In 1901 a physician, Archibald Garrod, observed a patient with black urine. He used this simple observation to demonstrate that a single mutant gene can produce a discrete block in a biochemical pathway, which he called an “inborn error of metabolism”. Garrod’s brilliant insight anticipated by 40 years the one gene-one enzyme concept of Beadle and Tatum. In similar fashion the chemist Linus Pauling and the biochemist Vernon Ingram, through study of patients with sickle cell anemia, showed that mutant genes alter the amino acid sequences of proteins. Clearly, many fundamental advances in biology were spawned by perceptive studies of human genetic diseases (1).

We began our work in 1972 in an attempt to understand a human genetic disease, familial hypercholesterolemia or FH. In these patients the concentration of cholesterol in blood is elevated many fold above normal and heart attacks occur early in life. We postulated that this dominantly inherited disease results from a failure of end-product repression of cholesterol synthesis. The possibility fascinated us because genetic defects in feedback regulation had not been observed previously in humans or animals, and we hoped that study of this disease might throw light on fundamental regulatory mechanisms.

Our approach was to apply the techniques of cell culture to unravel the postulated regulatory defect in FH. These studies led to the discovery of a cell surface receptor for a plasma cholesterol transport protein called low density lipoprotein (LDL) and to the elucidation of the mechanism by which this receptor mediates feedback control of cholesterol synthesis (2,3). FH was shown to be caused by inherited defects in the gene encoding the LDL receptor, which disrupt the normal control of cholesterol metabolism. Study of the LDL receptor in turn led to the understanding of receptor-mediated endocytosis, a general process by which cells communicate with each other through internalization of regulatory and nutritional molecules (4). Receptor-mediated endocytosis differs from previously described biochemical pathways because it depends upon the continuous and highly controlled movement of membrane-embedded proteins from one cell organelle to another in a process termed...
A Receptor-Mediated Pathway for Cholesterol Homeostasis

Many of the mutations in the LDL receptor that occur in FH patients disrupt the movement of the receptor between organelles. These mutations define a new type of cellular defect that has broad implications for normal and deranged human physiology.

In this lecture we first discuss the peculiar problem of plasma cholesterol transport. We then present some historical aspects of FH and the origin of the LDL receptor concept. Next, we summarize current knowledge of this receptor and the mechanism by which it functions in cells. Finally, we relate these findings to the pathogenesis of FH and to the common clinical problem of high blood cholesterol levels and atherosclerosis in human subjects.

THE PROBLEM OF CHOLESTEROL TRANSPORT

Cholesterol is the most highly decorated small molecule in biology. Thirteen Nobel Prizes have been awarded to scientists who devoted major parts of their careers to cholesterol (5). Ever since it was first isolated from gallstones in 1784, almost exactly 200 years ago, cholesterol has exerted a hypnotic fascination for scientists from the most diverse domains of science and medicine. Organic chemists have been fascinated with cholesterol because of its complex four-ring structure. Biochemists have been fascinated because cholesterol is synthesized from a simple two-carbon substrate, acetate, through the action of at least 30 enzymes, many of which are coordinately regulated. Physiologists and cell biologists have been fascinated with cholesterol because of its essential function in membranes of animal cells, where it modulates fluidity and maintains the barrier between cell and environment, and because cholesterol is the raw material for the manufacture of steroid hormones and bile acids. And finally, physicians have been fascinated because elevated levels of blood cholesterol accelerate the formation of atherosclerotic plaques leading to heart attacks and strokes. The studies of cholesterol therefore embrace almost all disciplines of modern biology. If the role of cholesterol in biomedicine is to be elucidated, all of these disciplines must be employed.

Cholesterol is a Janusfaced molecule. The very property that makes it useful in cell membranes, namely its absolute insolubility in water, also makes it lethal. For when cholesterol accumulates in the wrong place, for example within the wall of an artery, it cannot be readily mobilized, and its presence eventually leads to the development of an atherosclerotic plaque. The potential for errant cholesterol deposition is aggravated by its dangerous tendency to exchange passively between blood lipoproteins and cell membranes. If cholesterol is to be transported safely in blood, its concentration must be kept low, and its tendency to escape from the bloodstream must be controlled.

Multicellular organisms solve the problem of cholesterol transport by esterifying the sterol with long-chain fatty acids and packaging these esters within the hydrophobic cores of plasma lipoproteins (Fig. 1). With its polar hydroxyl group esterified, cholesterol remains sequestered within this core, which is essentially an oil droplet composed of cholesteryl esters and triglycerides, solubilized by a surface monolayer of phospholipid and unesterified cholesterol and stabilized by protein. The small amounts of unesterified cholesterol on the
Fig. 1. Structure of plasma LDL (left) and its cholesterol and cholesteryl ester components (right). LDL is a spherical particle with a mass of 3X10^6 daltons and a diameter of 22 nanometers. Each LDL particle contains about 1500 molecules of cholesteryl ester in an oily core that is shielded from the aqueous plasma by a hydrophilic coat composed of 800 molecules of phospholipid, 500 molecules of unesterified cholesterol, and 1 molecule of a 387,000-dalton protein called apoprotein B-100 (129). Elevations in blood cholesterol are usually attributable to an increase in the number of LDL particles.

The major classes of plasma lipoproteins were delineated in the 1950's and 1960's through work in many laboratories, most notably those of Oncley (6), Gofman (7), and Fredrickson (8). The four major classes are very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). A schematic representation of LDL, the most abundant cholesterol-carrying lipoprotein in human plasma, is shown in Fig. 1.

Packaging of cholesteryl esters in lipoproteins solves the problem of non-specific partitioning of cholesterol into cell membranes, but it creates another problem, namely one of delivery. Cholesteryl esters are too hydrophobic to pass through membranes. How then can esterified cholesterol be delivered to cells? The delivery problem is solved by lipoprotein receptors, of which the prototype is the LDL receptor (9). Strategically located on the surfaces of cells, these receptors bind LDL and carry it into the cell by receptor-mediated endocytosis. The internalized lipoprotein is delivered to lysosomes where its cholesteryl esters are hydrolyzed. The liberated cholesterol is used by the cell for the synthesis of plasma membranes, bile acids, and steroid hormones, or stored in the form of cytoplasmic cholesteryl ester droplets. Two properties of the receptor - its high affinity for LDL and its ability to cycle multiple times in and out
of the cell - allow large amounts of cholesterol to be delivered to body tissues, while at the same time keeping the concentration of LDL in blood low enough to avoid the buildup of atherosclerotic plaques. When LDL receptor function is inappropriately diminished as a result of genetic defects or in response to regulatory signals, the protective mechanism is lost, cholesterol builds up in plasma, and atherosclerosis ensues (10).

**Familial Hypercholesterolemia:**

**Origin of the LDL Receptor Concept**

As a disease, FH has a rich clinical history. It was first described in 1938 by Carl Müller, a clinician at the Oslo Community Hospital in Norway, as an "inborn error of metabolism" that produced high blood cholesterol levels and myocardial infarctions in young people (11). Müller astutely concluded that FH is transmitted as a single gene-determined autosomal dominant trait. In the mid 1960's and early 1970's, Khachadurian (12) at the American University in Beirut, Lebanon, and Fredrickson and Levy (13) at the National Institutes of Health showed that FH exists clinically in two forms: the less severe heterozygous form and the more severe homozygous form.

FH heterozygotes, who carry a single copy of a mutant LDL receptor gene, are quite common, accounting for 1 out of every 500 persons among most ethnic groups throughout the world (14). These individuals have a two-fold increase in the number of LDL particles in plasma from the time of birth. They begin to have heart attacks at 30 to 40 years of age. Among people under age 60 who suffer myocardial infarctions, about 5% have the heterozygous form of FH, a 25-fold enrichment over the incidence in the general population (15-17).

The attractiveness of FH as an experimental model stems from the existence of homozygotes. These rare individuals, who number about 1 in 1 million persons, inherit two mutant genes at the LDL receptor locus, one from each parent. Their disease is much more severe than that of heterozygotes. They have six to ten-fold elevations in plasma LDL levels from the time of birth, and they often have heart attacks in childhood (12-14). The severe atherosclerosis that develops in these patients without any other risk factors is formal proof that high levels of plasma cholesterol can produce atherosclerosis in humans. Experimentally, the availability of FH homozygotes permits study of the manifestations of the mutant gene without any confounding effects from the normal gene.

At the time that our studies began in 1972, it was generally felt that all important events in cholesterol metabolism take place in the liver or intestine (18). It was obviously impossible to perform meaningful studies in livers of humans with FH. Our only chance to explain its mysteries depended on the mutant phenotype being faithfully manifest in long-term cultured cells such as skin fibroblasts. Techniques for growing such cells had been established over the preceding two decades. Moreover, inherited enzyme defects were known to be expressed in cultured fibroblasts from patients with rare recessive diseases such as galactosemia, the Lesch Nyhan syndrome, and Refsum's syndrome. By
1970, Neufeld's classic studies of the mucopolysaccharidoses, a form of lysosomal storage disease, were beginning to establish the value of cultured skin fibroblasts in elucidating complex cellular pathways (19).

