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Alessandra Pani and Sandra Dessì

Cell Growth and Cholesterol Esters

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Dedication

To Giovanni, Mario and Laura

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PREFACE

In recent years, understanding the molecular mechanisms involved in intracellular cholesterol homeostasis has radically changed to include an increasing number of structurally diverse receptors and carriers. The latest additions have led to the implication of cholesterol in fundamental cell functions such as cellular signaling and growth regulation. It appears that, at least in some instances, adaptive regulation of cholesterol metabolism does not protect cells indefinitely. Changes in this fine homeostatic regulation may occur leading to pathologic consequences. The challenge of this book has been to provide a useful point of reference on the mechanisms that link cholesterol esters to cell growth and division. Particular attention has been dedicated to the alterations in cholesterol esterification in two important proliferative processes such as cancer and atherosclerosis.

The data presented in this book provide in vivo and in vitro evidence of a strong relationship between cholesterol esterification and rate of cell proliferation, and suggest that changes in the cholesterol esterification pathway might represent fundamental events in developmental growth processes. Although much progress has been made in tumor and atherosclerosis research, and remarkable therapeutic successes have been achieved in both these pathologies, several questions on the mechanisms underlying changes in cholesterol metabolism and cell proliferation remain to be answered. Therefore, the data acquire particular significance in view of the possibility that the overall process of cholesterol esterification could play a key role in regulating cell proliferation.

We hope that this book provides valuable information not only for physicians, but also for teachers and students. As for the Editors, we would like to look at this book as a milestone of a path started some seven years ago when two friends, even though not so young any more, still deeply fascinated by science and the potentiality of biology, put together their scientific competence to face important matters such as the pharmacological control of pathological proliferative processes from a novel perspective.

Alessandra Pani
Sandra Dessì

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Chapter 1

Overview

Intracellular Cholesterol Homeostasis: Old and New Players

Sandra Dessì and Barbara Batetta

Cholesterol is an extremely important biological molecule being a major component of cell membrane as well as a precursor for the synthesis of a number of essential vitamins, steroid hormones and bile acids. It is for this reason that an adequate supply of cholesterol must be constantly insured to all cells of an organism. This is achieved mainly by two ways, either cholesterol is synthesized de novo within the cell, or it is supplied by an extra-cellular source. Nevertheless, since cholesterol overaccumulation may be toxic, the cells have developed a series of complex responses that work together to control the intracellular levels of cholesterol. These multiple responses are referred to as mechanisms of cholesterol homeostasis and make cholesterol one of the most controlled molecule inside the cells. Although regulation of cholesterol homeostasis was recognized nearly 30 years ago, over the intervening years several new mechanisms have been discovered. This chapter focuses on common and novel themes of cholesterol homeostasis paying particular attention to the complex network of proteins involved in the regulation of intracellular cholesterol trafficking.

Acquisition of Cholesterol by Peripheral Cells

Cholesterol is a biological molecule of vital importance for mammalian cell structure and function. Lacking cholesterol invariably leads to cell death. Unless specialized cells, such as hepatocytes which require cholesterol for the synthesis of lipoproteins and bile acids and steroidogenic cells for steroid hormone production, other cells demand cholesterol for the proper functioning of cell membranes where it has a major role in regulating fluidity of the lipid bilayer.

The cellular requirement of cholesterol is satisfied mainly from two sources:

- endogenously, by synthesis from acetyl-coenzyme-A (CoA) through mevalonate;
- exogenously, from receptor-mediated uptake of low-density lipoproteins (LDLs)

Endogenous Synthesis

Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetate group of acetyl-CoA (for review see ref. 1).

The process has five major steps:

1. Acetyl-CoAs are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase.
2. HMG-CoA is converted to mevalonate by HMG-CoA reductase (HMG-CoA-R). The reaction catalyzed by this enzyme is the rate limiting step of cholesterol biosynthesis, this enzyme being subject to complex regulatory controls.

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3. Mevalonate is converted to the isoprene based molecule, isopentenyl pyrophosphate (IPP), with the concomitant loss of CO_2
4. IPP is converted to squalene
5. Squalene is converted to cholesterol in the endoplasmic reticulum (ER).

From its site of synthesis, cholesterol is transported to other cellular destinations, prevalently plasma membranes, where 70-90 % of cellular cholesterol resides. It is reported that cholesterol reaches the cell surface within 10-20 min after synthesis in the ER.[2-4] Recent evidences indicate caveolae as the initial site on the cell surface where new cholesterol appears.[5,6] Caveolae are distinctive, flask-shaped invaginations of the plasma membrane found in many cells. In contrast to coated pits, which are constitutively endocytosed, caveolae remain attached to the plasma membrane with their release being affected by unknown signal. They have a characteristic lipid composition, rich in cholesterol and glycosphingolipids, and are associated with the presence of a 22 kD protein called caveolin-1.[7,8] It is also reported that caveolin-1 is required for the translocation of newly synthesized cholesterol from the ER directly to caveolae. The arrival of new cholesterol in caveolae is followed by the immediate movement of the sterol to noncaveolae membrane and possibly out of the cell.[9]

The fate and the transport of newly synthesized cholesterol in mammalian cells is shown in Figure 1.1.

Exogenous Uptake: The LDL Receptor Pathway

Exogenous cholesterol is mainly obtained by the internalization of cholesteryl-rich lipoproteins via LDL receptor pathway.[10] This pathway is known since 1973 and its discovery led to the award of the Nobel Prize to Brown and Goldstein in 1985.

It is well known that cholesterol is a hydrophobic molecule quite insoluble in water. Thus, it cannot pass from the liver and/or the intestine to the cells simply dissolved in blood and in extracellular fluid. Instead it is carried in tiny droplets of lipoprotein. The most abundant cholesterol carriers in human are the LDLs.

LDL particles are spheres with a core containing mainly cholesterol esters covered with a single layer of phospholipid molecules with their hydrophilic heads exposed to the watery fluid (e.g., blood) and their hydrophobic tails directed into the interior. Over a thousand molecules of cholesterol are bound to the hydrophobic interior of LDL particles. A protein, called apolipoprotein B-100 (ApoB-100) is exposed at the surface of each LDL particle. The first step in acquiring LDL particles is their binding to LDL receptors that are localized at the cell surface in pits coated with clathrin, a protein involved in the formation of transport vesicles from membranes. LDL receptor is a cell surface glycoprotein with a molecular weight of 164 kDa having a site that recognizes and binds to the ApoB-100 on the surface of the LDL. Once LDL binds the receptor, ligand and receptor are collected in the coated pits, and internalized to form coated vesicles. The clathrin coat is then removed and the uncoated vesicle fuses with endosomes to form an early sorting endosome. Here, ATP-dependent proton pumps lower the pH which causes the LDL to separate from its receptor.

The vesicle then pinches apart into two smaller vesicles: one containing LDLs (late endosome); the other containing receptors (endocytic recycling compartment, (ERC)). The ERC returns to and fuses with the plasma membrane, turning inside out as it does so. In this way the LDL receptors are returned to cell surface for reuse. The late endosome fuses with lysosomes to form a late endosome/lysosome where the LDL undergoes digestion. This results in degradation of apoproteins and hydrolysis of cholesterol esters by a specific acid cholesterol esters hydrolase, (aCEH) in fatty acids and free cholesterol. This process, apparently complex, is extremely necessary for that cholesterol may be used by cell for membrane structure purpose. Cholesterol esters, the form by which cholesterol is found in LDL particles, are, in fact, highly non polar molecules and for this solubility characteristic they can not be able to become a

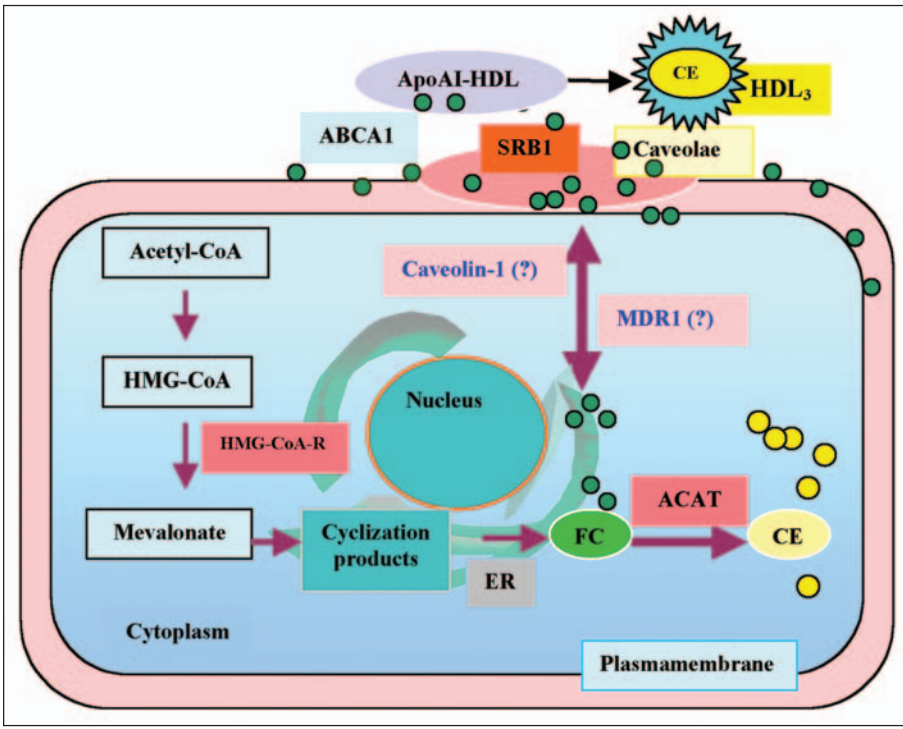

Fig. 1.1. Fate and transport of cholesterol endogenously synthesized.

- Cholesterol synthesis occurs in cytoplasm and endoplasmic reticulum (ER) from Acetyl CoA HMG-CoA-reductase, which resides in the ER, catalyzed the rate limiting step of cholesterol synthesis (conversion of HMG-CoA to mevalonate).
- Through a complex series of reactions (cyclization products), mevalonate is converted to free cholesterol (FC).
- Within 10-20 min after synthesis in the ER, FC appears to move directly to the plasma membrane.
- Evidence has been presented that caveolae are the initial site on the cell surface where new cholesterol appears and that caveolin-1 is required for the translocation of newly synthesized cholesterol from the ER directly to caveolae.
- The arrival of new cholesterol in caveolae is followed by the immediate movement of the sterol to noncaveolae membrane, or out of the cell via HDL receptors (ABC1-SRB1) or it is again transported to the ER (MDR1 ?) for esterification by ACAT.

member of cell membranes. By contrast, free cholesterol is a relatively polar lipid and integrates well in the phospholipid bilayers (for review see ref. 11).

Free cholesterol liberated into lysosome is promptly transported at cell surface caveolae from where it may be used by the cells for the synthesis of new membrane and/or for the normal membrane turnover. It has been found that a maximum of 40-50 min is required for transport of LDL-cholesterol from lysosomes to plasma membrane.[12] Thus, intracellular transport of LDL derived free cholesterol begins at clathrin-coated pits and terminates at cell surface caveolae.[6]

Recently, it has been speculated that a protein called NPC1 is involved in free cholesterol efflux from the late endosome/lysosome and that the NPC1 protein is responsible for transport of cholesterol to the trans Golgi network and then to plasma membranes. This idea comes from the discovery that cells obtained from patients with a rare, fatal, and presently untreatable

autosomal recessive disorder (Niemann Pick type C1 disease) accumulate massive amounts of free cholesterol in late endosomes which also expand, filling with whorls of membrane.[13,14] More recently, however, Lange and Coll.[15] provided evidence that functional NPC1 protein is not required for the exit of cholesterol from NPC1 lysosomes, being the rate of movement of cholesterol from lysosomes to plasma membrane in NPC1 cells at least equal to that observed in normal cells. They conclude that, the build up of cholesterol in NPC1 lysosomes was not a physiological response to cholesterol overload. Rather, it results from an imbalance in the brisk flow of cholesterol among membrane compartments. Based on these results the mechanisms mediating free cholesterol transport from lysosomes to the plasma membrane remain to be established. The LDL receptor pathway is summarized in Figure 1.2.

Mechanisms of Cholesterol Homeostasis

Within the cell, the distribution of cholesterol is highly compartmentalized, with most of the cellular cholesterol concentrated in the plasma membranes.

However, to preserve the integrity of cells, membrane FC mass must be maintained within narrow limits. The excess cholesterol is then stored as cholesterol esters in form of cytoplasmic lipid droplets. The esterification process is catalyzed by a membrane bound enzyme located in the ER called acyl-coenzyme A: cholesterol acyltransferase (ACAT). To maintain optimal content of cholesterol within the cells, the LDL uptake process, the endogenous sterol synthesis process, and the sterol esterification process are coordinately regulated. This is achieved by a series of complex responses referred to as mechanisms of cholesterol homeostasis.

When plasma membrane free cholesterol exceeds a threshold level, cholesterol beyond the cell's need is rapidly transported to the ER where homeostatic proteins reside.[16] Although cholesterol transport from the plasma membrane to ER seems to represent a crucial point of homeostatic activities, it is still unclear how sterols are transported to the ER and how this transport is regulated.

