sites that atheroma are observed most frequently (254). The prominent role of platelets in the cellular proliferative response to arterial endothelial injury has been suggested by the results of work with platelet factors in cell cultures (Section I.E.2.) and with drugs that affect platelet function *in vivo*. Among the drugs used, dipyrimadole, which inhibits platelet aggregation, was shown by Harker *et al*. to prevent the formation of smooth muscle cell lesions in baboon arteries that were subjected to endothelial desquamation (255).

Intimal cells *in vivo* are not proliferating continuously (256), are not exposed normally to platelet factors (257), but are probably exposed continuously to plasma LDL (Section I.C.5.). From the present work and that of others (see above), it is known that LDL can augment the proliferation of SMC and skin fibroblasts in culture. It is also known that low molecular weight constituents of platelets are potent mitogens in culture although the dependency on these factors for proliferation is variable among cell types (Section III.D.1).

The results of the present work showed that the removal of serum components of low molecular weight from PPLFS by ultrafiltration produced no change during one week in the increase in cell numbers when LDL was absent, but abolished the increase in cell proliferation expected when LDL was added to the medium (Section III.D.4.). This is a new finding. While it is not possible to rule out a co-factor for LDL-stimulated proliferation that is neither platelet-derived nor mitogenic by itself, the results are consistent with the hypothesis that fibroblasts have no absolute requirement for the platelet factor for cell division but that it is required in order for LDL to exert its stimulating effect on proliferation.

Arterial SMC in culture are more dependent on platelet factors for proliferation than are fibroblasts as shown by the absence of any significant proliferation in cultures of simian SMC and human SMC in medium containing platelet-poor serum (Section III.D.5). The addition of LDL to this medium failed to increase significantly the cell number in cultures of human SMC (122) although LDL has been shown by others to have an effect of enhancing animal SMC proliferation when added to serum from which only lipoproteins had been removed (15).

These results suggest, therefore, that the release of platelet factors is both necessary and sufficient for SMC proliferation but that the degree of proliferation may be

elevated by LDL. In vivo, this effect of LDL could amplify the normal response to injury of the arterial wall and could possibly compromise the subsequent repair.

It is improbable, however, that cell proliferation in itself can cause atherosclerosis (30). At some point in the early stages of atherosclerosis the cholesterol balance of the intimal cells must be disturbed so that cholesterol accumulates and produces the typical "foam cell".

C. The Effect of LDL on Cellular Cholesterol Content

The results of studies in vivo show that cholesterol in the intima originates almost entirely in the plasma (Section I.C.). About two-thirds of the plasma cholesterol is found in the LDL (Section III.H.3.). It is likely, therefore, that much of the cholesterol that accumulates in intimal "foam cells" originates in the LDL.

Lipoprotein-bound, free cholesterol in culture medium becomes associated with the cell either by a process of exchange with cholesterol in the plasma membrane (39, 123), or by a process of uptake that involves the internalization of the lipoprotein at a specific receptor site, or by bulk-phase pinocytosis (123). Free cholesterol may be incorporated into membrane, may be esterified and stored, or may be excreted by the cell (possibly by the exchange mechanism mentioned above) (136, 258). Esterified cholesterol does not appear to exchange with membrane cholesterol and must enter the cell, therefore, by endocytosis (39, 136, 258). Cholesterol esters

are either hydrolyzed or are stored within lysosomes (39, 136, 258).

Cells that are cultured in medium supplemented with animal sera contain several times as much free cholesterol as esterified cholesterol (39). The results of replacing whole serum by lipoprotein-free serum are to reduce the total content of cholesterol in the cells and to increase the ratio of free cholesterol : esterified cholesterol (123). If LDL is then added to this medium, the total cholesterol content per cell increases and relatively greater elevations occur in the esterified fraction. These changes have been observed in cultures of canine SMC (126), porcine SMC (126), mouse fibroblasts (124), and human fibroblasts (123, 125). Similar results were obtained when hyperlipidemic serum replaced fetal calf serum in cultures of rabbit SMC (127) and monkey SMC (45). When homologous sera were used, absolute increases in free cholesterol were always higher, however, than absolute increases in esterified cholesterol (123, 126).