There was some reason to believe that the FH derangement might be manifest in cultured skin fibroblasts. Studies in the 1960's by Bailey (20) and Rothblat (21) had demonstrated that several types of cultured animal cells synthesize cholesterol and that this synthesis is subject to negative feedback regulation. When serum was present in the medium, cultured cells produced little cholesterol from radioactive acetate. When serum lipoproteins were removed from the culture medium, cholesterol synthesis increased.

**Regulation of HMG CoA Reductase by LDL in Fibroblasts**

We began our work by setting up a micro-assay for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the rate-determining enzyme of cholesterol biosynthesis. This assay was used to measure HMG CoA reductase activity in extracts of cultured fibroblasts (2,22). Earlier studies in rat livers by Bucher and Lynen (23) and by Siperstein (24) had shown that the activity of this enzyme was reduced when rats ingested cholesterol and that this reduction limited the rate of cholesterol synthesis. We soon found that the activity of HMG CoA reductase was subject to negative regulation in fibroblasts (2,22). As shown in Fig. 2A, when normal human fibroblasts were grown in the presence of serum, HMG CoA reductase activity was low. When the lipoproteins were removed from the culture medium, the activity of HMG CoA reductase rose by at least 50-fold over 24 hr period. The induced enzyme was rapidly suppressed when lipoproteins were added back to the medium (Fig. 2B).

Not all lipoproteins could suppress HMG CoA reductase activity. Of the two major cholesterol-carrying lipoproteins in human plasma, LDL and HDL, only LDL was effective (22,25). This specificity was the first clue that a receptor might be involved. The second clue was the concentration of LDL that was required. The lipoprotein was active at concentrations as low as 5 µg of protein per ml, which is less than 10^{-8} molar (22,25). A high affinity receptor mechanism must be responsible for enzyme suppression.

The key to this mechanism emerged from studies of cells from patients with homozygous FH (2,25). When grown in serum containing lipoproteins, the homozygous FH cells had HMG CoA reductase activities that were 50 to 100-fold above normal (Fig. 2A). This activity did not increase significantly when the lipoproteins were removed from the serum, and there was no suppression when LDL was added back. Clearly, the genetic defect was expressed in cell culture (Figs. 2A and 2B).

The simplest interpretation of these results was that FH homozygotes had a defect in the gene encoding HMG CoA reductase that rendered the enzyme resistant to feedback regulation by LDL-derived cholesterol. This working hypothesis was immediately disproved by the next experiment. Cholesterol, dissolved in ethanol, was added to normal and FH homozygote cells. When
A. AFTER REMOVAL OF LIPOPROTEINS

Fig. 2. Regulation of HMG CoA reductase activity in fibroblasts from a normal subject (○) and from an FH homozygote (●). Panel A: Monolayers of cells were grown in dishes containing 10% fetal calf serum. On day 6 of cell growth (zero time), the medium was replaced with fresh medium containing 5% human serum from which the lipoproteins had been removed. At the indicated time, extracts were prepared and HMG CoA reductase activity was measured. Panel B: 24 hours after addition of 5% human lipoprotein-deficient serum, human LDL was added to give the indicated cholesterol concentration. HMG CoA reductase activity was measured in cell free extracts at the indicated time. (Reprinted from ref. 2.)

mixed with albumin-containing solutions, cholesterol forms a quasi-soluble emulsion that enters cells passively, apparently by diffusion through the plasma membrane. When cholesterol was added in this form, the HMG CoA reductase activities of normal and FH homozygote fibroblasts were suppressed at the same rate and to the same extent (25).

Clearly, the defect in the FH homozygote cells must reside in their ability to extract cholesterol from the lipoprotein, and not in the ability of the cholesterol, once extracted by the cells, to act. But how do normal cells extract the cholesterol of LDL? The high affinity of the process suggested that a cell surface receptor was involved. The existence of cell surface receptors for protein hormones and other chemical messengers had been known for many years. It was generally thought that these receptors acted by binding the ligand at the
surface and then generating a “second messenger” on the intracellular side of the plasma membrane. The classic second messenger was cyclic adenosine monophosphate (cyclic AMP) (26). Perhaps LDL was binding to a receptor and generating some second messenger that suppressed HMG CoA reductase.

**Delineation of the LDL Receptor Pathway**
The existence of an LDL receptor was confirmed when LDL was radiolabeled with $^{125}$Iodine and incubated with normal and FH homozygote fibroblasts. These studies showed that normal cells had high affinity binding sites for $^{125}$I-LDL, whereas FH homozygote cells lacked high affinity receptors (3,27). This seemed to explain the genetic defect in FH, but it did not reveal how LDL generated the signal that suppressed HMG CoA reductase. The answer came from studies of the fate of the surface-bound $^{125}$I-LDL. Techniques were developed to distinguish surface-bound from intracellular $^{125}$I-LDL (28), and these revealed that the receptor-bound LDL remained on the surface for less than 10 min on average (Fig. 3A). Within this time most of the surface-bound LDL particles entered the cell; within another 60 min the protein component of $^{125}$I-LDL was digested completely to amino acids and the $^{125}$I, which had been attached to tyrosine residues on LDL, was released into the culture medium as $^{125}$I-monoiodotyrosine (27,28). Meanwhile, the cholesteryl esters of LDL were hydrolyzed, generating unesterified cholesterol which remained within the cell (29).

The only cellular organelle in which LDL could have been degraded so completely and rapidly was the lysosome. Originally described by de Duve (30), lysosomes were known to contain a large number of acid hydrolases that could easily digest all of the components of LDL. The hypothesis of lysosomal digestion of LDL was confirmed through the use of inhibitors such as chloroquine (31), which raises the pH of lysosomes and inhibits lysosomal enzymes (32), and through studies of cultured fibroblasts from patients with a genetic deficiency of lysosomal acid lipase (29). Cells from the latter patients bound and internalized LDL but failed to hydrolyze its cholesteryl esters, even though they were able to degrade its protein component.

The cholesterol that was generated from LDL within the lysosome proved to be the second messenger responsible for suppressing HMG CoA reductase activity. We now know that cholesterol (or an oxygenated derivative that is formed within the cell) acts at several levels, including suppression of transcription of the HMG CoA reductase gene (33) and acceleration of the degradation of the enzyme protein (34). The LDL-derived cholesterol also regulates two other cellular processes in a coordinated action that stabilizes the cell’s cholesterol content. It activates a cholesterol-esterifying enzyme, acyl CoA: cholesterol acyltransferase (ACAT), so that excess cholesterol can be stored in the cytoplasm as cholesteryl ester droplets (35). It also suppresses synthesis of LDL receptors by lowering the concentration of receptor mRNA (36,37). The latter action allows cells to adjust the number of LDL receptors to provide sufficient cholesterol for metabolic needs without causing cholesterol overaccumulation (9). Through these regulatory mechanisms, cells keep their level of
Fig. 3. Internalization and degradation at 37°C of \(^{125}\)I-LDL previously bound to the LDL receptor at 4°C in fibroblasts from a normal subject (Panel A) and from J.D., a patient with the internalization-defective form of FH (Panel B). Each cell monolayer was allowed to bind \(^{125}\)I-LDL (10 µg protein/ml) at 4°C for 2 hr, after which the cells were washed extensively. In one set of dishes, the amount of \(^{125}\)I-LDL was determined by measuring the amount of \(^{125}\)I-LDL that could be released from the surface by treatment with heparin. Each of the other dishes then received warm medium, after which they were incubated at 37°C. After the indicated interval, the dishes were rapidly chilled to 4°C, and the amounts of surface-bound (heparin-releasable) \(^{125}\)I-LDL [ ], internalized (heparin-resistant) \(^{125}\)I-LDL (A), and degraded (trichloroacetic acid-soluble) \(^{125}\)I-LDL (m) were measured. (Reprinted with permission from ref. 41.)
unesterified cholesterol remarkably constant despite wide fluctuations in cholesterol requirements and exogenous supply.

Receptor-Mediated Endocytosis of LDL: Binding Coupled to Internalization in Coated Pits

The rapidity of internalization of receptor-bound LDL and the completeness with which the protein of LDL was hydrolyzed implied that fibroblasts have a special mechanism for transport of the lipoprotein from the cell surface to the lysosome. The likely mechanism was endocytosis, the process by which surface membranes pouch inward and pinch off to form vesicles that eventually fuse with lysosomes. Endocytosis was first demonstrated by cinematography of phagocytic cells in the 1930's, and its universal occurrence in all cells was established in the 1950's by the electron microscopic studies of Palade (38). Endocytosis was felt to be a nonspecific process that transported bulk fluid and its contents into cells. There was no precedent for entry of specific receptors into cells by this route.