Possible mechanisms that have been involved, include aqueous diffusion, vesicle-mediated transport, and soluble carriers, which may work together or separately to mobilize cholesterol within the cell. More recent evidences raise the possibility of a mechanistic role for MDR1-P-glycoprotein (P-gp) in cholesterol transport.[17-19]

Involvement of MDR1-P-gp in Cholesterol Transport

P-gps (140-180 kDa) are members of the large ATP-binding cassette (ABC) superfamily of transport proteins also called traffic ATPase. They are composed of two homologous halves joined by a flexible linker region, each one containing six transmembrane domains and an ATP binding-utilization domain. ATP-binding and hydrolysis appear to be essential for the proper functioning of P-gps. One member of P-gps family, encoded by MDR1 gene was originally identified for its ability to confer resistance against unrelated cytotoxic drugs in tumor cells. This phenomenon termed "multidrug resistance" represents today one of the main causes of cancer therapy failure[20] and will be discussed in more detail in the next chapters.

Although, MDR1-P-gp is over-expressed in tumors following chemotherapy, it is also normally expressed in many different tissues raising the question of the physiologic function(s) of this protein. In addition to the prospected roles for P-gp in outward translocation of substrates,[21-24] several recent studies suggest a function for MDR1-P-gp in the trafficking of sterol within cells. A series of amphiphilic agents, known to modulate MDR activity, blocked transport of cholesterol substrate from the plasma membrane to ER in a cultured rat hepatoma cell line.[16] Likewise, in CaCo2 cells and in a human hepatoma cell line, compounds known to inhibit MDR1 P-gp also inhibited the movement of cholesterol from the plasmamembrane to the ER.[25] Using a series of steroid hormones, Debry et al[18] found that inhibition of cholesterol

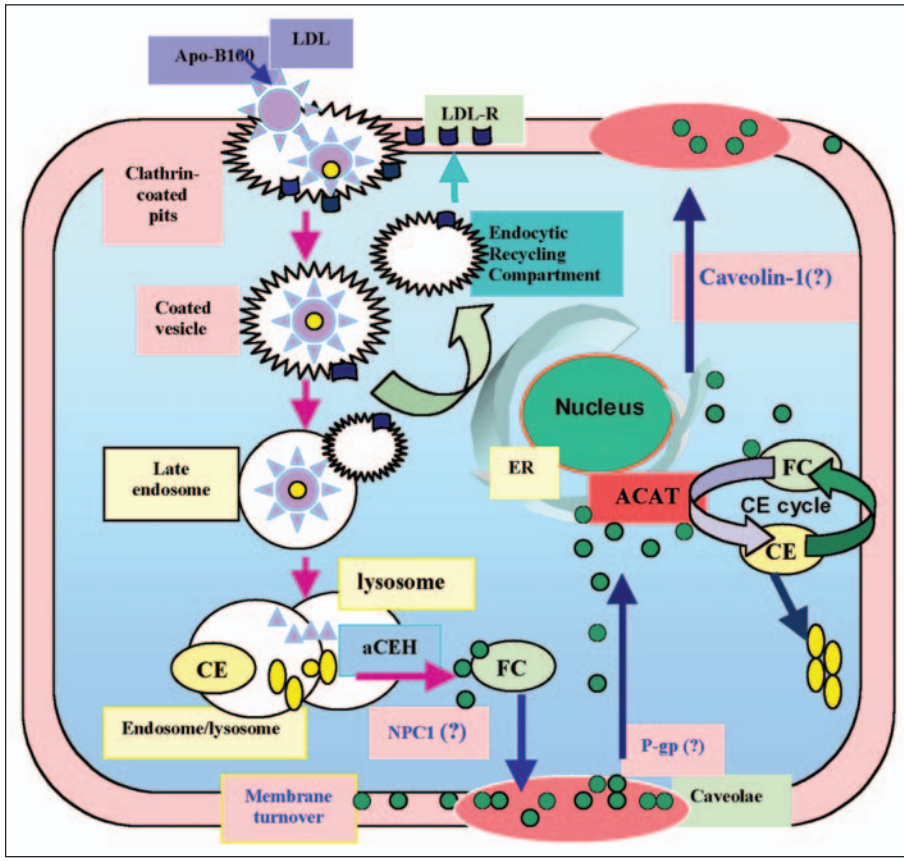

Fig. 1.2. The LDL receptor pathway.

- The first step in acquiring LDL particles is their binding to LDL receptors (LDL-R) that are localized at the cell surface in pits coated with clathrin.
- Once LDL binds the receptor, ligand and receptor are collected in the coated pits, and internalized to form coated vesicles. The clathrin coat is then removed and the uncoated vesicle fuses with endosomes to form an early sorting endosome. Here, ATP-dependent proton pumps lower the pH which causes the LDL to separate from its receptor.
- The vesicle then pinches apart into two smaller vesicles: one containing LDLs (late endosome); the other containing receptors (endocytic recycling compartment (ERC). The ERC returns to and fuses with the plasma membrane, turning inside out as it does so. In this way the LDL receptors are returned to cell surface for reuse.
- The late endosome fuses with lysosomes to form a late endosome/lysosome where the LDL undergoes digestion. This results in degradation of apoproteins and hydrolysis of cholesterol esters (CE) by a specific acid cholesterol esters hydrolase, (aCEH) in fatty acids and free cholesterol (FC).
- Most of the LDL-bound cholesterol released from the lysosome rapidly emerges at cell surface caveolae, from where it may be used by the cells for the synthesis of new membrane and/or for the normal membrane turnover or it is transported to ER for the esterification by ACAT.
- Cholesterol flux in and out of the ER may serve to regulate ACAT and other cholesterol sensors in the ER. Under high cholesterol trafficking, a dynamic cholesterol-cholesteryl ester cycle exists.

trafficking from the plasma membrane to the ER correlated directly with the hydrophobicity of each steroid and its potency in reversing the effect of P-gp on drug accumulation. Although the mechanism by which P-gp may affect cholesterol trafficking within cells is currently unknown, overall, these data support an additional physiologic function for P-gp in cholesterol transport from the plasma membrane to the ER.

Sterol Regulatory Systems in the ER

As mentioned above cellular cholesterol levels are controlled by a diverse set of homeostatic activities that are all located in the ER. These include enzymes for sterol biosynthesis (e.g., HMGCoA reductase) and esterification (acyl-CoA: cholesterol acyltransferase, ACAT) as well as precursors for transcription factors that control the expression of other regulatory elements. It seems that all cholesterol homeostatic proteins are orchestrated by a common regulatory signal: the level of ER cholesterol in their vicinity. The expression of genes for the LDL receptor, HMGCoA-synthase, HMGCoA reductase and other regulatory of cholesterol abundance such as farnesyl diphosphate synthase and squalene synthase is under the positive control of a family of ER membrane-bound proteins called sterol regulatory element-binding proteins (SREBPs), of which three different isoforms are currently recognized. They activate genes involved in the synthesis of cholesterol and its uptake from plasma lipoproteins by acting as transcription factors after proteolytic cleavage. This cleavage is regulated by a polytopic membrane protein called SREBP cleavage-activating protein (SCAP), which forms complexes with SREBPs in membranes of the ER and also serves as a sterol sensor, losing its activity when sterols overaccumulate in the ER.

When cholesterol levels in the ER are low, the NH2-terminal domains of the SREBPs are released from ER membranes by two sequential proteolitic cleavages, catalyzed by two different proteases, site-1 protease (S1P) and site-2 protease (S2P). S1P is a membrane-bound serine protease that cleaves the SREBPs at a leucina-serine bond within a hydrophilic loop that projects into ER lumen, dividing the SREBPs in two halves. SCAP facilitates cleavage of SREBPs by S1P. After the two halves of the SREBP have separated, a second protease, designated S2P, cleaves the NH2-terminal intermediate fragment at a site that is just within its membrane–spanning domain. Active fragments of SREBP leave the ER membrane and translocate to the nucleus where they bind to a 10 bp sterol regulatory element-1 (SRE-1) sequence contained in the promoters of sterol-regulated genes and activate gene transcription. On the contrary, when cholesterol levels in the ER rise, the proteolitic release of SREBPs from ER membranes is blocked and SERBP remains membrane-bound. As a result of these events, transcription of sterol-regulating genes declines, and sterol synthesis and uptake are suppressed.[26-27]

Current evidence indicates that cholesterol blocks the proteolytic release process by selectively inhibiting cleavage by S1P. S2P does not appear regulated directly by sterols since this enzyme cannot act until the two halves of SREBP have been separated through the action of S1P.

The build-up of cholesterol in the ER also results in an increased rate of cholesterol esterification by the ER-bound enzyme, ACAT, which converts excess free cholesterol to cholesterol esters that accumulate in the cytoplasm as cholesteryl esters droplets. To minimize interference by ACAT in the sterol translocation process between the ER and the plasma membrane, significant esterification by ACAT does not occur unless the cholesterol concentration in the ER exceeds a certain critical threshold that is higher than that required to induce SREBPs. Based on these observations it has been proposed that the main role of ACAT is to guard against excessive buildup of cholesterol in the ER.[28]

Figure 1.3 depicts the current knowledge about the two-step proteolytic cleavage of membrane bound SREBPs.

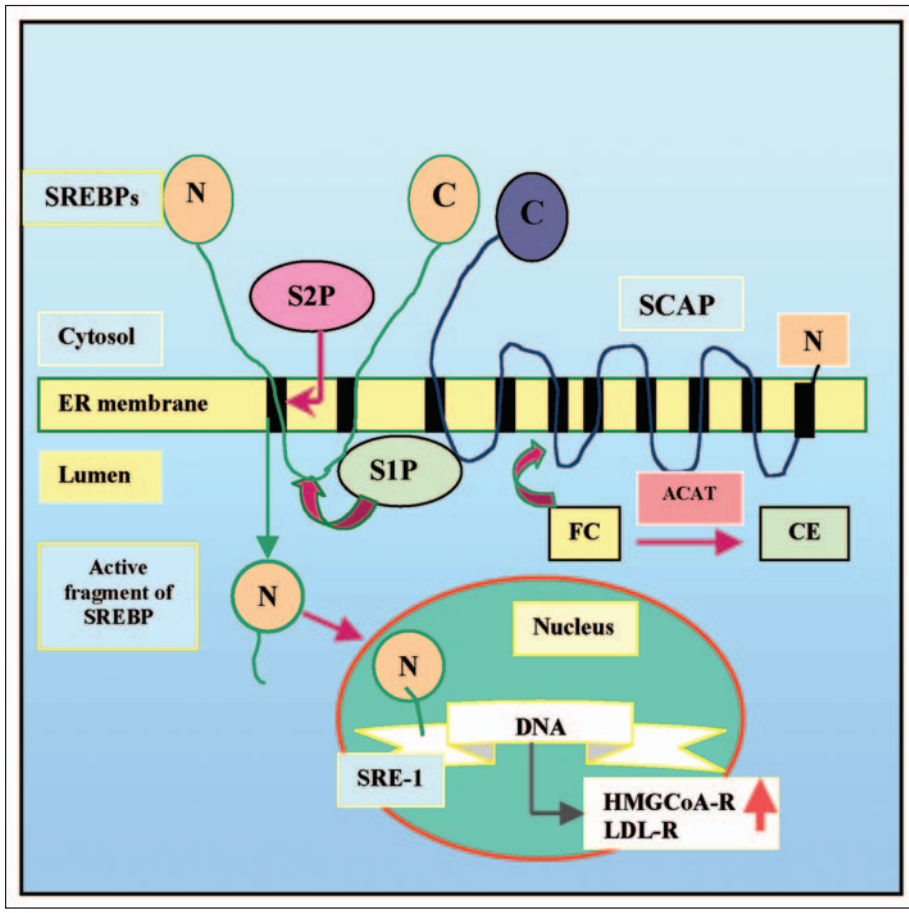

Fig. 1.3. Sequential proteolytic cleavage of SREBPs.

- The molecular basis of coordinate cholesterol regulation has been unraveled by the finding, made in the laboratory of Brown and Goldstein[26,27], that sterol regulatory element binding proteins (SREBPs) serve as sterol-specific transcription factors.
- The precursor forms of SREBPs exist as integral membrane proteins in the endoplasmic reticulum (ER); a protein called SCAP interacts with the SREBP in the ER. The N-terminal segment of the SREBP precursor contains the domain necessary for recognizing the SRE in order to activate transcription; this segment is held from entering the nucleus by an anchor segment formed by two transmembrane sequences and a short loop segment in the ER lumen.
- When cells are deprived of cholesterol, the SREBP precursor undergoes two sequential proteolytic cleavages. The first cleavage occurs within the lumen of ER, producing an intermediate that contains the SRE-recognition segment but remains attached to the ER thorough the first transmembrane domain. This cleavage, by the protease designated as Site-1 protease (S1P), is sensitive to cholesterol. SCAP is believed to be the sterol sensitive component involved in the S1P cleavage step. A second cleavage occurs within the first transmembrane domain of the intermediate, by Site-2 protease (S2P), that is insensitive to sterol, causing the release of the mature SREBP from the membrane.
- After the second cleavage, the mature SREBP (active fragment) leaves the ER membrane and enters the nucleus, where it activates target genes controlling cholesterol synthesis and uptake.