Even though substantial increases have been measured in the cellular cholesterol content, neither LDL nor hyperlipidemic serum has been shown to convert cells in culture into "foam cells". The increases in cholesterol content per fibroblast found in the present work were shown to be related to the concentration of LDL in the medium and were further correlated with increases in protein content per cell produced by LDL (Section III.B.4.b.). Cholesterol accumulation was, however,

unrelated to cell proliferation (Section III.D.4.). Although substantial increases occurred in cellular cholesterol levels, a constant ratio of about 0.1 was maintained at all times with cellular protein levels. It was inferred from this that cholesterol was being incorporated into cellular structures and was not accumulating to any significant degree in a storage form such as within lysosomes or within vacuoles. The accumulation of cholesterol in a storage form that causes an increase in the cholesterol : protein ratio of fibroblasts has been demonstrated by Stein et al. (125). Human skin fibroblasts were incubated with high levels of LDL and chloroquine, a drug that inhibits the degradation of LDL within lysosomes without affecting LDL binding and internalization by the cell (139). A similarly substantial increase in the cellular cholesterol : protein ratio is apparent in the data of Brown and Goldstein and associates who observed the formation of multiple cytoplasmic droplets apparently composed mainly of esterified cholesterol, when fibroblasts and SMC were exposed to LDL modified so as to carry a net positive charge (147, 148). Brown and Goldstein have presented the hypothesis that two functionally different types of cell are present in tissues: one type is protected by the "LDL pathway" (Section I.E.4.) from accumulating excessive amounts of cholesterol; another type, the "scavenger cell", is phagocytic, accumulates cholesterol in an unregulated way, and becomes the "foam cell"

of atherosclerosis (259). It is reasonable, however, to expect that the "scavenger cell" would appear in cultures and since "foam cells" have not been reported in cultures of skin fibroblasts or SMC (136), it is equally reasonable to suggest another explanation for their absence. The polycationic LDL used by Brown and Goldstein and associates produces "foam cells" readily in cultures of fibroblasts and SMC whereas high levels of native LDL do not. It is implied, therefore, that a factor which may determine the accumulation of intercellular cholesterol is the form in which cholesterol is presented to the cells. When non-lipoprotein-bound, free cholesterol is added in alcoholic emulsions to fibroblasts in culture, the cholesterol is accumulated to levels which are unapproachable by the addition of native LDL (123). This result agrees with the earlier findings of Rutstein et al. who detected by a qualitative method, similar levels of lipid deposited in human aortic cells that were incubated with LDL-cholesterol and much smaller amounts of non-lipoprotein-bound cholesterol in ethanolic solutions (149). It is significant perhaps that cells from patients with the homozygous form of familial hypercholesterolemia (patients who develop severe atherosclerosis very early in life) accumulated non-lipoprotein-bound cholesterol to a level about twice that of the control cells (123).

The form in which cholesterol is present in the intima may vary. Some cholesterol is likely to be associated with

free LDL molecules while some may be present in LDL molecules that have formed complexes with GAG or glycoproteins (Section I.D.3.b.). Since some of these complexes are insoluble and since GAG may reduce the solubility of free LDL by steric exclusion and sieving (Section I.D.3.a.), a part of the intimal extracellular cholesterol may in consequence be in an insoluble and immobile form which may be taken up by cells by a process of phagocytosis and result in cholesterol accumulation. It remains to be determined also what effect the formation of a soluble complex with GAG has on the metabolism of LDL particularly regarding the effect of these complexes on cholesterol accumulation.

The results of the present work and of published work have indicated, therefore, that in the presence of native LDL fibroblasts and SMC <u>in vitro</u> accumulate a limited amount of cholesterol and that the amount accumulated is related to the extracellular concentration. The present work showed also that the cholesterol was accumulated in a form that was probably structural. On the basis of these results and others which showed that these cells accumulate an uncontrolled amount of cholesterol when it is presented in a non-lipoprotein or modified lipoprotein form, it is suggested that the presence of cholesterol in the intima in a non-intact lipoprotein structure could lead to its accumulation in SMC and, in sufficient amounts, to the conversion of these cells to "foam cells".

D. The Effect of LDL on Protein Content and Size of Cells

In the present work, it was found that LDL increased both the protein content (Section III.B.) and the size of human skin fibroblasts (Section III.F.) and that because these increases are correlated with each other (Section III.F.), it is likely that the accumulated protein is structural. The additional protein in LDL-treated cells was found to be the result of a drop in the rate of protein degradation (Section III.C.4.).