To determine whether endocytosis was involved in LDL uptake, we began in 1975 a collaboration with Richard G.W. Anderson, a cell biologist at our medical school in Dallas. Through the use of LDL coupled to electron-dense ferritin, we found that receptor-bound LDL was internalized by endocytosis. More important, however, these morphological studies explained the efficiency of internalization: efficiency was contingent upon the clustering of the LDL receptors in small pockets on the surface called coated pits (39). Coated pits had been described in detail by Roth and Porter (40) in 1964 during electron microscopic studies of the uptake of yolk proteins by mosquito oocytes. These investigators showed that coated pits pinch off from the surface to form coated endocytic vesicles that carry extracellular fluid and its contents into the cell.

The finding that LDL receptors were clustered in coated pits raised the general possibility that these structures serve as gathering-places for cell surface receptors that are destined for endocytosis (4). Other cell surface proteins, being excluded from coated pits, could not rapidly enter the cell. This interpretation of coated pit function was strengthened by study of fibroblasts from a unique FH homozygote. Cells from most of these subjects simply failed to bind LDL. But cells from one FH patient, whose initials are J.D., bound LDL, but failed to internalize it (Fig. 3B and refs. 41 and 42). In collaboration with Anderson, we showed that the receptors in these mutant cells were excluded from coated pits (43). This was an important finding, for it established the essential role of coated pits in the high efficiency uptake of receptor-bound molecules (4).

Figure 4 summarizes the sequential steps in the LDL receptor pathway as deduced from the biochemical, genetic, and ultrastructural studies performed between 1972 and 1976. Figure 5 shows the striking "all-or-none" biochemical differences in the metabolism of LDL and its regulatory actions in fibroblasts derived from a normal subject and from an FH homozygote with a complete deficiency of LDL receptors.

Soon after the initial studies of the LDL receptor pathway, Pearse (44)
Fig. 4. Sequential steps in the LDL receptor pathway of mammalian cells. HMG CoA reductase denotes 3-hydroxy-3-methylglutaryl CoA reductase; ACAT denotes acyl-CoA: cholesterol acyl-transferase. Vertical arrows indicate the directions of regulatory effects. (Reprinted from ref. 130.)

Fig. 5. Actions attributable to the LDL receptor in fibroblasts from a normal subject (■) and from a homozygote with the receptor-negative form of FH (▲) incubated with varying concentrations of $^{125}$I-LDL or unlabeled LDL at 37°C for 5 hr. Assays were performed in growing cells in monolayers as previously described (128). All data are normalized to 1 mg of total cell protein. The units for each assay are as follows: Binding, µg of $^{125}$I-LDL bound to cell surface; Internalization, µg of $^{125}$I-LDL contained within the cell; Hydrolysis of apoprotein B-100, µg of $^{125}$I-LDL degraded to $^{125}$I-monoiodotyrosine per hr; Hydrolysis of cholesteryl esters, nmol of [3H]cholesterol formed per hr from the hydrolysis of LDL labeled with [3H]cholesteryl linoleate; Cholesterol Synthesis, nmol of [14C]acetate incorporated into [14C]cholesterol per hr by intact cells; Cholesterol esterification, nmol of [14C]oleate incorporated into cholesteryl [14C]oleate per hr by intact cells. (Reprinted from ref. 130.)
purified coated vesicles and found that the cytoplasmic coat was composed predominantly of a single protein that she named clathrin. At the same time, Cohen and his collaborators performed their elegant studies of the action of epidermal growth factor (EGF) on cultured fibroblasts (45). They found that this peptide hormone was internalized by cells in a manner that was indistinguishable from that of LDL. Similar observations were made by Terris and Steiner (46) with insulin in hepatocytes, by Neufeld and coworkers (47) and by Sly and coworkers (48) with lysosomal enzymes in fibroblasts, and by Ashwell, Morell, and coworkers (49) with asialoglycoproteins in hepatocytes. Moreover, Helenius, Simons, and their coworkers (50) showed that several lipid-enveloped viruses enter cells by this route. Clearly, receptor-mediated endocytosis did not exist solely for cholesterol delivery: it was a general process by which cells internalized and degraded many extracellular molecules (4,51). In all instances in which adequate morphologic studies were performed, this internalization was attributable to clustering of receptors in coated pits. Indeed, Pastan and Willingham (51) and Carpentier, et al. (52) showed that receptors for several different ligands co-localize in the same coated pit.

The early LDL receptor studies also exposed another feature of receptor-mediated endocytosis - namely, that receptors can be recycled (4,28). After internalization the receptors dissociate from their ligands. From the work of Maxfield (53) and of Helenius and coworkers (54), we now know that such dissociation is triggered by a drop in pH within a special class of endocytic vesicles called endosomes (discussed below). After dissociation the receptors find their way back to the cell surface. The LDL receptor makes one round trip into and out of the cell every 10 min for a total of several hundred trips in its 20-hr lifespan (4,28).

THE LDL RECEPTOR: STRUCTURE ADAPTED TO FUNCTION

The LDL receptor is a cell surface glycoprotein that contains approximately two asparagine-linked (N-linked) oligosaccharide chains of the complex type and approximately 18 serine/threonine-linked (O-linked) oligosaccharide chains (55,56). About two-thirds of the O-linked sugars are clustered in one region of the molecule (57). The LDL receptor binds two proteins: 1) apo B-100, the 387,000-dalton glycoprotein that is the sole protein of LDL (27); and 2) apo E, a 34,000-dalton protein that is found in multiple copies in intermediate density lipoprotein (IDL) and a subclass of HDL (58,59). Innerarity and Mahley (59) demonstrated that lipoproteins which contain multiple copies of apo E bind to LDL receptors with up to 20-fold higher affinity than LDL, which contains only one copy of apo B.

Figure 6 illustrates the circuitous itinerary followed by the LDL receptor from its site of synthesis to its site of internalization in coated pits and its site of recycling in endosomes. The receptor is synthesized in the rough endoplasmic reticulum (ER) as a precursor (60) that contains high mannose N-linked carbohydrate chains and the core sugar (N-acetylgalactosamine) of the O-linked chains (56). The O-linked core sugars are added before the mannose
residues of the N-linked chains are trimmed, i.e., while the receptor is still in the endoglycosidase H-sensitive stage. Thus, the O-linked sugars must be added either in the ER or in a transitional zone between the ER and the Golgi apparatus. The receptor precursor migrates on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis as a single band corresponding to an apparent molecular weight of 120,000 (60).

Within 30 min after its synthesis, the LDL receptor decreases in mobility on SDS gels. The apparent molecular weight increases from 120,000 to 160,000 (60). This change is coincident with the conversion of the high-mannose N-linked oligosaccharide chains to the complex endoglycosidase H-resistant form (56). At the same time, each O-linked chain is elongated by the addition of one galactose and one or two sialic acid residues (56). The amount of carbohydrate is not sufficient to account for an increase in molecular mass of 40,000 daltons. Rather, the decrease in electrophoretic mobility is primarily caused by a change in conformation of the protein that results from the elongation of the clustered O-linked sugars (56,57).

About 45 min after synthesis, LDL receptors appear on the cell surface, where they gather in coated pits. Within 3 to 5 min of their formation, the coated pits invaginate to form coated endocytic vesicles. Very quickly, the clathrin coat dissociates. Multiple endocytic vesicles then fuse to create larger

Fig. 6. Itinerary of the LDL receptor in mammalian cells. The receptor begins life in the endoplasmic reticulum from which it travels to the Golgi complex, cell surface, coated pit, endosome, and back to the surface. HMG CoA reductase denotes 3-hydroxy-3-methylglutaryl CoA reductase; ACAT denotes acyl-CoA: cholesterol acyltransferase. Vertical arrows indicate the direction of regulatory effects. (Reprinted from ref. 131 with permission.)
sacs of irregular contour called endosomes or receptosomes (4,61). The pH of
the endosomes falls below 6.5, owing to the operation of ATP-driven proton
pumps in the membrane (53,54,61). At this acid pH, the LDL dissociates from
the receptor. The latter returns to the surface, apparently by clustering with
other receptors in a segment of the endosomal membrane that pinches off to
form a recycling vesicle. Once it reaches the surface, the receptor binds another
lipoprotein particle and initiates another cycle of endocytosis (4). Each LDL
receptor makes one round-trip every 10 min in continuous fashion whether or
not it is occupied with LDL (4,62). The LDL that dissociates from the receptor
is delivered to a lysosome when the membranes of the endosome and lysosome
fuse. There the protein component of LDL is hydrolyzed to amino acids and
the cholesteryl esters are hydrolyzed by an acid lipase, liberating cholesterol
(discussed above).

The striking feature of this pathway is that it requires the continuous
movement of a membrane embedded protein from one organelle to another in a
highly ordered fashion. Each time it moves, the receptor must be segregated
from neighboring membrane proteins that do not follow the same route. This
raises a crucial question: What are the signals that direct the highly selective
movement of receptors from one membrane organelle to another? Clearly, the
signals must lie in the structures of the receptors. What do we know about the
structure of the LDL receptor?