Metabolic Fate of Cytoplasmic Lipid Droplets

Thus, ACAT is the enzyme responsible for the intracellular formation of cholesterol esters from cholesterol and long-chain fatty acids derived from acyl CoA and requires ATP for its synthesis. Beside its role in cholesterol detoxification, cholesterol esters are believed to be of physiological relevance also because they represent the form by which cholesterol is stored inside the cells, and thus a pool of cholesterol promptly available for cellular needs.[28] Cholesterol esters droplets, despite their quiescent appearance in electron micrographs, are metabolically active, undergoing continuous hydrolysis and re-esterification in an apparently futile cycle that wastes ATP; a process that has been termed the "cholesteryl ester cycle".[29] The enzyme responsible for the hydrolytic phase of the cholesterol ester cycle is a neutral cholesterol ester hydrolase (nCEH) located in the cytoplasm nearby the outer face of ER membrane. nCEH is distinct from lysosomal aCEH involved in LDL degradation, in that it localizes in the cytosol, it has neutral pH optima, it can be activated by cAMP and it hydrolyzes CE endogenously formed by ACAT.[30]

Until now not much information is available on the fate of free cholesterol released from hydrolysis of cholesteryl ester lipid droplets. It has been reported that if the free cholesterol generated by hydrolysis is not removed efficiently from the CE cycle it undergoes re-esterification by ACAT, without a change in cellular CE mass. Alternatively, it can move between the intracellular pool(s) and the plasma membrane where it may be re-utilized by the cells or become available for efflux via extracellular acceptor particles.

In summary it seems that, according to cellular needs, free cholesterol generated from hydrolysis of CE in droplets moves to the ER, from where it again becomes a substrate for ACAT or it is transported to the plasma membrane. In the latter compartment, cholesterol may be utilized for membrane biogenesis and turnover or it is eliminated from the cells via high density lipoprotein (HDL) efflux.

Efflux of Cholesterol

Efflux of cholesterol is the first step in "reverse cholesterol transport", a process firstly proposed by Glomset in 1968,[31] by which excess cholesterol is removed from peripheral cells and delivered to the liver for excretion from the body. For most types of cells this is the only mechanism available for removal of cellular cholesterol, being cells unable to catabolize it. It is thought that small, nascent, lipid poor HDL particles and other forms of phospholipid-rich-HDL are the referred acceptors of cellular cholesterol.

HDL Metabolism

HDL metabolism has been awarded much attention because the HDL plasma concentration negatively correlates with atherosclerosis. Growing evidence indicates that the protective effect against atherosclerosis relates to the HDL-facilitated reverse cholesterol transport of cholesterol from extrahepatic tissues to the liver.

HDLs represent a heterogeneous group of lipoprotein particles, which can be divided in subfractions, mainly HDL_2 and HDL_3, with different protein and lipid composition, continuously modulated by lipases, lipid transfer proteins and receptors.[32]

They are secreted from the liver or the small intestine as discoidal nascent particles (HDLn), mostly comprising phospholipid and apolipoprotein A-1 (APO-AI). Similar HDL particles can be also directly generated in the plasma compartment following the lipolysis of triglyceride-rich lipoproteins. HDLn promote the removal of excess free or unesterified cholesterol from cells of peripheral tissues. Cholesterol that is transferred to nascent HDL is esterified by the plasma-enzyme lecithin cholesterol acyl transferase (LCAT) to cholesteryl esters, which, by virtue of their hydrophobicity, move into the core of the HDL particle giving rise to the

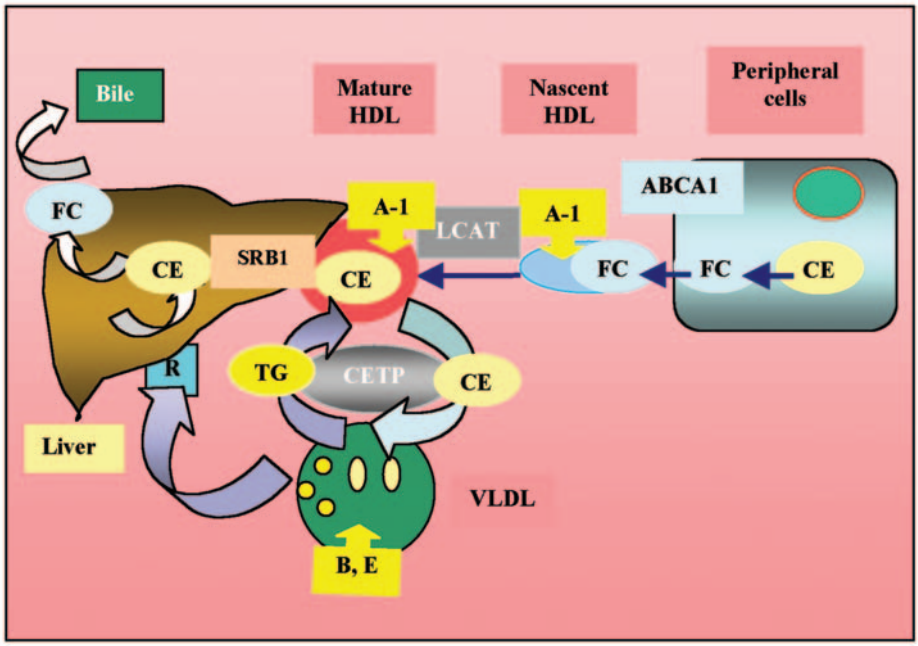

Fig. 1.4. HDL metabolism "reverse cholesterol transport"

- Reverse cholesterol transport starts when HDL and its major protein, known as apoA-1, interact with cells promoting the removal of excess free or unesterified cholesterol through a protein known as ABCA1 to the nascent HDL particles, which then undergoes some changes.
- Free cholesterol is converted to cholesterol ester by the action of the plasma enzyme lecithin/cholesterol acyltransferase (LCAT), which is activated by apolipoprotein A-1, and incorporated into the core of HDLn that are transformed into spherical mature HDL.
- Mature HDL can be taken up into the liver via an HDL receptor known as SR-B1 and then the cholesterol ester recycled and sent back out to the tissues in VLDL and any excess converted to bile salts (direct pathway),
- Alternatively, HDL can transfer, by the action of the cholesteryl ester transfer protein (CETP), the acquired cholesterol esters to VLDLs, which are further metabolized through cell-surface receptor-mediated endocytosis in the liver (indirect pathway).

spherical mature HDL able to transfer both cholesterol-ester and free cholesterol indirectly or directly to the liver from where cholesterol can be excreted from the body.

In the indirect pathway, a protein called cholesteryl ester transfer protein (CETP) transfers cholesterol from HDL to triglyceride-rich lipoproteins, such as the very-low-density lipoproteins (VLDLs), which are further metabolized through cell-surface lipoprotein receptor-mediated endocytosis. Alternatively, HDL can deliver its cholesterol directly to the liver by selective uptake of cholesterol, in which cholesterol in receptor-bound HDL is transferred from the surface of cells without internalization and disassembly of the entire HDL particle[33] (see Fig. 1.4).

HDL Receptors

Selective uptake of CE from HDL is characterized by the initial movement of CE into a reversible, plasma membrane pool and the subsequent internalization to a nonreversible, intracellular pool. In 1996, the scavenger receptor B type I (SR-BI), a member of the CD36 protein

superfamily, was reported as the first molecularly well-defined and functionally active cell-surface HDL receptor capable to mediate the selective uptake of CE.[34] The detailed mechanisms involved in SR-Bl-mediated cholesterol transfer between HDL particles and the cell surface remain mostly unknown. More recent studies have demonstrated that SR-B1 resides in caveolae, and thus that these clathrin-free cell surface invaginations, rich in FC and sphingolipids are the initial sites of SR-B1 selective cholesterol ester uptake.[35]

The identification and characterization of the HDL receptor SR-B1 raised the possibility that this receptor might also participate to cholesterol efflux.[36] Really, several studies demonstrated that overexpression of SR-B1 in cultured cells increases the rate of cholesterol efflux from the cell to HDL particles strongly supporting the concept that SR-B1 could potentially contribute to the net cholesterol efflux in some circumstances in vivo. Thus, SR-BI might ultimately facilitate both the initial step (HDL-mediated cholesterol efflux from peripheral cells) as well as the final step (hepatic uptake of HDL-CE) of the reverse cholesterol transport pathway.[37] Several lines of evidences now identify caveolae as probable intermediates in the HDL SR-B1 mediated FC efflux pathway and caveolin-1, the key structural protein of caveolae, as possible carrier of excess cholesterol from the ER to the caveolae-plasma membrane where it becomes substrate for efflux.

Overall these notions suggest a functional relationship between caveolin-1, cellular FC transport, caveolae, FC efflux and CE-HDL selective uptake and thus that caveolin-1 and caveolae might have an important role in maintaining cellular cholesterol homeostasis. In agreement with this conclusion, separated studies by Fielding and Hailstones[38,39] found that caveolin-1 is regulated by cholesterol in the same direction of ACAT activity and in the opposite direction to that of the HMG-CoA reductase, cholesterol synthetase, and the LDL, which are all up-regulated after cholesterol depletion.

As caveolin-1 is a cholesterol-binding protein, one possible function of increasing caveolin-1 expression under conditions of high cholesterol in the cell, would be to provide a "sink" for excess cholesterol in the membrane. Conversely, when the cells are depleted of cholesterol, the lower expression of caveolin-1 may result in a reduction of cholesterol efflux from the cell.

Another membrane protein that binds HDL and mediates cellular free cholesterol efflux is the ATP-binding cassette transport 1 (ABC1). A breakthrough in our understanding of this protein came from studies of Tangier disease and familiar HDL deficiency, in which the molecular defect was shown to be a series of mutations in the ABC1 gene.[40] Tangier disease is a rare recessive genetic disorder characterized by extremely low HDL levels, accumulation of cholesterol esters in macrophages and, in some cases, premature coronary heart disease. ABC1 is a 240-kDa protein belonging to a large family of conserved transmembrane proteins that transport a wide variety of substrates, including ions, drugs, peptides, and lipids, across cell membranes.[41,42] In contrast to SR-B1 that seems binds more avidly to relatively CE-rich HDL particles such as HDL_3, ABC1 is thought to actively mediated cholesterol and phospholipid efflux onto apo AI indicating that lipid-free Apo A-I is the metabolic substrate for ABC1. Like a typical ABC transporter, ABC1 might form a pore that is open to the outside of the cell. Cholesterol might be translocate from intracellular sites on ApoA1 sitting in the ABC1 pore. Recent studies indicate that cholesterol and sphingomyelin-rich membrane rafts do not provide lipid for efflux promoted by lipid free Apo AI through the ABC1-mediated lipid secretory pathway and that ABCA1 is not associated with this domain.[43]

Conclusions

In recent years, understanding of the molecular mechanisms involved in intracellular cholesterol homeostasis has radically changed to include an increasing number of structurally diverse receptors and carriers. The latest additions have led to the implication of cholesterol in fundamental cell functions such as cellular signaling and growth regulation. It is apparent that,

at least in some instances, adaptive regulation of cholesterol metabolism does not protect cells indefinitely, and eventual changes in this fine homeostatic regulation may occurs leading to pathologic consequences. Challenge of the following chapters will be to provide a useful point of references on the mechanisms that link cholesterol esters to cell growth and division. Particular attention will be dedicated to the possible implications of alterations in cholesterol esterification in two important proliferative processes such as tumors and atherosclerosis.

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Chapter 2

Role of Mevalonate Derivatives in Cell Cycle Progression

Sandra Dessì

The mevalonate pathway is a sequence of enzymatic reactions leading to farnesyl pyrophosphate (-PP), which is the common substrate for the biosynthesis of cholesterol, as well as for nonsterol isoprenoid compounds that are vital for diverse cellular function (Fig. 2.1). It is unrivaled in nature for the chemical diversity of the compounds it produces, most of which are rate limiting for cellular growth and have regulatory functions in cell proliferation. As an example, isopentenyl adenine is involved in DNA synthesis, dolichol is an important intermediate in protein glycosylation, and cholesterol is a constitutive membrane component. Ubiquinone is a membrane bound lipid participating in redox reactions necessary for ATP synthesis by membrane electron transport and an antioxidant protecting the cell membranes from oxidative damage. A novel critical role for this classic pathway emerged in recent years, due to the requirement of farnesyl-PP and geranyl-geranyl-PP in isoprenylation of various G proteins, most notable of which is the product of the Ras gene, p21, a key transducer of mitogenic signals. Mutations that constitutively activate Ras are found in about 30% of all human cancer. Finally, an emerging theme that tightly links mevalonate pathway to cell proliferation is the key role that cholesterol plays in the maintenance of microdomains in cell membranes, termed raft or caveolae, that are sites where signaling molecules involved in cell division are concentrated.

In this chapter we review the most significant discoveries in this area and discuss the relation between the end products of mevalonate pathway and cell proliferation in normal and tumor conditions.

Cholesterol and Cell Growth

Over the past 20 years, there has developed overwhelming evidence that the mevalonate pathway gives rise to end products which have important and distinct roles in the regulation of growth of all eucaryotic cells.