It was suggested previously that LDL may be a "preferred substrate" for protein degradation and could thus delay the degradation of some cellular proteins and cause their accumulation. Alternatively, a lower rate of degradation may be the most efficient way of providing more protein to incoporate internalized LDL-cholesterol into cellular structures (Section III.C.4.e).

It was observed that LDL caused increases in the average and the maximum cell size in the fibroblast cultures (Section III.F.3.). The appearance of large cells, that are similar morphologically to macrophages, has been reported in cultures of human skin fibroblasts (232). Martin and Sprague have also observed clones of large cells in cultures of simian aortic SMC and of dermal fibroblasts (150). It remains to be established that these large cells are present in tissue in vivo. The finding of a correlation between cell protein

and cell cholesterol contents implies, however, that the "large cells" observed in the present work did not accumulate an abnormal amount of cholesterol.

E. The Effect of LDL on GAG

Changes in the amounts of GAG in the intima of atherosclerotic arteries have been reported (Section I.D.2.). There is indirect evidence, obtained mainly from studies in vitro that changes in the relative amounts of individual GAG may alter the mobility of LDL in the arterial mesenchyme either by increasing the probability of forming LDL-GAG complexes (that may vary in their solubility) or because of the effects of steric exclusion or molecular sieving (Section I.D.3). Changes in the GAG also might alter the accessibility of SMC to LDL that has infiltrated the intima since sulphated-GAG have been shown to compete with cells in culture for LDL (260).

The causes of alterations in the amounts of GAG in the intima are not known. Some studies have revealed that cells in culture produce amounts of GAG that vary with phases in the growth cycle of cells. However, in some cell types the production is maximum while the cells prepare for division whereas in others GAG production is greatest during proliferation (Section I.E.).

One of the many questions remaining unanswered in atherosclerosis research concerns the sequence of changes in GAG composition and cholesterol deposition in the intima:

Are changes in GAG composition the cause of conditions that promote LDL precipitation and cholesterol accumulation or does the presence of insoluble cholesterol or precipitated LDL provoke an inflammatory-reparative response in which the production of GAG differs from that under normal conditions? The present work yielded two results that are relevant to these questions: LDL was found to increase the accumulation of GAG in the medium and the proliferation of cells was not a factor in the accumulation of GAG in the medium (Section III.E.3.). The first of these results is a novel finding that reveals an effect of LDL on GAG metabolism which could be significant in the early stages of atherogenesis. Since the GAG may immobilize LDL and may compete with the cell surfaces for LDL, an increased amount of extracellular GAG could restrict the access of LDL to the cell. While this may be thought of as a protective action of the cell to prevent the overwhelming of its capacity to degrade LDL, a possible hazard is that LDL may change into a form that promotes the accumulation of cholesterol extracellularly (such as insoluble complexes of LDL-GAG or precipitates of LDL caused by steric exclusion or molecular sieving) or intracellularly (it is not known, for example, if LDL in LDL-GAG complexes is internalized and degraded in the same way as free LDL).

The second result showed that the proliferation of cells (that would occur in an inflammatory-reparative response) did not influence the total amounts of GAG recovered from the

medium. This result is consistent with the absence of a relation between cell proliferation and GAG production that has been reported by others (Section I.F.5.). It is possible that changes occurred in the relative amounts of individual GAG but these were not measured.

F. The Effect of Diabetes Mellitus on Atherosclerosis

The results of a variety of clinical and post-mortem studies have shown that atherosclerosis is frequently more severe and appears earlier in diabetics (Section I.F.). Although hyperlipoproteinemia is an established risk factor in atherosclerosis, there is no conclusive evidence at present that a greater frequency of hyperlipoproteinemia exists in the diabetic population (Section I.F.). Maturity-onset diabetes mellitus is inherited; the exact nature of genetic transmission is, however, unknown although there is support for a multigenic pattern (189). It is suspected, but remains to be proved, that premature atherosclerosis is a part of the syndrome of diabetes mellitus.

In the present work, the responses of skin fibroblasts in culture from maturity-onset diabetics and normal controls were compared for differences in proliferation, cholesterol accumulation, and protein accumulation. In another study, the compositions of plasma lipoproteins in the serum of maturity-onset diabetics were analyzed. These two investigations were carried out to examine further the relation between diabetes and atherosclerosis.