The LDL Receptor: A Multi-Domain Protein
The LDL receptor was purified from bovine adrenal cortex by Wolfgang
Schneider in our laboratory (55). A partial amino acid sequence was obtained,
and this sequence was used by David Russell and Tokuo Yamamoto to isolate a
full-length cDNA for the human LDL receptor (37,63). Biochemical studies of
the receptor protein, coupled with the amino acid sequence that was deduced
from the nucleotide sequence of the cDNA, have provided insight into
the structural domains of the LDL receptor (Fig. 7 and refs. 63-65).

At the extreme NH₂-terminus of the LDL receptor, there is a hydrophobic
sequence of 21 amino acids that is cleaved from the receptor immediately after
it is translated. This segment functions as a classic signal sequence to direct the
receptor-synthesizing ribosomes to the ER membrane. Because it does not
appear in the mature receptor, the signal sequence is omitted from the structur-
al domain that is described below. The mature receptor (without the signal
sequence) consists of 839 amino acids (63).

The first domain of the LDL receptor consists of the NH₂-terminal 292 amino
acids, which is composed of a sequence of 40 amino acids that is repeated with
some variation seven times (65,66). Studies of anti-peptide antibody binding to
intact cells revealed that this domain is located on the external surface of the
plasma membrane (67). Each of the seven 40-amino acid repeats contains six
cysteine residues, which are in register for all of the repeats. The receptor
cannot be labeled with [³H]iodoacetamide without prior reduction, suggesting
that all of these cysteines are disulfide-bonded (65). This region of the receptor
must therefore exist in a tightly cross-linked, convoluted state. This explains the extreme stability of the binding domain of the receptor; the receptor can be boiled in strong denaturants and still retain its binding activity as long as the disulfide bonds are intact (65).

A striking feature of each cysteine-rich repeat sequence is a cluster of negatively-charged amino acids near the COOH-terminus of each repeat (65,66). The charges on these sequences are complementary to a cluster of positively-charged residues that are believed to occupy one face of a single α-helix in apo E, the best studied ligand for the LDL receptor (68). Elegant studies by Mahley and Innerarity (68) with mutant and proteolyzed forms of
apo E and with monoclonal antibodies against different regions of apo E showed that the positively-charged region contains the site whereby this protein binds to the LDL receptor. It is therefore tempting to speculate that the negatively-charged clusters of amino acids within the cysteine-rich repeat sequence of the LDL receptor constitute multiple binding sites, each of which binds a single apo E molecule by attaching to its positively-charged $\alpha$-helix (65).

The second domain of the LDL receptor, consisting of $\sim$ 400 amino acids, is 35% homologous to a portion of the extracellular domain of the precursor for epidermal growth factor (EGF) (63,64,69). The EGF precursor is a molecule of 1217 amino acids that appears to span the plasma membrane one time like the LDL receptor (69-72). Analysis of the amino acid sequence of the EGF precursor, as revealed from the sequence of the cloned cDNA (70,71), suggests that EGF, a peptide of 53 amino acids, is liberated from the EGF precursor by proteolysis. The sequence of EGF is not homologous to the LDL receptor. Rather, the homology involves a part of the EGF precursor that is on the NH$_2$-terminal side of EGF itself. The function of this region in either the LDL receptor or the EGF precursor is unknown.

The third domain of the LDL receptor lies immediately external to the membrane-spanning domain and consists of a stretch of 58 amino acids that contains 18 serine or threonine residues (63,66). This domain is encoded within a single exon (see below). Proteolysis studies reveal that this region contains the clustered O-linked sugar chains (64).

The fourth domain consists of a stretch of 22 hydrophobic amino acids that span the plasma membrane, as demonstrated by proteolysis experiments (63,64). Comparison of the amino acid sequences of the human and bovine LDL receptors reveals that the membrane-spanning region is relatively poorly conserved (65). Of the 22 amino acids in this region, 7 differ between human and cow, but all of the substitutions retain a hydrophobic character.

The fifth domain is the cytoplasmic tail. The human and bovine LDL receptors each contain a COOH-terminal segment of 50 amino acids that projects into the cytoplasm (63,64). Localization of this domain to the cytoplasmic side of the membrane was determined through use of an anti-peptide antibody directed against the COOH-terminal sequence (64). When inside-out membrane vesicles containing receptor were digested with pronase, the antibody-reactive material was removed, and the molecular weight of the receptor was reduced by approximately 5000. The cytoplasmic sequence is strongly conserved among species. Of the 50 amino acids in this region, only 4 differ between human and cow, and each of these substitutions is conservative with respect to charge (65).

The cytoplasmic domain of the LDL receptor plays an important role in clustering in coated pits, either through interaction with clathrin itself or with some protein that is associated with clathrin on the cytoplasmic side of the membrane (4). This conclusion is based on a molecular analysis of three naturally-occurring mutations at the LDL receptor locus that produce receptors that bind LDL normally but fail to cluster in clathrin-coated pits. All three
of these mutations produce defects in the cytoplasmic tail (discussed below and ref. 65 and 73).

**The LDL Receptor: A Mosaic Gene**

The haploid human genome contains a single copy of the LDL receptor gene (66) on chromosome 19 (74). Sequences representing almost the entire gene were isolated from bacteriophage lambda and cosmid libraries by Thomas Südhof and David Russell (66). The position of each intron within the gene was mapped, and the sequence of each exon-intron junction was determined.

The LDL receptor gene spans ~ 45 kilobases and is made up of 18 exons separated by 17 introns (66). There is a striking correlation between the exons in the gene and the functional domains of the protein (Fig. 8). The first intron is located just at the end of the DNA encoding the cleaved signal sequence. The binding domain is encoded by exons 2 to 6. Within this domain (which contains the seven cysteine-rich repeats), introns occur precisely at the ends of repeats I, II, V, VI, and VII (Fig. 8). Repeats III, IV, and V are included in one exon. The binding domain is terminated by an intron at amino acid 292, the last residue in the seventh repeat. Thus, the binding domain is composed of a single exon that has been duplicated multiple times to produce seven repeats of a single 40 amino acid sequence. Each of these seven repeats in the LDL receptor is strongly homologous with a stretch of 40 amino acids that occurs in the middle of the C9 component of complement, a plasma protein of 537 amino acids that participates in the complement cascade (66,75).

The next eight exons in the LDL receptor gene (exons 7 to 14) encode the region that is homologous with the EGF precursor (Fig. 8). The gene for the EGF precursor contains the same eight exons (69). These exons form a cassette that has been lifted out of some ancestral gene during evolution and placed in the middle of the EGF precursor gene and the LDL receptor gene. Three of these exons have also been used by another class of genes. These exons encode a cysteine-rich sequence of 40 amino acids (labeled A, B, and C in Fig. 8) that is repeated three times in the LDL receptor and occurs once in several proteins of the blood clotting system, including factor IX, factor X, and protein C (69,76). Thus, these exons have been used by members of at least three different gene families.

The O-linked sugar domain is also encoded by a single exon (exon 15). However, not all domains of the protein are encoded by single exons. Thus, the membrane-spanning region is encoded by parts of two exons (exons 16 and 17). The cytoplasmic tail is also encoded by two exons (exons 17 and 18) (Fig. 8).

The sharing of exons between the LDL receptor gene and other genes provides strong evidence to support Gilbert’s hypothesis concerning the nature and function of introns (77). As originally proposed by Gilbert, introns permit functional domains encoded by discrete exons to shuffle between different proteins, thus allowing proteins to evolve as mosaic combinations of preexisting functional units. The LDL receptor is a vivid example of such a mosaic protein (66,78). It seems likely that other cell surface receptors will also be found to be mosaic structures assembled from exons shared with other genes.
Fig. 8. Correlation of exon organization with protein domains in the human LDL receptor. The domains of the protein are delimited by thick black lines and are labeled in the lower portion. The seven cysteine-rich, 40-amino acid repeats in the LDL binding domain (Fig. 7) are assigned roman numerals I to VII. Repeats IV and V are separated by eight amino acids. The three cysteine-rich repeats in the domain that is homologous with the EGF precursor are lettered A to C. The positions at which introns interrupt the coding region are indicated by arrowheads. Exon numbers are shown between the arrowheads. (Reprinted with permission from ref. 66.)

GENETIC DEFECTS IN THE LDL RECEPTOR

The mutations in the LDL receptor gene in FH patients have helped to delineate the crucial steps of receptor-mediated endocytosis. At the time of this writing, we have studied fibroblasts from 110 patients with the clinical phenotype of homozygous FH. All of them show evidence of defects in the LDL receptor, but not all defects are the same. At least 10 different mutations can be distinguished by structural criteria (65). These fall into four classes as shown in Fig. 9. Many of the apparent FH homozygotes are actually compound heterozygotes who inherit different mutant alleles from each parent.