The first evidence of a possible relationship between cholesterol biosynthetic pathway and cell growth was represented by the finding that rapidly growing tissues such as the brains of newborn rats actively synthesize cholesterol, whereas the adult brain, which has a slow cell turnover, carries out de novo cholesterol synthesis at an extremely slow rate.[1]

This observation anticipated an explosion of subsequent studies which demonstrated that in general, an excellent correlation exists between rate of cell replication and rate of cholesterol synthesis. Thus, rapidly proliferating tissues, such as the intestine have high levels of cholesterol synthesis and 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase activity. By contrast, an organ such as the kidney, which undergoes only very slow cell turnover, is virtually

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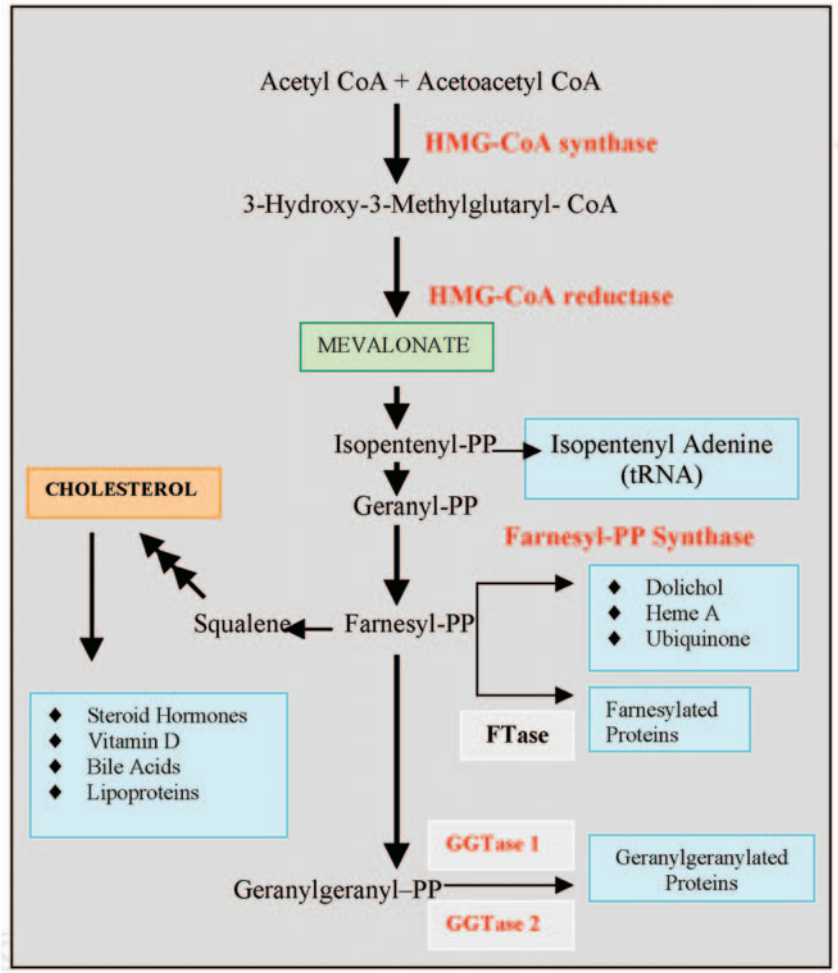

Fig. 2.1. The mevalonate pathway.

- Two moles of acetyl-CoA are condensed in a reversal thiolase reaction, forming acetoacetyl-CoA.
- One acetoacetyl-CoA, along with a third mole of acetyl-CoA are converted to HMG-CoA by the action of HMG-CoA synthase.
- HMG-CoA is converted to mevalonate by HMG-CoA reductase. The reaction catalyzed by HMGCoA reductase (which is bound to the endoplasmic reticulum) is the rate limiting step in cholesterol biosynthesis. This enzyme is subject to complex regulatory control.
- Mevalonate is then activated by three successive phosphorylations, yielding 3-phospho-5-pyrophosphomevalonate. From this, an ATP dependent decarboxylation yields isopentenyl pyrophosphate (PP) , from which generate geranyl pyrophosphate (PP).
- Then one geranyl -PP condenses with one isopentenyl-PP to form farnesyl pyrophosphate (PP).
- The NADPH-requiring enzyme, squalene synthase catalyzed the head-to-tail condensation of two molecules of farnesyl PP, yielding squalene.
- Squalene then undergoes a two-step cyclization to yield lanosterone.
- Finally, through a series of nineteen additional reaction, lanosterol is converted to cholesterol.
- Farnesyl PP is also a critical substrate for other cellular metabolites such as dolichol, haem A, ubiquinone and for protein prenylation. FTase = farnesyltransferase, GGTase 1 and 2 = geranylgeranyltransferase.

devoid of de novo cholesterol synthesis and has very little HMG-COA reductase activity. The one exception to this rule is the adult liver, which has a slow cell turnover, but actively synthesizes cholesterol. It is assumed that, as the major source of serum cholesterol, de novo cholesterogenesis by the liver is required primarily for export function.[2] Nevertheless, liver cells undergoing proliferation synthesize cholesterol at higher rate than that observed under resting condition. In this context, it has been repeatedly shown in our laboratory that different experimental models of hepatic cell proliferation are all characterized by a very rapid rate of cholesterol synthesis irrespectively of the proliferative stimuli. The increase in cholesterol synthesis was in fact observed in liver regeneration after partial hepatectomy,[3] in liver hyperplasia occurring after a single administration of a potent mitogen, lead nitrate,[4] in liver of diabetic rats given insulin as well as in that of fasted refed rats.[5] In all of these models the peak of cholesterol synthesis clearly preceded the maximum incorporation of labeled thymidine into DNA.

The same trend of cholesterol biosynthesis modification was seen in preneoplastic liver nodules[6] as well as during the growth of a rat ascites hepatoma (Yoshida AH-130).[7]

Increase of cholesterol synthesis following mitogenic stimulation "in vivo" was observed not only in liver, but also in other organs such as kidney[8] and bone marrow.[9] In addition, the level of HMGCoA-reductase activity was found proportional to the degree of cell proliferation during fetal and neonatal development, in pancreatic regeneration after tissue loss as well as in fast and slow growing pancreatic tumors.[10]

The correlation between cell proliferation, cholesterol synthesis and HMG-COA reductase has been also demonstrated in a number of tissue culture systems. Chen, Heninger, and Kandutsch[11] for the first time showed that phytohemagglutinin-stimulated mouse lymphocytes undergo a sixfold increase in labeled acetate incorporated into cholesterol 24 hr before the cells entered the S-phase of the cell cycle. Habenicht, Glomset, and Ross[12] demonstrated a similar increase in HMG-COA reductase and cholesterol synthesis in both mouse fibroblasts and in smooth muscle cells following stimulation with platelet-derived growth factor (PDGF). Interestingly, Goldstein and Brown[13] and Witte et al[14] demonstrated an increase in low density lipoprotein receptors in cells undergoing proliferation, suggesting that such cells can satisfy at least some of their cholesterol requirements by an increased uptake of cholesterol-rich LDL.

Overall, these studies demonstrated a correlation between cholesterogenesis and cell proliferation, but they did not directly address the question of a requirement for cholesterol in cell replication. The most critical test of a true requirement for cholesterol in cell growth, therefore necessitated a method of specifically inhibiting the production of mevalonate in the absence of a supply of exogenous cholesterol.

The first evidence that inhibition of cholesterol synthesis would inhibit cell growth was provided in 1974 by Chen, Kandutsch, and Waymouth[15] who demonstrated that treatment of mouse L cells with either 25-hydroxy- or 20-hydroxycholesterol, two inhibitors of HMG-COA reductase, blocked the growth and proliferation of this cells. In the same year Brown and Goldstein[16] carried out similar experiments and noted that treatment of human fibroblasts with oxygenated sterols greatly reduced cell proliferation. Subsequently, the studies of Goldstein, Helgeson, and Brown[17] and of Kaneko, Hazama-Shimada, and Endo[18] showed that treatment of cultured cells with compactin, a very potent competitive inhibitor of HMG-COA reductase, prevented proliferation of cells. This series of experiments left little doubt that cholesterol and or mevalonate serve essential functions, primarily to provide the needed structural cholesterol in cell growth and proliferation.

In addition, Hazama-Shimada, and Endo[18] made also the important observation that, even in the presence of cholesterol, the addition of mevalonate was needed to restore proliferation to compactin-treated cells, an observation that was also confirmed by Brown and Goldstein.[19] These last two studies firstly indicated that other isoprenoid products of mevalonate are required for cell growth.

Role of Mevalonate in DNA Synthesis

As early as 1979 Quesney-Huneeus et al[20] first raised the possibility that the production of mevalonic acid, in addition to serving as a precursor for providing structural cholesterol needed for cell growth, might regulate cell proliferation by playing a direct role in the initiating phase of DNA replication.

Initially, these authors examined the relationship between HMG-COA reductase, and hence mevalonate, and DNA synthesis in synchronized BHK-2 cells during the various stages of the normal cell cycle. They demonstrated that there is a consistent increase in HMG-COA reductase at or just prior to each the S-phase of cell cycle, suggesting that HMG-CoA reductase might have a function in the replication of DNA. In fact, when HMG-COA reductase activity was blocked by treatment with compactin, a concomitant block of the S-phase was observed. The addition of mevalonate to compactin-inhibited cells rapidly caused a reinitiation of DNA replication. By contrast cholesterol, although added in amounts sufficient to cause significant intracellular cholesterol accumulation, had no effect upon DNA synthesis in the compactin-treated cells. It is noteworthy that, the effect of mevalonate was specific for S-phase DNA replication, with no influence of mevalonate in either G1 or G2 phases of the cell cycle. These data provided the first evidence that, independent of its function in cholesterol synthesis, the mevalonate synthesized by HMG-COA reductase plays a critical role in initiating DNA replication. A number of other investigators, have subsequently also shown that depletion of mevalonate inhibited DNA synthesis by a mechanism that was independent of cholesterogenesis since inhibition of DNA synthesis, caused by HMGCoA-reductase inhibitors was reversed by the addition of mevalonate but not of cholesterol.[20,21]

These findings have therefore indicated that mevalonate serves at least two functions in cell growth and proliferation. First, mevalonate can, when conditions require, provide sufficient amounts of structural cholesterol for the maintenance and synthesis of the plasma and intracellular membranes needed for cell growth. Second, independently by its role as a cholesterol precursor, mevalonate serves an essential role in the initiation of DNA replication.[22]

Loss of Feedback Control of Cholesterol Synthesis in Malignant States

The finding that the synthesis of mevalonic acid has an essential role in DNA replication rose the possibility that a derangement in mevalonate synthesis might play a role in carcinogenesis. It is well known that in normal tissues a negative feedback mechanism exists in which exogenous cholesterol depresses the de novo synthesis of endogenous cholesterol by inhibiting the synthesis of mevalonic acid. A number of evidence has accumulated starting from 1964 on the loss of cholesterol feedback control in cells that undergo malignant transformation. It was, therefore, important to determine whether this defect in feedback regulation followed or preceded the malignant transformation. A number of studies have demonstrated that the cholesterol feedback lesion is also present in the precancerous state. The consistency with which the loss of the cholesterol feedback system occurs in malignant cells, coupled with the finding that this defect precedes the appearance of cancers under premalignant conditions, supports the hypothesis that deranged cholesterogenesis may be involved in the early stages of carcinogenesis (for review see ref. 23).

In summary, there is now ample evidence that cell growth, as well as DNA replication, is closely linked to cholesterogenesis. These findings, coupled with the fact that loss of the cholesterol feedback control of mevalonic acid represents a consistent biochemical defect in the malignant and premalignant states, indicate that uncontrolled synthesis of mevalonate may play a role in deranged DNA synthesis and malignant transformation.

Mevalonate and Protein Prenylation

As outlined above a series of studies from 1980 demonstrated that cells cultured in the presence of inhibitors of HMGCoA-reductase, such as mevinolin or compactin, or mutant cells lacking HMG-COA synthase or reductase, did not grow unless large amount of mevalonate was added to the cells. These findings opened new and exciting areas of investigation leading to the identification of the mevalonate derivatives implicated in the initiating phase of DNA replication.

Mevalonate products such as "ubiquinone" or "dolichol" were considered unlikely candidates because cells should have a sufficient storage pool of these metabolites to allow at least one round of cell division. Thus, the studies were focused on a regulatory molecule that have to be synthesized at a precise phase of the cell cycle to allow DNA synthesis. A clue emerged in 1984 when Schmidt and coworkers,[24] using labeled mevalonate, discovered that cultured mammalian cells incorporated this compound into covalent linkage with proteins of relative molecular mass in the ranges of 20Kd-30Kd and 50Kd-70Kd. This observation was confirmed and extended by other groups.[25,26] However, some time would have to pass before the identities of the proteins that were prenylated and the nature of the modifying group were determined.