The literature at present contains several reports of differences in aspects of growth *in vitro* of pre-diabetic and diabetic fibroblasts compared with fibroblasts from age-matched normal controls. Thus, a lower number of individual cells were able to form colonies (192) and the replicative life-span of diabetic cells was shorter in the cultures of diabetic cells (188, 191). The number of colony-forming cells diminishes with cell age *in vitro* (192) and human skin fibroblasts possess a limited capacity for replication *in vitro* that is related inversely to the age of the donor (191). The diabetic cells appear, therefore, to have growth characteristics in culture of cells from normal donors of a greater age (191). The abnormal behaviour of diabetic cells in culture has been attributed hypothetically to an accelerated process of cell death and cell replacement *in vivo* that results from an inherited intolerance to cellular injury, and that is manifested in, among other pathological processes, a premature onset of vascular disease (188).

The results of the present work revealed no differences between diabetic and normal cells in their proliferative capacities in control medium (PPLFS) alone or with either level of LDL (Section III.B.4.a.). This finding concurs with that of Rowe *et al*. who found no difference in the proliferation rates of skin fibroblasts from age-matched diabetics and controls when these cells were incubated in medium containing fetal calf serum (261).

A possible cause of premature atherosclerosis is an abnormally rapid accumulation of cholesterol within the intima. The similar cholesterol contents of diabetic and control cells found in the present work gave no indication that the diabetic cells accumulated cholesterol with an abnormal avidity in culture (Section III.B.4.b.). Additional proof of the normality of the diabetic cells was found in the effect of LDL on protein content which was virtually identical in the two groups, even though substantial differences were found in the effects of different levels of LDL (Section III.B.4.c.).

It was concluded, therefore, that the proliferation, cholesterol accumulation, and protein accumulation of diabetic cells exposed to varying amounts of LDL were normal. In consequence, the evidence for a cellular defect that predisposes the diabetic to atherosclerosis remains, at best, circumstantial. At present, this evidence is based solely on findings of lower numbers of mean population doublings and of reduced plating efficiencies of diabetic cells in culture. The association of these with premature atherosclerosis *in vivo* remains to be established and explained.

The examination of the compositions of VLDL and HDL in the serum of diabetics revealed no major abnormalities: differences that were found could be attributed usually to hyperlipoproteinemias. Furthermore, Keen has expressed the view that there is no characteristic abnormality in the levels of lipoproteins of diabetics (172).

Much attention is being given at present to HDL in serum because of the association of depressed levels with coronary heart disease (34, 35, 186). It was found in the present work that cholesterol was distributed among the lipoproteins identically in normolipidemic diabetics and controls. The diabetics with elevated LDL were found, however, to carry a smaller amount of cholesterol in LDL (Section III.H.3.b.) and this result was in agreement with that of Howard et al. from a study of moderately hyperlipoproteinemic diabetics (174). These sub-groups of diabetics would thus be considered to be at a risk that is greater than normal of developing coronary heart disease.

An incidental observation of interest in a wider context of coronary heart disease in the general population was the correlation found between the cholesterol and apo E contents of VLDL (Section III.H.3.). This finding adds support to the suggestion of Shore and Shore that apo E (the arginine-rich protein) is associated primarily with cholesterol (250). An apo E-containing lipoprotein was shown to raise the cholesterol esterifying activity in human skin fibroblasts to a higher level than LDL (138). These investigators did not examine, however, the ability of the apo E-containing lipoprotein to produce "foam cells" in culture. Their results and the results of the present work suggest, nevertheless, a role for VLDL in the cholesterol metabolism of cells that may have significance in atherogenesis.

In summary, neither the existence of a cell defect nor abnormal compositions of lipoproteins that would suggest a cause of a predisposition to atherosclerosis in diabetes mellitus could be found in the studies described in this thesis. Although some reports tend to refute the idea (eg.,177), it is reasonable to assume for the present that the premature atherosclerosis among diabetics is related to a higher frequency of elevations in the low density lipoproteins (VLDL and LDL) or to some unrelated factor such as a higher frequency of hypertension (Section I.F.) in this group. It is not established that hyperlipoproteinemia, in itself, is a cause of atherosclerosis but evidence from a variety of sources indicates that plasma cholesterol levels play a major role in determining the incidence and severity of the disease (Section I.C.1.).