Class I Mutations: No Receptors Synthesized
This is the most common class of mutant alleles, accounting for approximately half of the mutations so far analyzed. These genes produce either no LDL receptor protein or only trace amounts as determined by reaction with polyclonal or monoclonal antibodies. One of these alleles has been analyzed by molecular cloning; the gene bears a large deletion that extends from exon 13 to an $Alu$ repetitive element in intron 15 (79). This deletion is easily recognized on Southern blots of genomic DNA. We have not found evidence of a similar deletion in any other individual with the receptor-negative phenotype, so this particular deletion must be rare.

Class II Mutations: Receptor Synthesized, But Transported Slowly From ER to Golgi
This is the second most common class of mutations. These alleles produce receptors that are synthesized as precursors whose apparent molecular weights vary from 100,000 to 135,000. Most have an apparent molecular weight similar to that of the normal precursor (120,000). These receptors contain high mannose N-linked sugars and the core N-acetylgalactosamine of the O-linked sugars (56,80). However, the N-linked sugars are not converted to the complex endoglycosidase H-resistant form nor are the O-linked sugar chains elongated.
Fig. 9. Four classes of mutations that disrupt the structure and function of the LDL receptor and cause FH. Each class of mutation affects a different region in the gene and thus interferes with a different step in the process by which the receptor is synthesized, processed in the Golgi complex, and transported to coated pits. Each class of mutation can be further subdivided into different mutant alleles that are described in detail in ref. 65. (Reprinted with permission from ref. 132.)

These mutant receptors do not appear on the surface of the cell; rather, they seem to remain in the ER until they are eventually degraded. Some mutations in this class are complete, i.e., there is no detectable processing of carbohydrate. Others are partial, i.e., some of the receptors are processed and move to the surface at a rate that is one-tenth of normal (80,81). The molecular defect in this class of mutations has not been determined.
Class III Mutations: Receptors Processed and Reach Cell Surface, But Fail to Bind LDL Normally

In the mature form, these mutant receptors can have a normal apparent molecular weight of 160,000 or aberrant apparent molecular weights of 140,000 or 210,000 (65). They are all synthesized as precursors that appear to be 40,000 daltons smaller than the mature form. They all undergo normal carbohydrate processing and reach the cell surface, and they bind a variety of antibodies directed against the LDL receptor. However, they have a markedly reduced ability to bind LDL. We suspect that these mutations may involve amino acid substitutions, deletions, or duplications in the cysteine-rich LDL binding domain or the EGF precursor region, but none has yet been fully elucidated at the molecular level.

Class IV Mutations: Receptors Reach Cell Surface and Bind LDL, But Fail to Cluster in Coated Pits

Study of these internalization-defective mutations at the cellular level originally revealed the importance of coated pits in receptor-mediated endocytosis (42,43). Three of the mutations have now been elucidated in molecular detail. All involve alterations in the cytoplasmic tail of the receptor, i.e., the 50 amino acids at the COOH-terminus that project into the cytoplasm (Fig. 10). The mutations have been unraveled through the preparation of genomic DNA libraries and the subsequent isolation and sequencing of exons 17 and 18, which encode the cytoplasmic domain. In the most drastic case a tryptophan codon has been converted to a nonsense (stop) codon at a position that is 2 residues distal to the membrane-spanning region (73). This produces a recep-

Fig. 10. Mutations affecting the cytoplasmic domain of the LDL receptor in three FH homozygotes with the internalization-defective form of FH.
tor with only 2 amino acids in the cytoplasmic tail. Another mutation involves a duplication of 4 nucleotides following the codon for the 6th amino acid of the cytoplasmic tail (73). This duplication alters the reading frame and leads to a sequence of 8 random amino acids followed by a stop codon. This receptor has only 6 of the normal amino acids in the cytoplasmic domain. Protein chemistry studies have confirmed that these two proteins lack the normal COOH-termi- 
nus (73).

The third mutation is the most informative. In this patient, who was the original internalization-defective subject to be described (J.D., Fig. 3), a single base change leads to the substitution of a cysteine for a tyrosine residue at position 807, which is in the middle of the cytoplasmic tail domain (Fig. 10). We have recently reproduced this amino acid substitution in the normal LDL receptor cDNA by oligonucleotide-directed mutagenesis. When the altered cDNA was introduced into Chinese hamster ovary cells by gene transfer techniques, it produced a receptor that bound LDL but did not cluster in coated pits, confirming that the single base change is responsible for the internalization defect in J.D.’s cells (82).

Inasmuch as all three internalization-defective mutations involve the cyto-
plasmic tail, this region must normally play a crucial role in the clustering of LDL receptors in coated pits. It is likely that the cytoplasmic tail binds to clathrin or some other protein that is itself linked to clathrin. The puzzling feature at the moment is that other cell surface receptors that cluster in coated pits do not show obvious homology with the LDL receptor in the amino acid sequences of their cytoplasmic tails (65). Thus, the precise structure that links receptors to coated pits remains a mystery.

We have identified several interesting variants of the Class IV mutations in which the mutant genes produce LDL receptors that are secreted into the culture medium. In two mutants of this class (each from an unrelated family), the responsible mutation is a large deletion that results from a recombination between two repetitive Alu sequences, one in intron 15 and the other in the 3’ untranslated region of exon 18. The deletion joints in the two mutants are similar but not identical, indicating that the two mutations arose by independ- ent events (83 and unpublished observations). In each mutant, the deletion removes the exons encoding the membrane-spanning region as well as the cytoplasmic tail. Presumably these prematurely terminated proteins have a short random sequence of amino acids at the COOH-terminus, owing to read-
through of an unspliced mRNA. The receptors are transported to the surface, where some of them remain bound to the membrane. The vast majority, however, are released into the culture medium (83 and unpublished observa-
tions). The few receptors that remain on the surface bind LDL, but do not migrate to coated pits, thus giving rise to an internalization-defective pheno-
type. These findings emphasize the importance of the membrane-spanning region in anchoring the LDL receptor to the plasma membrane.

Figure 11 shows the location of nine mutations in the LDL receptor gene that have been analyzed at the molecular level. Each FH family examined to date has had a different mutation, and multiple types of mutational events have
Fig. 11. Location of mutations in the LDL receptor gene. To date, nine mutations have been identified by molecular cloning and DNA sequence analysis or by restriction endonuclease analysis of genomic DNA. Five of the nine mutations are described in detail in refs. 73, 79, 82, and 83. kb denotes kilobases; nt denotes nucleotides.

Occurred. Of the nine mutations, two involve single base substitutions, two involve insertions (one small and one large), and five involve large deletions. Many of the deletion joints occur in Alu repetitive elements.

FUNCTIONS OF THE LDL RECEPTOR IN THE BODY

The LDL receptor was elucidated by an investigative route that is opposite to the one usually employed to uncover metabolic pathways in animals. These pathways are usually observed first in intact animals or tissues and then they are studied in isolated cells. The LDL receptor was first observed in a totally artificial environment - namely, tissue culture. The question immediately arose: What tissues express LDL receptors in the body, and how do they work?

We knew at the outset that the receptor must play some role in the body as evidenced by the devastating consequences of LDL receptor deficiency in FH homozygotes and the proportionately less severe abnormalities in FH heterozygotes. Clearly, the receptor must be functioning somewhere. But where?

Detection of LDL Receptor Expression In Vivo

The first cells that were demonstrated to have LDL receptor activity in vivo were circulating blood lymphocytes. In the initial studies carried out with Y.K. Ho in 1975, we isolated lymphocytes from the bloodstream and incubated them for 67 hr in vitro in the absence of exogenous cholesterol so as to "derepress" receptor synthesis (84). Under these conditions the lymphocytes expressed abundant LDL receptors as determined by measurements of the high affinity uptake and degradation of $^{125}$I-LDL (Fig. 12A). Lymphocytes from FH homozygotes did not express detectable LDL receptor activity, and lymphocytes from FH heterozygotes had an intermediate level consistent with the presence of only a single functional gene (85). LDL receptors were also detectable on
lymphocytes immediately after their isolation from the bloodstream, although the level of activity was lower than it was after derepression for 67 hr (85). Thus, LDL receptors were expressed in at least one cell type in vivo.

Another early clue to the function of LDL receptors in vivo came from studies of the rate of disappearance of intravenously-injected $^{125}I$-LDL from plasma (Fig. 12B). Such LDL is removed from the circulation more slowly in FH heterozygotes than it is in normals (86,87). In FH homozygotes the removal defect is even more profound (87-89). The sluggishness of LDL catabolism in vivo correlates with the relative deficiency of LDL receptors as determined in isolated lymphocytes.