Protein Prenylation

Protein prenylation is a post-translational lipid modification of a peptide chain. The lipid modification can be the addition of a 15-carbon farnesyl chain (farnesylation), or a 20-carbon geranylgeranyl chain (geranylgeranylation). The isoprenoid group is attached to a specific cysteine residue located at the C-terminus of certain proteins, increasing the hydrophobicity of the molecule. This enhances the affinity of protein for cell membrane and enables it to associate with other hydrophobic proteins.

The first evidence that isoprenoids were covalently attached to proteins was provided by studies on the structure of certain fungal mating factor peptides.[27,28] Shortly thereafter, prenylation was shown to occur in mammalian cells.[29,30] The first mevalonate-labeled animal protein to be identified was lamin B.[29] A feature common to both lamin B and the fungal mating factors was the presence of a so-called "CAAX-motif' at their carboxyl terminus. The discovery that the CAAX-motif could direct addition of isoprenoids to proteins containing it, lead to a re-examination of other proteins containing this motif to determine if they too were prenylated. Many of the proteins with this motif have been now identified and many participate in important regulatory functions, particular signal transduction pathways. Prenylated proteins include many small G proteins, trimeric G proteins, and some kinases that are involved in diverse signaling events. It is of interest to mention that the 21 KD membrane associated guanine nucleotide-binding proteins (P21) encoded by Ras protooncogenes, require isoprenylation to occur in order to become membrane-associated, an essential condition in the induction of signaling pathways crucial in the control of cell proliferation. Since activated oncogenic forms of Ras proteins are associated with a wide range of human cancers, the finding that farnesylation of Ras by farnesyl protein transferase is essential for Ras-induced cellular transformation, has opened up a promising new approach to the development of antitumor agents based on their ability to inhibit HMGCoA-reductase and/or farnesyl transferase proteins.[31,32]

In the following paragraphs we summarize recent findings concerning the members of the family of G proteins and the complex interrelationships that closely related these proteins to mitogenic signaling. We suggest to readers a number of recent review articles (refs. 32-35), in which most of the mechanistic aspects of the protein prenylation pathways are discussed in more details.

Family of GTP-Binding Proteins (G Proteins)

GTP-binding proteins have been intensively studied as important regulators of many physiologic process such as mitogenic signaling, membrane and protein trafficking, nuclear functions, cytoskeletal organization and apoptosis. Two major classes have been identified: the monomeric (small) G proteins, made up of the Ras superfamily, and the heterotrimeric G proteins.

Ras proteins play a central role in the integration of regulatory signals that govern processes within cell cycle and modulate cellular proliferation. They are produced as biologically inactive precursor molecules in the cytoplasm of cells and are dependent on posttranslational modifications for their activation, one of significant interest is farnesylation.

Ras Superfamily

The family of Ras proteins, encoded by three different genes, includes H-Ras, N-Ras, K-ras4A and K-ras4B (the two K-Ras proteins are made as a result of alternative splicing). Mutated versions of the three human Ras genes have been detected in 30% of all human cancers, implying an important role for aberrant Ras function in carcinogenesis. The frequency is not uniform with respect to tumor type, suggesting that Ras mutations contribute to the development of some, but not all, tumors.

The Role of Farnesylation in Ras Function

The carboxyl terminus of all Ras proteins contains two signal sequences that promote their association with the plasma membrane (Fig. 2.2). The carboxy-terminal CAAX motif (where C is a cysteine residue to which the isoprenoid is attached, A an aliphatic amino acid and X is methionine or serine) signals three post-translational modifications: farnesylation, AAX proteolysis and carboxymethylation. The "second signal", provided either by carboxy-terminal modification by the palmitate fatty acid (H-Ras, N-Ras and K-ras4A) (palmitoylation), or by a lysine-rich polybasic sequence (K-ras4B) (polybasic region), completes the localization of Ras to their specific sites in the plasma membrane.

Ras proteins that have undergone CAAX-signaled modifications alone are targeted stably to the endoplasmic reticulum, rather than remaining in the cytosol as was once believed. Palmitoylation of H-Ras and N-Ras and the polybasic region of K-ras4B may direct these proteins along different routes to the plasma membrane. Although the subcellular site where palmitoylation occurs is still not known, H-Ras and N-Ras transit the Golgi and become palmitoylated before they arrive to the plasma membrane. In contrast K-ras4B was seen to traverse more quickly from the ER to the plasma membrane that may not involve passage through Golgi membranes. The end result, however, is that all Ras proteins are localized at the plasma membrane where they convey signals into the cell.

Ras Encoded G Protein Signaling Cascade

Once in the plasma membrane, normal Ras proteins typically reside in the inactive, GDP-bound state. Upon binding at the cell surface of a variety of extracellular stimuli such as growth factors, hormones, neurotransmitters and cytokines to tyrosine kinase receptors, Ras proteins are activated to stimulate the exchange of bound GDP for GTP to form the active, GTP-complexed Ras protein. This active state is transient, and Ras GTPase activating proteins (GAP) stimulate the intrinsic GTP hydrolysis of Ras to cycle it back to the inactive GDP-complexed state. Upon activation, Ras relays the stimulus by activating a cascade of protein kinases (e.g., Raf and mitogen activated protein (MAP) kinases) that in turn initiated further cytoplasmic and nuclear events leading to cell growth and differentiation.

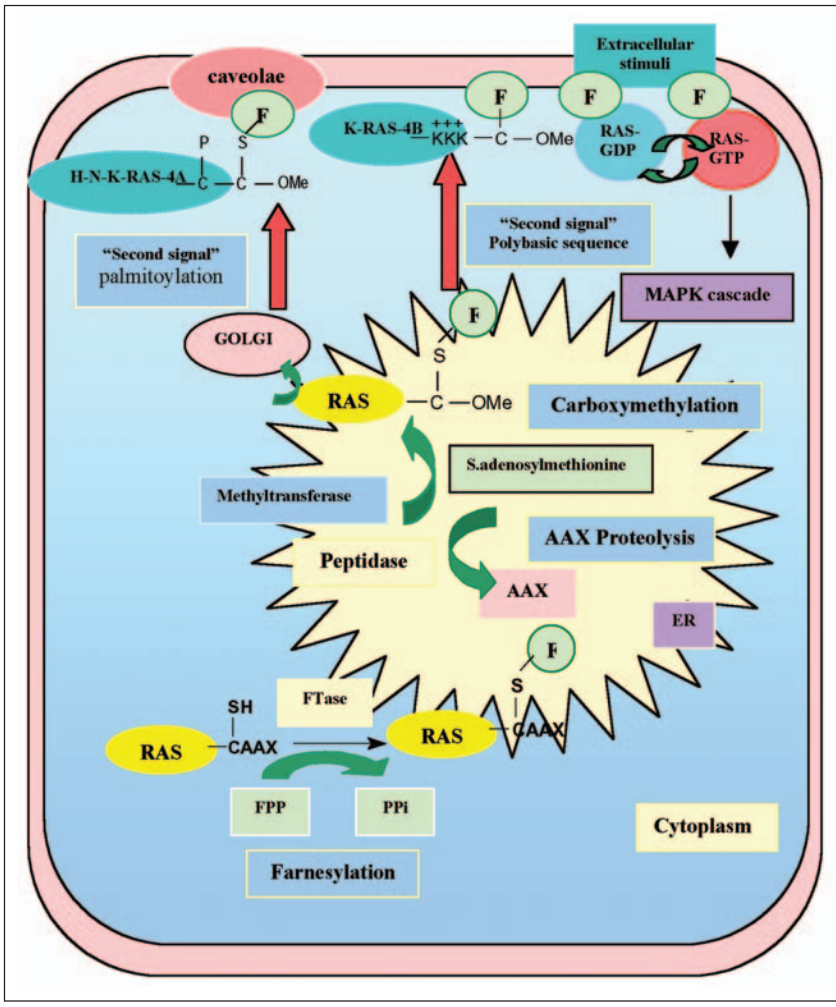

Fig. 2.2. Processing and membrane association of Ras proteins.

- Ras proteins are initially synthesized as precursor proteins and are found in the cytoplasm of cells.
- Within minutes, they undergo a series of CAAX-signaled posttranslational modifications that promote translocation and stable association with the inner surface of the plasma membrane.
- Ras first undergoes farnesylation (covalent addition of the 15-carbon farnesyl group, via a thioether linkage from the C1 carbon to the thiol of the cysteine residue of CAAX box). The cytosolic farnesyltransferase (FTase) enzyme catalyzes this reaction using farnesyl diphoshate (FPP) and the CAAX box as cosubstrates. FPTase preferentially farnesylates CAAX proteins having a terminal serine or methionine residue.
- After farnesylation, the three COOH-terminal residues (AAX) are removed by a peptidases in (ER) (AAX proteolysis) and the farnesyl-cysteine residue is methylated in a S-adenosylmethionine (SAM)–dependent reaction (carboxymethylation).
- H-Ras and N-Ras then transit the Golgi; it remains unclear if K-Ras does too.
- The CAAX-signaled modifications alone are not sufficient to traffic Ras proteins to the plasma membrane and carboxy-terminal 'second signal' present in sequences immediately upstream of the CAAX sequence are required for this.

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Fig. 2.2., *continued.*

- H-Ras, N-Ras and K-Ras4A undergo covalent addition of palmitoyl group, a 16-carbon lipid chain (palmitoylation) to cysteine residues that are positioned immediately upstream of the CAAX motif. This completes their transit to the plasma membrane and confers additional hydrophobic properties to Ras for better membrane binding. The K-Ras4B compensates for the absence of a hydrophobic palmitoyl moiety with a positive charged lysine-rich region near the CAAX box (polybasic sequence) to enhance membrane interactions with negatively charged phosholipids.
- These different targeting mechanisms may result in their localization to functionally distinct microdomains of the plasma membrane.
- The palmitate modification may target Ras proteins to the caveolin-containing, cholesterol-rich plasma membrane pits (caveolae), whereas the lysine-rich sequences of K-Ras4B may result in a more general localization with the plasma membrane.
- Once in the plasma membrane, normal Ras proteins typically reside in the inactive, GDP-bound state.
- Upon binding at the cell surface of a variety of extracellular stimuli such as growth factors, hormones, neurotransmitters and cytokines to tyrosine kinase receptors, Ras are activated to stimulate the exchange of bound GDP for GTP to form the active, GTP-complexed Ras protein.

Oncogenically mutated forms of Ras are found in many human cancers. The mutated Ras genes present in human tumors encode single amino acid mutations that render these oncogenic Ras mutant proteins insensitive to GAP stimulation. Hence, they remain constitutively active in the absence of external stimuli.

Overall these studies led to an intense area of research to identify inhibitors of Ras function as possible therapeutic agents in the treatment of cancers where Ras plays a role.

Role of Cholesterol in the Formation of Rafts and Signal Transduction

An emerging theme that further links mevalonate pathway to cell proliferation is the key role that cholesterol plays in the maintenance of microdomains in cell membranes, termed raft or caveolae. Caveolae are cholesterol and sphingolipid rich specific membrane microdomains, first discovered as clathrin-free invaginations, on surface of endothelial and epithelial cells and represent about 1-4% of the total plasma membrane surface area. Historically, caveolae were defined solely based on an invaginated morphology, however, this definition has recently been expanded to include flat membranes, as well as a subclass of vesicles that have a similar lipid composition and similar biophysical properties. The structure and function of caveolae depend on the amount of cholesterol associated with the domain. A number of recent evidences suggest a central role of intracellular cholesterol in controlling the structure and the function of caveolae (for review see ref. 36).

It has been well documented that caveolae are sites where signaling molecules involved in cell division are concentrated and thus that caveolae play a crucial part in signal transduction by assembling inactive signaling complexes ready for rapid activation in response to growth factors. The list of signaling molecules apparently localized to caveolae has increased and includes Src family kinase, nitric oxide synthases, epidermal growth factor receptor, platelet derived growth factor receptor, p21 Ras and most proteins involved in the MAPK cascade.

Caveolin 1 is the major structural protein of caveolae and it has been suggested that it directly interact with many of these signaling molecules via a conserved 20 amino-acid domain termed the caveolin scaffolding domain (CSD), residues 82-101 of caveolin-1.[37] In the majority of cases the interaction with the CSD appears to hold the signaling proteins in an inactive conformations, indicating that caveolin-1 plays a role as a negative regulator of a number of signaling molecules involved in cell growth and mitogenesis.

Caveolin-1 expression is also tightly linked to cell transformation. Caveolin is downregulated, and caveolae are reduced in number, in transformed NIH3T3 cells. Conditional expression of caveolin-1 in these cells, at levels sufficient to induce caveolae formation, abrogates the transformed phenotype. These data are consistent with caveolin operating as a direct negative regulator of signaling proteins.

However, an alternative interpretation is that the activation of signaling molecules necessitates disassembly of cholesterol in the caveolae, and thus that at least some of the effects exerted by caveolin on signal transduction are indirect being mediated through its influence on cholesterol transport.[38] It has been in fact reported that caveolin-1 is an important regulator of cholesterol trafficking. It is needed for the transport of cholesterol from the ER to the plasmamembrane[39] and increasing caveolin expression causes an increase in efflux of free cholesterol derived either from the de novo synthesis or low density lipoproteins to the plasmamembrane.[40] These results suggest that the major function of caveolin is to regulate the cholesterol content of plasma membrane surface domains, in this way regulating signal transduction. Such a model would link the postulate role of caveolae in cholesterol transport with their function in signaling. In support of this, in a study of 1998 Furuki and Anderson[41] showed that there is a direct link between caveolar domain, cholesterol levels and MAPK pathway. They used Rat-1 cells, which synthesize very little cholesterol after growing for 24-48 h in absence of serum. Cholesterol depletion to these cells caused a reduction in components of the MAPK cascade associated with a caveolar fraction. Remarkably, cholesterol depletion alone causes a reversible stimulation of MAPK suggesting that reduced cholesterol in caveolae is sufficient to activate pathway leading to cell division.