It was suggested above that an important factor in the accumulation of cholesterol in the intima was the physical state of cholesterol in the arterial wall. Native LDL does not produce "foam cells" in cultures of skin fibroblasts and arterial SMC and the results of the present work supported the suggestion that these cells can accomodate an increased load of cholesterol by reducing the rate of protein degradation and using the additional protein made available by this process to incorporate cholesterol into cell structures. In this way, it would be possible for the intimal cell to accomodate moderate elevations in plasma LDL.

The results of the present work also showed that LDL promoted the accumulation of GAG in the medium in cultures of human skin fibroblasts. Some, but not all, investigators find an increase in the total GAG in the intima during the early stages of atherosclerosis (Section I.D.2.). GAG appear to affect the solubility of LDL by complex formation, molecular sieving, and steric exclusion (Section I.D.3.). Elevations in plasma LDL probably cause elevations in intimal LDL concentrations and, by being present at higher concentrations in the intima and by stimulating the SMC to produce more GAG, LDL may increase the probability of its precipitation. LDL precipitated in the intima may then present the cells with a form of cholesterol different from that in intact native LDL. Since cholesterol that is not in intact native LDL has been shown to accumulate in an apparently uncontrolled way in cells in culture, it is proposed that an excessive precipitation of LDL in the intima may be an initiating event in atherosclerosis. It is also possible that soluble GAG-LDL complexes modify the metabolism of LDL so as to promote cholesterol accumulation.

In conclusion, several observations made during this work suggest future studies that may be profitable to the understanding of atherosclerosis. The results in general, must be confirmed in cultures of human arterial SMC and ultimately, if possible, <u>in vivo</u>. Of particular interest, would be a confirmation of the finding that native LDL reduces the rate of protein degradation in SMC and that the cholesterol : protein ratio of these cells remains constant (as it does in skin

fibroblasts) over a range of concentrations of extracellular LDL. Of equal interest, would be the demonstration that LDL promotes GAG accumulation in the medium in cultures of human arterial SMC as it does in the fibroblast cultures used in the present work. The effect of each of the various GAG on cholesterol accumulation of cells in culture that are incubated with LDL would be valuable information since some of the GAG appear able to form complexes with LDL and the effect of these complexes on the cellular metabolism of LDL is presently unknown. It may be speculated that "foam cells" arise <u>in vivo</u> partly because of the inability of the cells to degrade LDL when it is internalized as part of an LDL-GAG complex.

V. CONTRIBUTION TO KNOWLEDGE

The work described in this thesis yielded the following contributions to knowledge:

(i) LDL was shown to augment the proliferation of human skin fibroblasts in culture;

(ii) LDL-augmented proliferation was shown to be dependent on a low molecular weight component of human serum;

(iii) LDL produced an increase in the content of protein per cell;

(iv) The elevated protein content per cell was shown to be caused by a diminished rate of protein degradation;

(v) LDL increased the average and the maximum size of cells;

(vi) The cell size index (defined in Section III.F.2.) was shown to be proportional to the protein content per cell in all cell cultures;

(vii) The cholesterol content per cell was shown to be proportional to the protein content per cell in all experimental cultures. Increases in cholesterol contents of cells incubated with LDL were accompanied by proportional increases in the protein content per cell. This result supported the proposal that LDL-derived cholesterol was incorporated into cellular structures. It was suggested that "foam cells" in culture have not been observed when mammalian cells are incubated with intact, unchanged LDL because LDL-cholesterol is not accumulated in a storage form;

(viii) LDL was shown to cause an elevation in the amounts of GAG that accumulated in the culture medium;

(ix) LDL effects on cell protein accumulation, cell cholesterol accumulation, and GAG accumulation in the medium were shown to be independent of cell proliferation;

(x) LDL effects on cell proliferation, protein accumulation, and cholesterol accumulation were quantitatively similar in cultures of skin fibroblasts from diabetics and non-diabetics. These results indicated that the response of diabetic cells to LDL was normal;

(xi) No abnormalities were found in the compositions of serum lipoproteins from normolipidemic diabetics;

(xii) A correlation was found between the amounts of apo E and cholesterol in VLDL. This finding supports the suggestion in the literature that apo E preferentially binds cholesterol-rich particles.

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