More detailed demonstrations of LDL receptor function in vivo have been obtained in experimental animals. Together with Sandip K. Basu, we established an assay for the binding of $^{125}I$-LDL to membranes from homogenates of cultured cells and various tissues of the cow and other animals (90). Using this assay, Petri Kovanen found that most tissues of the cow had detectable high affinity $^{125}I$-LDL binding; the adrenal gland and ovarian corpus luteum had the highest activity on a per gram basis (92). When the weight of the organ was taken into consideration, the liver was found to produce by far the largest number of LDL receptors. Similar results were obtained in studies of human fetal tissues (91). In collaboration with Havel’s laboratory, we showed that $^{125}I$-LDL was taken up by perfused rat livers by a high affinity receptor-mediated process that could be markedly accelerated by administration of the estrogenic hormone, $17a$-ethynyl estradiol (92).

![Figure 12](image-url)

**Fig 12.** Measurement of the number of LDL receptors in blood lymphocytes (Panel A) and in living subjects (Panel B). Panel A: Lymphocytes were isolated from venous blood of 32 normal subjects (e), 15 FH heterozygotes (a), and 4 FH homozygotes (0). After incubation for 67 hr at 37°C in medium containing 10% lipoprotein-deficient serum, LDL receptor activity was assessed by measurement of the high affinity degradation of $^{125}I$-LDL at 37°C. (Data replotted from ref. 85).

Panel B: In the whole-body assay, a tracer amount of $^{125}I$-LDL was injected intravenously, and the radioactivity remaining in the circulation over the next 16 days was measured in samples of venous blood (87,89). The higher the number of LDL receptors on body cells (Panel A), the faster the removal of $^{125}I$-LDL from the blood (Panel B).
High levels of hepatic LDL receptors were also observed when radiolabeled LDL was injected into the circulation of experimental animals and its uptake into various tissues was compared. Steinberg and coworkers (93) and Dietschy and coworkers (94) showed that approximately 70% of the total-body uptake of radiolabeled LDL took place in the liver by LDL receptor-dependent pathways, but that the highest rates of uptake on a weight basis were seen in the adrenal gland. Various other tissues also showed receptor-mediated uptake of LDL in excess of that seen with nonspecific bulk phase markers such as radiolabeled albumin.

Measurements of receptor-mediated LDL uptake by tissues of animals were made more practical as a result of two developments: 1) Steinberg and coworkers developed a method to label LDL with radioactive sucrose and later with tyramine-cellobiose (95). In contrast to ¹²⁵I-labeling of tyrosines, the latter methods produced a radioactive marker that remained trapped in lysosomes after uptake and degradation, thus allowing slow rates of uptake to be quantified cumulatively over long periods. 2) Shepherd and Packard (96) showed that LDL whose arginine residues were modified by reaction with cyclohexanedione was cleared from the human circulation much more slowly than was native LDL. The rationale for these latter studies lay in previous work from our laboratory (97) and from Mahley’s laboratory (98), which showed that modification of arginine or lysine residues on LDL abolished its ability to bind to the LDL receptor. These observations provided a crude estimate of the fraction of LDL clearance that was attributable to LDL receptors.

We had earlier estimated the fraction of total LDL clearance that was receptor-dependent by comparing the rate of catabolism of intravenously injected ¹²⁵I-LDL in normal individuals and in FH homozygotes (99). The fractional catabolic rate for LDL, i.e., the fraction of the total plasma pool of LDL removed per unit time, was 3-fold higher in normal subjects than in FH homozygotes (87). From this observation we reasoned that approximately two-thirds of LDL clearance is normally mediated through the LDL receptor (99). This conclusion has generally been borne out by a number of studies comparing the degradation rates for native versus lysine-modified or arginine-modified LDL both in normal human subjects and in a wide variety of experimental animals (100).

The WHHL Rabbit and the Role of the LDL Receptor in Clearance of IDL

One of the most important functions of LDL receptors in vivo was appreciated only in the past few years as a result of studies performed in Watanabe Heritable Hyperlipidemic (WHHL) rabbits. This strain of mutant rabbits was discovered in the late 1970’s by Yosio Watanabe, a veterinarian in Kobe, Japan (101). These rabbits have a mutation in the LDL receptor gene that is similar to the Class II mutations in human FH (81,102). When present in the homozygous form, this mutation gives rise to extremely high LDL-cholesterol levels; the rabbits develop atherosclerosis early in life (101,102).

The WHHL rabbits proved invaluable in explaining a previously puzzling feature of homozygous FH. Kinetic studies of ¹²⁵I-LDL metabolism by Myant
and coworkers (88), and by Bilheimer and Grundy (87,89) indicated that FH subjects have a dual defect. In addition to degrading LDL more slowly, FH homozygotes and heterozygotes also appeared to overproduce LDL. How does a genetic defect in the LDL receptor lead simultaneously to overproduction and reduced degradation of LDL? The answer lies in the complex biosynthetic pathway for LDL.

Early studies by Gitlin (103) and later those of Bilheimer, Levy, and Eisenberg (104) suggested that LDL is not secreted directly from the liver, but rather produced in the circulation from a blood-borne precursor, very low density lipoprotein (VLDL) (Fig. 13A). VLDL is a large, triglyceride-rich lipoprotein that is secreted by the liver; it transports triglyceride to adipose tissue and muscle. The triglycerides in VLDL are removed in capillaries by the enzyme lipoprotein lipase, and the VLDL returns to the circulation as a smaller particle with a new name, intermediate-density lipoprotein (IDL). The IDL particles have lost most of their triglyceride, but they retain cholesteryl esters. Some of the IDL particles are rapidly taken up by the liver; others remain in the circulation where they undergo further triglyceride hydrolysis and are converted to LDL. A distinguishing feature of the IDL particles is their content of multiple copies of apo E in addition to a single copy of apo B-100. The multiple copies of apo E allow IDL to bind to the LDL receptor with very high affinity. When IDL is converted to LDL, the apo E leaves the particle and only apo B-100 remains. Thereafter, the affinity for the LDL receptor is much reduced (102).

With Toru Kita, we showed that the apparent overproduction of LDL in WHHL rabbits is due to the failure of IDL to be removed from the plasma (102,105) (Fig. 13B). Thus, when ^125I-VLDL was administered to WHHL rabbits, the resultant IDL was not taken up by the liver, as it was in normal rabbits (105). Rather it remained in the circulation and was converted in increased amounts to LDL. These findings strongly suggest that IDL is normally cleared from plasma by binding to LDL receptors in the liver. Although experiments of similar detail cannot be carried out in humans, the observations of Soutar, Myant, and Thompson (106) are consistent with the notion that enhanced conversion of IDL to LDL also occurs in FH homozygotes, thus accounting for much of the apparent overproduction of LDL.

Figure 13A illustrates the dual role of the LDL receptor in LDL metabolism as determined from the studies of WHHL rabbits. First, the receptor limits LDL production by enhancing the removal of the precursor, IDL, from the circulation. Second, it enhances LDL degradation by mediating cellular uptake of LDL. A deficiency of LDL receptors causes LDL to accumulate as a result both of overproduction and of delayed removal (Fig. 13B). By this quirk of dual functionality, LDL receptors become crucially important modulators of plasma LDL levels in humans and animals.
Receptors

Fig. 13. Schematic model of the mechanism by which LDL receptors in the liver control both the production and catabolism of plasma LDL in normal human subjects (Panel A), in individuals with FH (Panel B), and in individuals consuming a diet rich in saturated fats and cholesterol (Panel C). VLDL denotes very low density lipoprotein; IDL denotes intermediate density lipoprotein. (Modified from ref. 132 with permission.)
PERSPECTIVES

Receptor Regulation: Therapeutic Implications

Knowledge of the fundamental properties of the LDL receptor has important implications for the therapy of FH and other hypercholesterolemic states. This knowledge also provides fuel for certain speculations about the role of the LDL receptor as a protective factor against atherosclerosis in human beings.

The therapeutic implications of the LDL receptor studies center on strategies for increasing the production of LDL receptors in the liver, thereby lowering plasma LDL-cholesterol levels. In FH heterozygotes this goal can be attained by stimulating the normal gene to produce more than its usual number of LDL receptors, thus compensating for the defective allele (107). The rationale for such therapy emerged from studies of cultured fibroblasts, which showed that the production of LDL receptors is driven by the cell’s demand for cholesterol (9,36). When demands for cholesterol are high, the cells have high levels of mRNA for the LDL receptor. Conversely, when demands for cholesterol are reduced, excess cholesterol accumulates in cells, and the amount of receptor mRNA falls (36,37).

Inasmuch as the liver is the major site of expression of LDL receptors, the therapeutic problem is reduced to the development of methods to increase hepatic demands for cholesterol. This can be achieved by two techniques: 1) inhibition of the intestinal reabsorption of bile acids; and 2) inhibition of cholesterol synthesis. These techniques can be used alone or in combination, as illustrated in Fig. 14.