Taken together these findings raise the possibility that caveolin-1, by playing a role as a cholesterol transporter involved in maintaining caveolae cholesterol, might specifically influence raft-dependent signal transduction, and thus cell proliferation, by regulating the amount of cholesterol available for raft formation in the plasma membrane.

A simplified scheme of the role of cholesterol in the formation of rafts and signaling transduction illustrating the two pathways whereby caveolin-1 may block or activate signal transduction involved in cell growth and division is shown in Figure 2.3.

Conclusions

Researches summarized herein leave no doubt that the classic pathway from mevalonate to cholesterol is of great importance for cell growth. The better known mevalonate derivatives identified in mammalian cells include cholesterol, steroid hormones, bile acid, retinoids, heme a, ubiquinone, prenylated proteins, isopentenylated t RNAs, and long-chain isoprenoids such as dolichols, most of them are rate limiting for cellular growth and have regulatory functions in cell cycle progression.

Of particular functional interest is the role of prenylation in facilitating protein-protein interactions and membrane-associated protein trafficking. The loss of proper localization of Ras proteins when their farnesylation is inhibited has also permitted significant progress in the development of farnesyltransferase inhibitors as potential anti-Ras drugs. While there is significant evidence to support the anti-tumor action of these inhibitors, much remains to be understood about how these inhibitors may target Ras function and how effective they will be for cancer treatment.

An emerging theme that further link mevalonate pathway and cell cycle progression is the close functional relationship between cholesterol, caveolin and caveolae. While much further research is needed, the balance of evidence indicates that cholesterol and caveolin may represent important elements in the control of the composition of the plasma membrane, and the transmission of information between it and the nucleus.

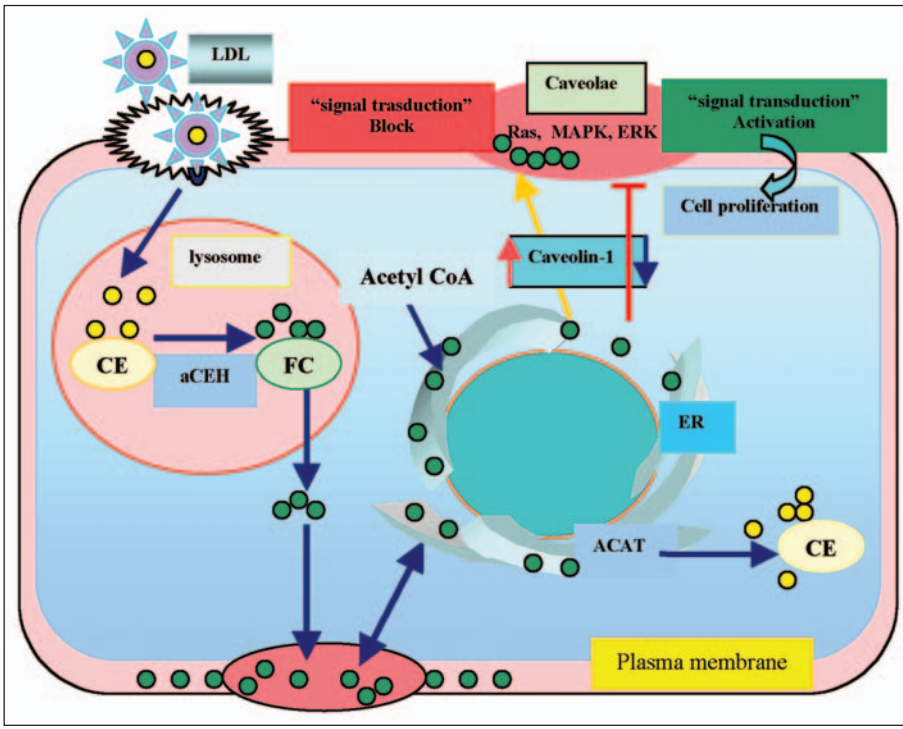

Fig. 2.3. Role of cholesterol in the formation of caveolae and signaling transduction

- Cellular cholesterol is derived either from de novo synthesis in the endoplasmic reticulum or by uptake from the extracellular environment (LDL).
- Once cholesterol is acquired, it is distributed within minutes in a highly asymmetric way among various cellular membranes.
- When plasma membrane free cholesterol exceeds a threshold level, cholesterol is shifted by a putative sensor to the ER, where it is converted into cholesterol esters (CE) by the action of ACAT or it is transported in small invaginations of plasma membrane called caveolae from where it is excreted by the cells via HDL receptors. Caveolin-1, an abundant component of caveolae, is the protein responsible for the transport of cholesterol from the ER to the caveolae and for the efflux of cholesterol from the plasma membrane.
- Caveolae are sites where signaling molecules involved in cell division are concentrated (Ras, erk, MAPK) and play a crucial part in signal transduction by assembling inactive signaling complexes ready for rapid activation in response to growth factors.
- It has been suggested that caveolin-1 might specifically influence caveolae-dependent signal transduction by regulating the amount of cholesterol available for caveolae formation in the plasma membrane.
- If caveolin-1 expression is high, much cholesterol is transported to caveolae and signal transduction is blocked. By contrast when cholesterol content is reduced in caveolae, pathways leading to cell division are activated.

In summary, this chapter emphasizes some of novel and important observations that have made in the area of lipid signaling by isoprenoid and sterols. It is an area of research that will continue to excite, challenge, and stimulate scientists for some time to come.

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Chapter 3

Cholesterol Esters and Cell Growth:

Coregulation in Animal Models

Sandra Dessì and Barbara Batetta

As outlined in Chapter 2, over the years considerable evidence has been accumulated indicating that cholesterol, and other isoprenoids produced throughout the mevalonate biosynthetic pathway, play a critical and essential role in the growth of eucaryotic cells. Modifications in cholesterol synthesis and metabolism are now well recognized as cellular events that occur in response to mitogenic stimuli.

In our laboratory, the relationship between cell growth and distribution of cellular and plasmatic cholesterol have been intensively studied "in vivo" in an attempt to understand the role of cholesterol metabolism in promoting cell growth and division. Different experimental models of normal and neoplastic cell proliferation have been used in order to provide information that might be relevant in humans. Our group has focused initially on hyperplastic and neoplastic liver models, this organ being the major producer of cholesterol in the body. Subsequently, we extended the studies to other organs such as kidney and pancreas devoid of any cholesterol synthesizing activity under normal resting conditions. This experimental approach provided opportunities to identify the presence in tissues and plasma of characteristic alterations in cholesterol ester metabolism, irrespectively to the nature of the mitogenic stimulus.

This chapter focuses on experimental evidence "in vivo" which links cholesterol esterification pathway to cell growth. Additionally, the possible biologic significance of these findings will be discussed.

Cholesterol Esters Regulation in Vivo

Before describing abnormalities in the metabolism of cholesterol esters which occur during cellular growth we briefly review physical properties of cholesterol esters and their role and distribution in the whole animal.

In mammals, cholesterol, the chief sterol found in vertebrates, exists both as a free sterol and as a component of cholesterol esters (cholesterol coupled through an ester bound to any several long chain fatty acid), which are synthesized by acyltransferase enzymes.[1-3]

Compared to free, cholesterol esters are more apolar and therefore not suited as membrane lipids. They have a tendency to form intracellular droplets and are found in the interior of lipoproteins.

In normal cells, the highest concentration of cholesterol (65-90%, depending of cell types) resides in the plasma membrane as free sterol, while only a limited amount of cholesterol esters (5-10%) are stored in cells as lipid cytosolic droplets. By contrast, in plasma only small amounts of cholesterol is free (about 20%) being the remaining incorporated in lipoprotein core as cholesterol esters.

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In vivo, an accumulation of cholesterol esters is a normal cellular process only in lipoprotein-secreting tissues, such as intestine and liver, and in steroid-hormone-secreting organs, while excess storage in other tissues can lead to certain pathologies, most notable atherosclerosis. Since cholesterol, but not cholesterol ester, is associated with membranes, the interconversion of these two forms is expected to regulate structure and function of the biological membrane.

In contrast to the multiplicity of free cholesterol sources, the synthesis by acyl-CoA: cholesterol acyltransferase (ACAT) enzymes is the only route for the formation of intracellular cholesterol esters (Fig. 3.1). They, in fact, cannot be directly obtained from extracellular lipoproteins, because cholesterol esters that are internalized in the form of lipoprotein complex are immediately hydrolyzed in the lysosomes (see Chapter 1).

By virtue of the critical role in the formation and progression of the atherosclerotic plaque, considerable knowledge concerning cholesterol ester metabolism has accumulated during the past century. However, rapid advances have occurred in the past 10 years since the cloning of ACAT.[4] This led to the subsequent identification of the ACAT family of acyltransferase, including $ACAT_1$, and $ACAT_2$.[5,6] These two enzymes are united with lecithin:cholesterol acyltransferase (LCAT) as the three known sources of cholesterol esters in animals.

$ACAT_1$ is ubiquitously expressed, whereas, $ACAT_2$, is prevalently localized in intestine and liver.[6]

Despite of these findings, however, what is the real function of the cholesterol esterification process and why there are several different enzymatic pathways for creating cholesterol esters remains, to be established.

Even though the majority of body cholesterol is synthesized by the liver and secreted as circulating lipoproteins, all cells in the body have genomic information for cholesterol biosynthesis, but they cannot degrade it. So, when cellular cholesterol is no longer required as a metabolic intermediate or for membrane stabilization, it is released from the cell or stored intracellularly in the form of cytosolic cholesterol ester droplets.

In instances in which cholesterol is in overabundance, such as in arterial macrophages during atherogenesis and in tumor progression, $ACAT_1$ may promote enough lipid storage to induce conversion of cells to lipid-laden foam cells.

The movement of cholesterol between peripheral tissues and liver, a process termed "reverse cholesterol transport" (RCT), requires another system of cholesterol-ester formation. Initially, cholesterol is transferred to high-density lipoproteins (HDL), where it is esterified by the plasma-enzyme LCAT. This enzyme is soluble in blood plasma and is mechanistically distinct from both $ACAT_1$ and $ACAT_2$ in that it uses phosphatidylcholine from HDL rather than acyl-CoA as the source of acyl chains for cholesterol esters. The cholesterol esters formed by LCAT maintain HDL particle structure by providing a source of core lipid, thereby supporting higher plasma HDL levels. The LCAT derived cholesterol ester of HDL is removed from plasma primarily by the liver through selective cholesterol-ester uptake and by whole HDL particle uptake. Thus, high-density lipoprotein-cholesterol (HDL-C) concentration is a marker for the RCT system (see also Chapter 1).

In many species, including man, the plasma cholesterol ester transfer protein (CETP) also contributes in RCT by transferring cholesterol esters from HDL to very low-density lipoprotein particles and chylomicrons. These lipoproteins are rapidly cleared in the liver by receptor-mediated endocytosis.

In liver, cholesterol esters taken into the hepatocytes are hydrolyzed to free cholesterol primarily in the lysosome. Hepatic free cholesterol can be used intracellularly to maintain membrane function or, after re-esterification by $ACAT_2$, for incorporation into cytosolic lipid storage droplets. $ACAT_2$-derived cholesterol ester can also be incorporated into hepatic ApoB containing very low density lipoprotein (VLDL), which are secreted into the plasma compart-

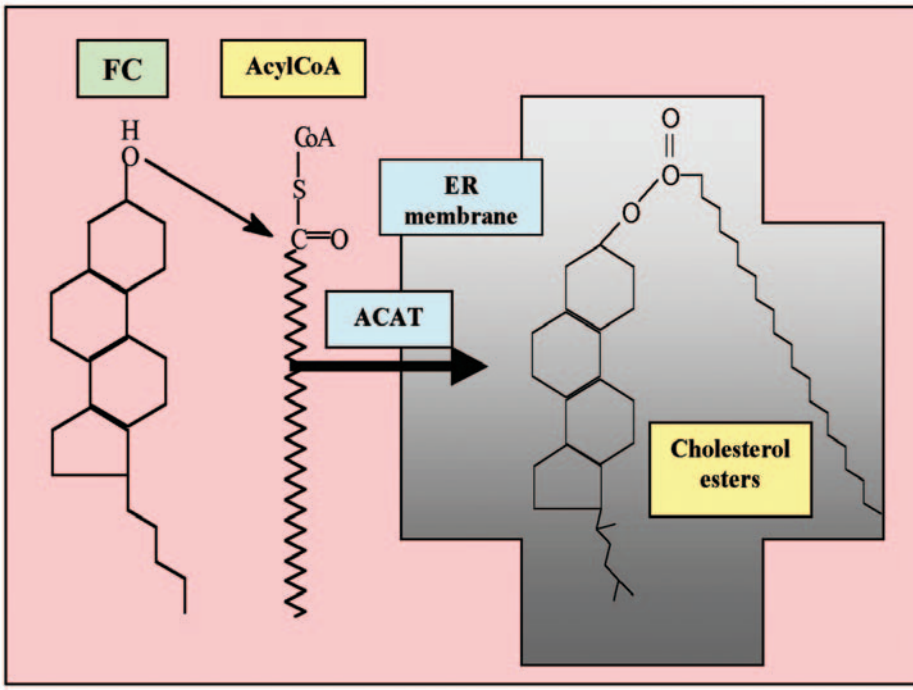

Fig. 3.1. The ACAT reaction. ACAT is located at the endoplasmic reticulum membrane, where it catalyzes a reaction in which specific sterols, such as cholesterol, are covalently joined to fatty acyl CoAs to form sterol esters.

ment. Some of the free cholesterol is also used for bile acid synthesis and for direct incorporation into bile micelles-mixtures of phospholipid, cholesterol and bile acids which are transported through the common duct into the intestine during fat digestion. The cholesterol secreted in micelles subsequently mixes in the intestinal lumen with dietary cholesterol and a portion gets absorbed into the mucosal cells.

Most of the absorbed cholesterol is esterified by $ACAT_2$ for incorporation into chylomicron particles, so that 75-80% of newly absorbed cholesterol transported into the body in chylomicrons enters as cholesterol ester. Chylomicron cholesterol esters are rapidly and selectively removed from plasma by the liver.

In summary, LCAT provides cholesterol esterification to fix cholesterol in the cores of HDL particles for reverse cholesterol transport, $ACAT_1$ provides membrane integrity in cells throughout the body, while $ACAT_2$ participates in cholesterol movement from the intestine to the liver, in regulation of cholesterol metabolism within the hepatocyte, and in cholesterol ester secretion and transport in plasma lipoproteins.[7]

A schematic view of the process of cholesterol esterification within the body is shown in Figure 3.2.

Cholesterol Esters and Cell Growth in Experimental Animals

The relationship between cholesterol metabolism and cell growth has been extensively studied in our laboratory using different experimental models of normal and neoplastic cell proliferation.

This chapter describes the experimental protocols and the models used to determine that alterations of cholesterol esterification in vivo, both in proliferating tissues and plasma, are part of the metabolic alterations occurring during cell proliferation.

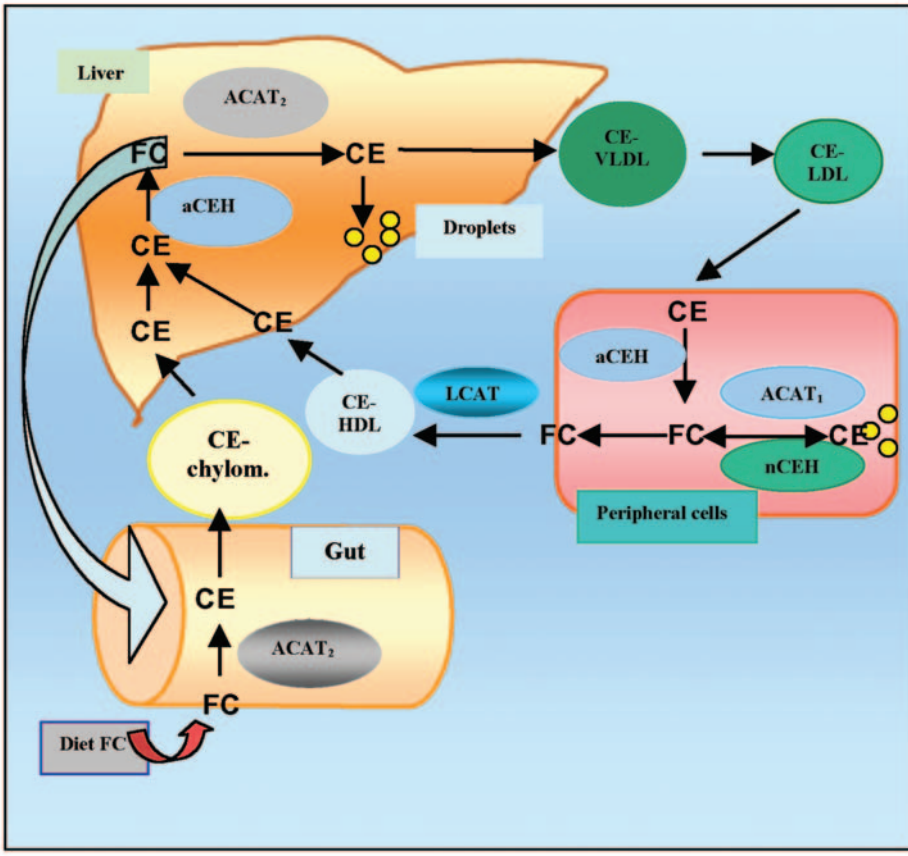

Fig. 3.2. Mechanisms of cholesterol esterification in the body.

- Dietary free cholesterol (FC) absorbed into the enterocyte is transformed into cholesterol esters (CE) by $ACAT_2$-catalyzed esterification. CE are incorporated into chylomicrons and through lymphatic fluid and plasma transported to the liver where they enter the hepatocyte through receptor-mediated uptake.
- In the hepatocyte, CE is hydrolyzed to FC by a lysosomal acid cholesterol ester hydrolase (aCEH). FC, can be used for bile acid synthesis or esterified back to CE by $ACAT_2$ for storage in lipid droplets or for incorporation into VLDL particles, which, secreted into the plasma compartment, are subsequently transformed into LDL particles.
- CE is thus transported to peripheral cells, where it is hydrolyzed by aCEH into FC. In peripheral cells, FC is utilized for membrane assembly and the excess re-esterified by $ACAT_1$ for storage in lipid droplets. Some of the CE can be hydrolyzed by a cytosolic neutral cholesterol ester hydrolase (nCEH) to FC, which can then be discharged from cells and bind to HDL particles.
- Much of the FC picked up by HDL is esterified by LCAT (in the plasma compartment) to form CE, and this CE is returned to liver for whole particle uptake and/or selective CE uptake, via SR-B1 receptors.

Cholesterol Ester Metabolism in Experimental Models Involving Normal Liver Growth Activation

The hepatocytes in normal adult rats are in a state of quiescence, or G0. They retain the capacity to proliferate, however, and re-enter the cell cycle after partial hepatectomy as well as in manipulations of the diet, administration of hormones, growth factors, toxic agents, and

other substances. This experimental approach offers a means of addressing in whole-animal experimentation in vivo, questions as to whether metabolic changes occurring during cell proliferation, follow a general trend, irrespectively to the nature of the mitogenic stimulus.

For this reasons, we examined cholesterol metabolism during the adaptation of rat liver cells to proliferation in a series of animal models of liver hyperplasia due to the following stimuli:

- Compensatory: liver regeneration after partial hepatectomy.[8]
- Chemical: lead-induced liver hyperplasia.[9]
- Hormonal: liver cell proliferation induced by insulin in streptozotocin diabetic rats.[11,12]
- Nutritional: liver cell proliferation induced by refeeding in 72-hr fasted rats.[10,11]

The first studies were conducted in rats using two different experimental models: liver regeneration after partial surgical hepatectomy and liver hyperplasia induced by a potent chemical mitogen, lead nitrate.

These two models were chosen since the first is a compensatory process subsequent to loss of parenchyma, while the latter is a process characterized by a net tissue gain with no apparent sign of liver injury. In both models the "de novo" cholesterol synthesis was measured in vivo by the incorporation of tritiated water into hepatic free and esterified cholesterol.

In liver regeneration after partial hepatectomy, no significant changes of labeling in free cholesterol and very high levels of labeled cholesterol esters were observed. The incorporation of tritiated water into free and esterified cholesterol increased after lead treatment, the percentage being higher in cholesterol esters than in free cholesterol. In these models the increase in cholesterol ester synthesis preceded the increase of DNA synthesis, or that of liver weight or the appearance of parenchymal mitoses.[8,9] The increase in cholesterol ester synthesis was followed by a massive accumulation of cholesterol esters but not of free cholesterol in both proliferating parenchyma in models. A recovery of the normal cholesterol ester metabolism was achieved together with the retrieve of hyperplasia, thus showing that the changes in cholesterol metabolism are synchronous with the different phases of cell proliferation.

Similar results were obtained when cholesterol ester metabolism was evaluated in other two models of hepatic cell proliferation such as liver hyperplasia occurring in diabetic rats given insulin and in fasted-refed rats.[10,11]

In line with these observations, studies from other laboratories showed by electron microscopy an accumulation of large droplets of lipid in the cytoplasm of the embryonic liver during the development of the chick embryo.[12] In addition, analysis of the lipid composition of livers indicated that this accumulation resulted from a dramatic increase in the cholesterol ester content of the liver during embryonic development. Significant high levels of ACAT activity were also expressed in the liver throughout the developmental period.

More recently, in a study conducted to evaluate the ontogeny of ACAT activity, Ding and Liburn[13] observed an increase in ACAT activity concomitant with a large quantity of CE in the yolk sac membrane, liver and intestine during embryonic development and early growth of turkeys, suggesting that total capacity for cholesterol esterification increases during development and shortly after hatching.

Cholesterol Ester Metabolism in Experimental Models Involving Extrahepatic Growth Activation

Like the liver, kidney, is an organ which has a very slow mitotic rate in adult rats, but it differs from the latter organ in having a very low capacity for synthesizing cholesterol. Since it has been shown that acute lead administration caused cell proliferation not only in the liver, but also in renal tubules of rats and mice, we investigated whether the association between cell proliferation and cholesterol esterification also occurred in the kidney following administration of lead nitrate. The results of this study showed that also lead-induced kidney hyperplasia is associated with an increased cholesterol ester synthesis which precedes that of DNA.[14]

A similar association between cell proliferation and cholesterol esterification has been found to occur in several other conditions of extrahepatic growth, such as in fetal and postnatal pancreas,[15] in fetal rabbit aorta,[16] in developing brain,[17] as well as during compensatory bone marrow hyperplasia induced by hemolysis in rats.[18]

The experimental evidence showing a strict relationship between cholesterol ester metabolism and cell growth activation irrespectively to the mitogenic stimulus or organ involved, led us to hypothesize a functional role for cholesterol esterification during cellular proliferation.

The Inhibition of Cholesterol Esterification Suppress the Proliferative Capacity of an Organ

Starting from the concept that if a biochemical event is essential for cell proliferation its inhibition could restrain the proliferative capacity of the organ or system involved, we evaluated cell growth in a series of experimental conditions in which cholesterol esterification was inhibited.

Cholesterol synthesis and esterification, actively operating in liver cells, are strongly depressed during fasting, we thus verified whether 72 hr of fasting was able to modify the hyperplastic response induced by different mitogenic stimuli. The results of these experiments showed that even though the liver of fasted rats maintained its capacity to proliferate following mitogenic stimulus, there was a decline of the magnitude of this response. The increase in cell proliferation, in fact, was clearly reduced as compared to mitogen-treated fed rats.[19,20] Similar findings were obtained when rats were treated with two different inhibitors of cholesterol esterification, chlorpromazine and bezafibrate.[21,22]

These data reinforced our idea that an endogenous source of cholesterol esters could be a limiting factor for cell proliferation.

Cholesterol Esterification during Tumor Growth

Aberrant cell proliferation is one of the hallmarks of cancer progression. The above finding indicating that cholesterol esterification appeared to play a part in normal cell proliferation, raised the possibility that a derangement in cholesterol ester metabolism might play a role in carcinogenesis. Consequently, we set up a series of studies using different experimental models of malignant cellular growth.

Initially, we studied the regulation of cholesterol esterification in two different pancreatic tumors: fast and slow growing tumors transplanted in nude mice.[15] Both tumors showed an increase of ACAT activity during the active tumoral growth phase, the enzymatic activity being significantly higher in fast than in slow growing tumors.

Subsequently, cholesterol metabolism was investigated in tumor cells during the growth of a highly deviated fast growing ascites hepatoma (Yoshida AH-130) in the rat. In this model the increase of cholesterol ester synthesis was associated with a progressive accumulation of cholesterol esters in growing AH-130 cells.[23]

These findings were consistent with several reports in the literature showing that cholesterol esters accumulate in tumor cells in a variety of experimental models.

Yet, the CE level reached about 300 times higher than that of controls and comprised 57.5% of the total lipid of testis of aging Fischer 344 with Leydig testis tumors.[24] An accumulation of cholesterol esters was observed in immature neuroblastoma cells,[25] in glioblastoma cells,[26] in renal carcinomas implanted into isogenic Wistar-Lewis rats[27] and in Syrian hamster[28] as well as in a slow growing hepatoma in vivo.[29]

These findings represent clear indications that excessive cholesterol ester synthesis and accumulation also occur during the growth of malignant cells.