The liver requires cholesterol for conversion into bile acids, which constitute the major route by which cholesterol is excreted from the body (18). However, only a fraction of the bile acids secreted by the liver actually leaves the body. The vast bulk of bile acids are reabsorbed in the terminal ileum and returned to the liver for reutilization. As a result, the liver converts only a minimal amount of cholesterol into bile acids (Fig. 14, left). The liver’s demand for cholesterol can be enhanced by the ingestion of resins that bind bile acids in the intestine and prevent their reabsorption. Since the liver can no longer re-use old bile acids, it must continually make new bile acids and the liver’s demand for cholesterol increases. In order to obtain this cholesterol, the liver makes a dual response: 1) it synthesizes increased amounts of cholesterol through an increase in the activity of HMG CoA reductase; and 2) it attempts to take up additional plasma cholesterol by increasing the production of LDL receptors. The increased LDL receptor activity causes plasma LDL levels to fall (Fig. 14, center). The problem with bile acid resin therapy (and the physiologically equivalent procedure of ileal bypass surgery) is that the effects are not profound. The increase in cholesterol production partially offsets the hepatic demand for cholesterol-and so there is only a 15 to 20% increase in the synthesis of LDL receptors and only a 15 to 20% drop in plasma LDL-cholesterol levels.

The second method for increasing LDL receptor production, namely, inhibition of hepatic cholesterol synthesis, is much more powerful than bile acid depletion. The technique emerged from the discovery in 1976 of a class of
fungal metabolites that inhibit HMG CoA reductase. The original compound, discovered by Akira Endo at the Sankyo Drug Company in Japan, is called compactin (108). A more recent version, developed by the Merck, Sharp and Dohme Research Laboratories in the United States, is called mevinolin (109). These two agents are potent competitive inhibitors of HMG CoA reductase; the inhibitory constant is approximately $10^{-9}$ molar (108).

When given to experimental animals, compactin or mevinolin initially inhibit cholesterol synthesis in the liver, and this triggers a complex regulatory mechanism that lowers the plasma LDL-cholesterol level. With Kovanen and Kita, we showed that the inhibition of cholesterol synthesis elicits a dual compensatory response: 1) hepatocytes synthesize increased amounts of HMG CoA reductase; and 2) they synthesize increased numbers of LDL receptors (110). When a new steady state is attained, the increase in HMG CoA reductase is almost sufficient to overcome the inhibitory effects of compactin. Total body cholesterol synthesis is only slightly reduced (111). Meanwhile, the plasma LDL level has fallen as a result of the increase in LDL receptors. The fall in plasma LDL levels is balanced by the increase in LDL receptors, and so the absolute amount of cholesterol entering the liver through the receptor pathway is the same as it was earlier. The difference, however, is that this delivery is now occurring at a lower plasma LDL level (107).

When given as a single agent to FH heterozygotes, mevinolin routinely produces a 30% fall in plasma LDL-cholesterol levels. When given together with cholestyramine, mevinolin blocks the compensatory increase in cholesterol synthesis, and the increase in LDL receptors is even more profound (Fig. 14, right). Plasma LDL cholesterol levels fall by 50 to 60% (112).

The important principle to emerge from these studies is that stimulation of LDL receptor activity lowers the plasma LDL-cholesterol level without grossly distorting cholesterol delivery (107,111). At present mevinolin and related
compounds are in the early stages of clinical testing. Their efficacy in lowering plasma LDL-cholesterol levels has been well established, but there is no information regarding long-term toxicity in man. If these drugs turn out to be non-toxic, they will have an important role in the therapy of FH heterozygotes and probably of other hypercholesterolemic individuals as well.

The principles applied to treatment of FH heterozygotes cannot, unfortunately, be applied to homozygotes, especially those who have totally defective LDL receptor genes. These individuals do not respond to the above-mentioned drugs because they cannot synthesize LDL receptors (113). Current therapy for these individuals involves removal of LDL from plasma extracorporeally through repeated plasmapheresis (114). Such procedures, which must be repeated every two to three weeks, are technically difficult and are very demanding of patient and physician.

Recently, a more direct therapeutic approach was taken in an FH homozygote, whose initials are S.J., and who has two mutant genes at the LDL receptor locus. This six-year-old girl, who is a patient of our colleague David Bilheimer in Dallas, had a total plasma cholesterol level over 1,000 mg/dl (greater than 6 times above normal limits), and she sustained repeated episodes of myocardial infarction. After she failed to respond to two coronary bypass procedures plus a mitral valve replacement, she was subjected to combined heart-liver transplantation by a team of surgeons led by Thomas E. Starzl at the University of Pittsburgh (115). The liver transplant was designed to provide a source of LDL receptors. The heart transplantation was necessitated because of the poor condition of her own heart as a result of the atherosclerotic process.

Immediately after the operation, S.J.’s total plasma cholesterol level fell from 1100 mg/dl to the range of 200 to 300 mg/dl, and it remained in that range for the succeeding 13 months (Fig. 15A). Thereafter she was started on the HMG

![Fig. 25. LDL metabolism in S.J., a patient with homozygous FH, before and after liver-heart transplantation. Panel A: Total cholesterol levels in plasma. Panel B: Plasma decay curves of 125I-LDL after intravenous injection of tracer amounts of 125I-LDL before (A) and after (A) liver-heart transplantation. (Data in Panel B reprinted with permission from ref. 115).](image-url)
CoA reductase inhibitor mevinolin, and her cholesterol fell further to the range of 150-200 mg/dl (Fig. 15A). Liver transplantation not only lowered the plasma cholesterol level but it also restored responsiveness to mevinolin, which requires a normal LDL receptor gene in order to act. Lipoprotein turnover studies performed six months after surgery confirmed that the new LDL receptors furnished by the transplanted liver were responsible for the dramatic drop in plasma cholesterol level (Fig. 15B). S.J. remains asymptomatic at the time of this writing, and her cutaneous xanthomas have resolved. However, she requires continuous therapy with cyclosporin to prevent rejection of the transplanted organs, and her long-term prognosis is uncertain.

The response to liver transplantation in S.J. underscores the importance of hepatic LDL receptors in vivo and raises the possibility that other FH homozygotes may respond to similar transplantation procedures. Hopefully, in appropriate cases liver transplantation can be performed before heart transplantation becomes necessary.

Speculations: LDL Receptors and the General Problem of Atherosclerosis

We now leave the realm of solidly established scientific fact and enter the much more controversial realm of speculation about the relation between cholesterol levels, LDL receptors, and atherosclerosis in the general population. After all, FH heterozygotes account for only 5% of myocardial infarctions in patients under the age of 60. What causes the other 95% of heart attacks?

Extensive epidemiologic studies performed in many populations in many countries over the past three decades have pointed strongly to a general association of high blood cholesterol levels with heart attacks. Among the most striking examples is the seven-country study of coronary artery disease directed by Ansel Keys (116). A similar correlation has been observed within a single population in the extensive studies in Framingham, Massachusetts (117).

These studies have all shown that the incidence of myocardial infarction rises in proportion to the plasma cholesterol level, more specifically the plasma level of LDL-cholesterol. When LDL-cholesterol levels are below 100 mg/dl (equivalent to a total plasma cholesterol level of ~170 mg/dl), heart attacks are rare. When LDL-cholesterol levels are above 200 mg/dl (equivalent to a total plasma cholesterol level of ~280 mg/dl), heart attacks are frequent. Controversy arises over the middle ground, i.e., individuals with plasma LDL-cholesterol levels between 100 and 200 mg/dl (total plasma cholesterol of 170 to 280 mg/dl). This is the range in which the vast bulk of heart attacks occur. Somewhere within this range there is a threshold value of cholesterol at which heart attacks begin to become more frequent. In this middle ground how much of the heart attack burden is attributable to plasma cholesterol? There is no definitive answer. In addition to cholesterol, heart attacks in this group are aggravated by smoking, hypertension, stress, diabetes mellitus, and poorly understood genetic factors. However, it seems reasonable to propose that plasma cholesterol does have something to do with heart attacks in these subjects, and that the incidence of heart attacks would be reduced if plasma cholesterol could be lowered (10).
The LDL receptor studies lend experimental support to the epidemiologists’ suggestion that the levels of plasma cholesterol usually seen in Western industrialized societies are inappropriately high (9). This support derives from knowledge of the affinity of the LDL receptor for LDL. The receptor binds LDL optimally when the lipoprotein is present at a cholesterol concentration of 2.5 mg/dl (28). In view of the 10 to 1 gradient between concentrations of LDL in plasma and interstitial fluid, a level of LDL-cholesterol in plasma of 25 mg/dl would be sufficient to nourish body cells with cholesterol (118). This is roughly one-fifth of the level usually seen in Western societies (Fig. 16 and ref. 119). Several lines of evidence suggest that plasma levels of LDL-cholesterol in the range of 25-60 mg/dl (total plasma cholesterol of 110 to 150 mg/dl) might indeed be physiologic for human beings. First, in other mammalian species that do not develop atherosclerosis, the plasma LDL-cholesterol level is generally less than 80 mg/dl (Fig. 16 and ref. 120). In these animals the affinity of the LDL receptor for their own LDL is roughly the same as the affinity of the human LDL receptor for human LDL, implying that these species are designed by evolution to have similar plasma LDL levels (9,119). Second, the LDL level in newborn humans is approximately 30 mg/dl (121), well within the range that seems to be appropriate for receptor binding (Fig. 16). Third, when humans are raised on a low fat diet, the plasma LDL-cholesterol tends to stay in the range of 50 to 80 mg/dl. It only reaches levels above 100 mg/dl in individuals who consume a diet rich in saturated animal fats and cholesterol that is customarily ingested in Western societies (116,122).

What is the mechanism for the high levels of plasma LDL that are so frequent in Western industrialized societies? Extensive evidence implicates two major factors: diet and heredity. When people habitually consume diets low in animal fats, their plasma LDL-cholesterol levels generally tend to remain low. When even moderate amounts of animal fat are introduced into the diet, the plasma cholesterol level rises (116,122). However, the level does not rise equally in every person. Clearly, genetic as well as dietary factors play a role.

How might a diet rich in animal fats and cholesterol elevate the plasma LDL-cholesterol level? Here we believe that two properties of the LDL receptor play a role - saturation and suppression. As the plasma LDL level rises, the receptors become saturated. This saturation of receptors sets an upper limit on the rate at which LDL can be removed efficiently from plasma (123). Each receptor can handle only one particle of LDL at a time. Once the receptors become saturated, the rate of removal of LDL can be accelerated only by an increase in clearance by non-receptor pathways that operate at low efficiency. In order to drive these alternate pathways, the LDL level must be quite high (99). At ordinary levels of LDL, the major factor that limits the removal of LDL from plasma is saturation of the LDL receptor (123).

Once LDL receptors become saturated, the removal rate of LDL is proportional to the number of receptors. Whenever the number of receptors is reduced, plasma LDL levels must rise. Experiments in animals indicate that the consumption of a high fat diet decreases the number of LDL receptors in the liver (123, 124). We believe that this mechanism operates through feedback
suppression as described above. That is, when excess dietary cholesterol accumulates in the liver, the liver responds by decreasing the production of LDL receptors (Fig. 13C). The entry of dietary cholesterol into the liver is mediated by a receptor, termed the chylomicron remnant receptor, whose activity is genetically distinct from the LDL receptor (125). The chylomicron remnant receptor is unaffected by cholesterol accumulation (126), and it causes cholesterol to accumulate to high levels in liver when the diet contains excess fat.

The combination of saturation and suppression of hepatic LDL receptors contributes in a major way to the buildup of LDL in plasma when a diet rich in saturated fats and cholesterol is ingested. Insofar as such a diet also may increase production of LDL in the face of a fixed or declining removal capacity, the LDL level would rise even higher.

If the LDL receptor does limit the removal of LDL from plasma, then maneuvers that increase LDL receptor activity might be effective in individuals who have high plasma LDL-cholesterol levels, but who do not have defective LDL receptor genes. Such therapy seems feasible with the development of HMG CoA reductase inhibitors. However, it is still too early to tell whether such therapy would decrease the incidence of myocardial infarctions in individuals with moderately elevated plasma LDL-cholesterol levels in the range of 100 to 200 mg/dl. There is much circumstantial evidence to expect such improvement (127), but unequivocal data are simply not there. Hopefully, with the availability of powerful receptor-stimulating drugs, the hypothesis should be susceptible to testing in the near future.

In considering the role of diet and drugs in treatment of high cholesterol levels, physicians and public health authorities must bear in mind the genetic variability between individuals. This variability exists at three levels: 1) The degree of increase in plasma cholesterol upon ingestion of a high cholesterol diet is variable. Not all people develop hypercholesterolemia. Some people, such as the Pima Indians, maintain low plasma cholesterol levels despite ingestion of a high fat diet (10). 2) Even when the plasma cholesterol level becomes elevated, the propensity for atherosclerosis varies. For example, a substantial proportion of FH heterozygotes (10 to 20%) escape myocardial infarction until the 8th or 9th decade despite pronounced hypercholesterolemia from birth (14). 3) Genetic susceptibility to contributory risk factors is variable. Some people can withstand hypertension and cigarette smoking for decades without developing atherosclerotic complications; others are highly sensitive.

In view of this genetic variability in susceptibility, dietary and drug recommendations must be individualized. The family history of the individual must be constantly borne in mind, particularly the familial incidence of premature heart attacks or strokes. An important goal of future research will be to dissect this genetic variability. Hopefully, it will become possible to identify the genes that determine such predispositions and to analyze them in each individual. For example, are there alleles that produce subtle defects in the LDL receptor that predispose to “garden-variety”, diet-responsive hypercholesterolemia? Are there subtle abnormal alleles at other loci such as those governing chores-
terol absorption, cholesterol synthesis, or bile acid synthesis? It seems likely that variability at such loci exists; the scientific challenge is to expose it.

**Receptor Recycling: A Novel Cellular Pathway**

The studies of the LDL receptor have revealed a new process by which membrane-embedded receptors cycle continuously into and out of cells. The receptors move from one organelle to another as a result of two sequential events: 1) segregation from other proteins by lateral movement in the plane of the membrane, and 2) pinching off of receptor-enriched membranes to form vesicles that eventually fuse with a different organelle. These receptors have been designated as “migrant” membrane proteins to distinguish them from “resident” membrane proteins that do not move in this fashion (4). The purpose of such intracellular traffic is to integrate the behavior of multiple organelles to form coherent biochemical pathways. Thus, the movement of the LDL receptor links the cell surface to the endosome and to the lysosome. The cholesterol liberated from LDL in lysosomes exerts regulatory effects in two other organelles, the endoplasmic reticulum and the nucleus. Selective movement of membrane proteins from one organelle to another allows such multi-organelle regulation to occur.

Fig. 16. Range of LDL levels in “normal” adults in Western industrial societies, indicated by the hump-shaped curve (127), is compared with the range in adult animals (120) and human infants (121) and with the levels seen in FH patients (14). Levels in the shaded region of the chart are above the threshold associated with accelerated atherosclerosis; more than half of the adults have LDL levels above this threshold. The LDL level is inversely associated with the number of LDL receptors. (Modified from ref. 10 with permission.)
What are the signals that dictate the path that each migrant membrane protein must follow? We are beginning to obtain some insight into the signals necessary for LDL receptors to be incorporated into one sorting structure, the coated pit. However, there is still no information with regard to signals that cause proteins to leave other organelles such as the endoplasmic reticulum and move to different organelles such as the Golgi complex. Delineation of these sorting signals is a major challenge facing the field of cell biology.

CONCLUDING REMARKS
In his Nobel Lecture of 1964, Konrad Bloch summarized his brilliant studies on the biological synthesis of cholesterol. At the end of his talk, Bloch predicted that the next era in cholesterol research would involve the elucidation of homeostatic control mechanisms (128). A decade later, in 1973, the LDL receptor concept was advanced to explain the homeostasis of plasma cholesterol and to account for regulatory abnormalities in cholesterol metabolism that were observed in patients with familial hypercholesterolemia. During the next 12 years, the LDL receptor was transformed from a genetic abstraction to a well-characterized protein whose structural domains have been defined. Studies of this receptor taught us about receptor-mediated endocytosis and the novel route by which receptors cycle in and out of cells. We have learned that receptors contain multiple functional domains that direct each step in this movement and that these domains are encoded on exons that can be shared among many proteins. We have learned that genetic defects in the receptor can cause cholesterol to accumulate in plasma, producing premature atherosclerosis. Together with others, we have also learned that the liver is the most important site of action of LDL receptors and that liver replacement can offer a successful form of therapy for children with homozygous FH. Finally, we have learned that regulation of this receptor through drugs and diet can profoundly change the LDL-cholesterol level and that saturation and suppression of receptors may contribute to the high incidence of hypercholesterolemia in industrialized society. It is hoped that these insights will lead to a deeper understanding of the biology of cells and thereby to more effective forms of treatment for diseases such as familial hypercholesterolemia.

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REFERENCES AND NOTES


5. Heinrich O. Wieland (1928), Adolf O.R. Windaus (1928), Leopold Ruzicka (1939), Robert Robinson (1947), and Otto P.H. Diels (1950) were awarded the Nobel Prize in Chemistry in part for work that led to the elucidation of the structure of cholesterol, a brilliant chapter in the history of organic chemistry. Konrad Bloch and Feodor Lynen were awarded the Nobel Prize in Chemistry in 1965 "for his outstanding achievement in the art of organic synthesis." Derek H.R. Barton and Odd Hassel were awarded the Nobel Prize in Chemistry in 1969 "for developing and applying the principles of conformation in chemistry," which included establishing the all chair conformation of cholesterol. John W. Cornforth, who collaborated with George Popjak to establish the orientation of all of the hydrogen atoms in the cholesterol molecule, received the Nobel Prize in Chemistry in 1975 "for his work on the stereochemistry of enzyme-catalyzed reactions."


A Receptor-Mediated Pathway for Cholesterol Homeostasis