Alterations of Cholesterol Esterification in Proliferating Tissues Are Associated with Peculiar Changes of Lipid Metabolism in the Plasma Compartment

Cholesterol metabolism in the body is regulated through a complex series of transport and biosynthetic mechanisms, which rely on the continuos exchange between tissues and blood. It is thus conceivable that any substantial alteration of cholesterol metabolism at the cellular level may entail changes in the plasmatic pool of cholesterol, and viceversa. All results reported above pointed to the fact that proliferating tissues possess some abnormalities in the intracellular metabolism of cholesterol, suggesting that these modifications could also influence the distribution of cholesterol in the plasma compartment. The performed studies clearly evidenced a strong decrease of cholesterol levels in HDL lipoprotein fraction as well as in LCAT activity in all models studied. In contrast, changes in total serum lipid concentrations or in other lipoprotein fractions did not reflect a general pattern associated with growth, being it variable and dependent on the type of hyperplastic or neoplastic growth.

In rats, a reduction of LCAT activity leading to a decrease of serum cholesterol esters and a concomitant reduction of cholesterol in high density lipoprotein (HDL-C) fraction was seen to occur during liver regeneration after partial hepatectomy,[8] in liver and kidney hyperplasia induced by lead nitrate,[30] in bone marrow hyperplasia induced by hemolysis.[18] Another observation from these studies was the apparent inverse correlation between the levels of HDL-C and the extent of cell proliferation in any of the experimental models considered. No consistent patterns of change beyond the control range were found for other lipid parameters or for other lipoprotein fractions. Low levels of HDL-C and LCAT activity were also observed under physiologic conditions of intense cellular growth as during rat fetal and neonatal development.[31]

In tumors, a decrease in LCAT activity, and a strong reduction of HDL-C were found in serum of nude mice transplanted with fast and slow growing pancreatic tumors,[15] as well as during the outgrowth of tumor in mice bearing a transplanted lymphoid tumors.[32] In addition, our studies on rats transplanted with a fast growing ascites hepatoma (Yoshida AH-130) showed that the host animals progressively developed marked changes in the level and distribution of serum cholesterol, with an increase in total cholesterol as well as a marked reduction of HDL-C. HDL-C steadily decreased over the course of tumor growth (day 4 and 7), then increased to near normal levels at day 10, a time coinciding with the stationary phase of tumor growth.[23]

These studies supported the idea that low HDL-C levels might represent a specific response to cell proliferation as a consequence of the greater utilization and storage of cholesterol esters by growing tissues. A major function attributed to HDL is to maintain normal cell cholesterol homeostasis by removing excess cholesterol from intracellular pools.[33] Since during proliferative processes cholesterol esters accumulate in proliferating tissues, it is possible that the observed decrease in HDL may reflect a reduced release of cholesterol from proliferating cells to HDL. Many in vitro studies supported these conclusions: exposure to HDL results in a net efflux of free cholesterol from various cultured cells,[34,35] this efflux being partially blocked in rapidly proliferating cells and in transformed cell lines[3.6,37]

Furthermore, Oram et al[38] demonstrated that Apo AI-HDL binds to cell surface receptors and promotes selective removal of excess cholesterol from intracellular pool. The activity of these receptors is regulated by both the availability of exogenous cholesterol and by the growth state of the cells.

Treatment of quiescent cells with serum growth factors suppresses both HDL receptor activity and HDL-mediated cholesterol efflux.[39] An opposite effect was obtained by the treatment of cultured fibroblasts with inhibitors of cell proliferation.[40]

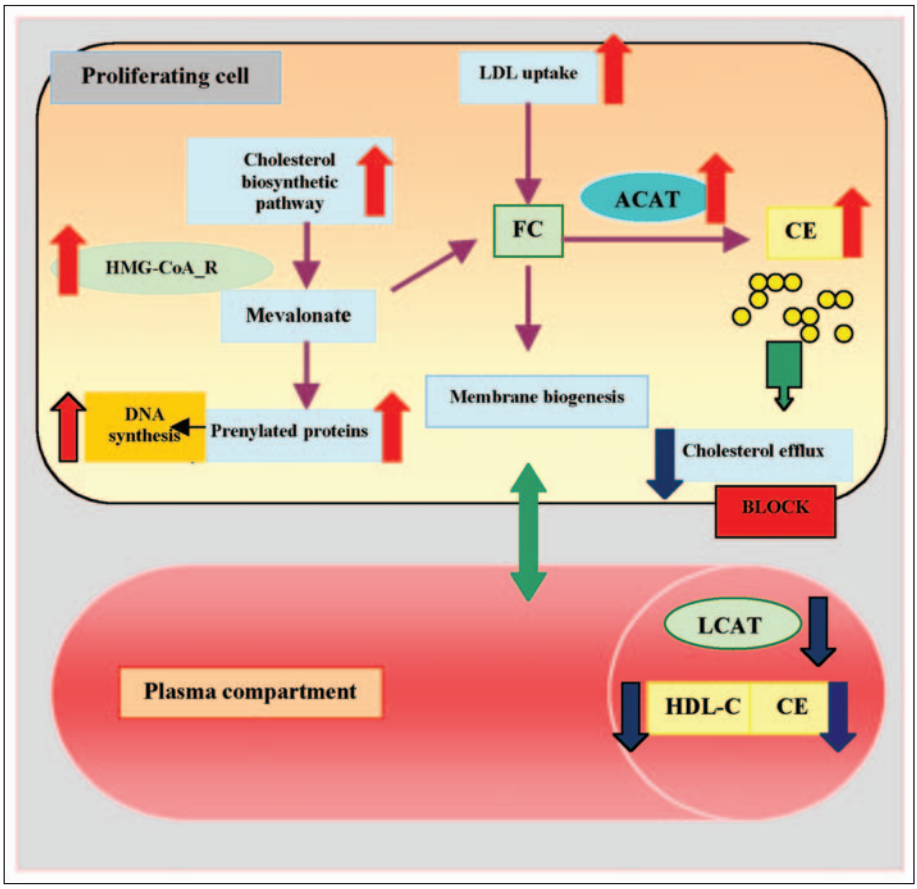

Fig. 3.3. Cholesterol metabolism during cell proliferation.

- A supply of cholesterol required by proliferating cells is achieved both endogenously (de novo synthesis) and exogenously (LDL uptake).
- The increase in cholesterol synthesis serves at least two essential functions during processes of cellular proliferation: (1) It provides free cholesterol (FC) for the biogenesis of new membrane; and (2) It promotes protein prenylation which is essential for signal transduction leading to DNA replication.
- The expansion of cellular free cholesterol pool stimulates esterification by acylCoA: cholesterol acyltransferase (ACAT) resulting in the storage of excess cholesterol in the form of cytosolic cholesterol ester (CE) droplets.
- The increased utilization, and the storage of cholesterol esters in the proliferating tissues, also results in a decreased efflux of cholesterol in the plasma compartment with consequent impairment of circulating cholesterol metabolism, as indicated by the decrease in LCAT activity and by the fall of cholesterol esters in HDL lipoprotein fraction.

In line with these data, the "in vivo" inhibition of cholesterol esters accumulation by a specific inhibitor of ACAT, strongly prevents the decrease of HDL normally found during proliferative processes,[21,41] supporting the hypothesis that HDL alterations in serum are dependent on the altered cholesterol metabolism in proliferating tissues.

The possible role of cholesterol metabolism during cell proliferation is summarized in Figure 3.3.

Conclusions

Although many questions regarding the mechanisms underlying changes in cholesterol metabolism and cell proliferation remain to be answered, the data presented in this chapter provide "in vivo" evidence of a strong relationship between HDL cholesterol levels, cholesterol esterification and rate of cell proliferation, and suggest that changes in cholesterol esterification pathway might be fundamental events in developmental growth processes.

These conclusions acquire particular significance especially in view of the possibility that cholesterol esterification could be a key player in regulating cell proliferation in human diseases like cancer and atherosclerosis, as will be discussed in the following chapters of the book.

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Chapter 4

Cholesterol Metabolism in Human Tumors

Sandra Dessì and Barbara Batetta

In humans, cholesterol metabolism, its production, transport and distribution in the body has been extensively studied mainly in relation to the atherosclerotic process. The relation ship between cholesterol and coronary heart disease has been a topic of intense research and considerable debate for a good part of the 20th century. In the last years, cholesterol metabolism has begun to being questioned also in cancer, not only from a scientific and experimental point of view but also for its medical importance in human tumor pathology. However, in spite of the growing evidence that alterations of cholesterol metabolism may be relevant in cancer patients, the possible contribution of circulating cholesterol to cancer risk in humans has been so far neglected, and alterations of lipoproteins metabolism are still commonly considered only as risk factors for atherosclerosis and cardiovascular diseases.

In this chapter a serious attempt was made to locate the most pertinent studies published in the research fields on cholesterol and human cancer and we apologize for any of the studies that have not been cited. Our aim was to verify whether lipoprotein metabolism has a biologic relevance in cancer and to provide an useful point of reference on the possible implications involving changes of cholesterol metabolism in human cancer.

Lipoprotein Metabolism and Human Cancer

As reported in Chapter 3, cholesterol metabolism has been a topic of extensive studies in our laboratory. Using different experimental models we found the presence in tumor bearers of peculiar changes in cholesterol metabolism not only in the proliferating tissues, but also in the plasma compartment. These studies raised questions regarding the possible contribution of cholesterolemia to human cancer. In a attempt to assess the relative contribution to the variability in serum cholesterol, which occurs both within and between different cancer populations, it is crucial to take into consideration the distribution of cholesterol in the different lipoprotein classes.

Thus, before describing pertinent studies on variations of serum lipid in cancer patients, we review briefly the physical properties of the various lipoprotein classes and those of their constituent apoproteins. At this stage, we will limit ourselves to discussing the distribution of plasma lipids within each of the lipoprotein classes, other features together with more details of their metabolism are reported in Chapters 1 and 3.

General Pathway of Lipoprotein Metabolism: An Overview

Most cholesterol in the body serves as a structural element in the membrane of cells, while much of the rest is in transit through the blood. The transport of cholesterol and other lipids through the circulatory system is facilitated by their packaging into lipoprotein carriers. There are at least 4 different classes of lipoproteins in the blood that are clinically important. They

Cell Growth and Cholesterol Esters, edited by Alessandra Pani and Sandra Dessì.

Table 4.1. The major lipoprotein complexes

Complex	Source and Function	Major Lipid Content
Chylomicron	Produced in the intestine. Transports dietary triglyceride from the gut to the liver, adipose tissue and muscle.	Dietary triglyceride and cholesterol
Chylomicron remnant	Chylomicrons after most of the triglyceride is removed within the capillary beds of muscle and adipose tissue by the action of lipoprotein lipase. Transports dietary cholesterol to the liver.	Dietary cholesterol
Very-low density lipoprotein (VLDL)	Produced in the liver. Transports mostly triglyceride, some cholesterol, from liver to the periphery.	Endogenous triglyceride
Intermediate-density lipoprotein (LDL)	Transient; Derived from VLDL in the capillaries of adipose tissue and muscle after the extractions of triglyceride by LPL in the capillary beds.	Endogenous cholesterol
Low-density lipoprotein (LDL)	Derived from VLDL. Transports cholesterol to peripheral tissues.	Endogenous cholesterol
High-density lipoprotein HDL_2 and HDL_3	Derived from nascent HDL as a result of the acquisition of cholesterol esters by LCAT. Involved in "reverse transport" of cholesterol from cells to the liver.	Excess cholesterol

vary in their function, densities, size and triglyceride/cholesterol ester ratios: VLDL, IDL, LDL and HDL. In addition chylomicrons and chylomicron remnants are present in the postprandial state (Table 4.1).

Lipoproteins are composed of a neutral core of cholesterol esters and triglycerides. These molecules are very hydrophobic and are coated with an outer shell of apoproteins, phospholipids and free cholesterol oriented so that their hydrophobic tails face the central core whereas their hydrophilic portions face the aqueous environment of the plasma. The lipids in the core are obtained from the diet (fat) or de novo synthesis.

The apoproteins are specific proteins attached to lipids. They have a variety of functions in aiding in the metabolism of lipids which includes:

- To provide structures for facilitation of transport out of the intestine and liver;
- To provide recognition sites for cell-surface receptors;
- To act as cofactors for enzymes involved in metabolism of lipoproteins.

Table 4.2 shows the main apoproteins, their lipoprotein association and function.

Chylomicrons are assembled in the intestinal mucosa as a means to transport dietary cholesterol and triglycerides to the rest of the body. They are transported across the intestinal mucosa to the blood where circulate only after a meal. The enzyme lipoprotein lipase (LPL) found on capillary endothelial cells in adipose tissue, skeletal muscle and the heart liberates free fatty acids which are taken up and remetabolized to triglycerides for storage. The remaining chylomicron remnant is taken up by the liver and metabolized to free fatty acids, glycerol, free

Table 4.2. Major apoproteins, lipoprotein association and function

Apoprotein–MW (Da)	Lipoprotein Association	Function
Apo-AI (29,016)	Chylomicrons, HDL	Major protein of HDL, activates lecithin:cholesterol acyltransferase, LCAT
Apo-AII (17,400)	Chylomicrons, HDL	Primarily in HDL, enhances hepatic lipase activity
Apo-AIV (46,000)	Chylomicrons and HDL	Present in triglyceride- rich lipoproteins
Apo-B48 (241,000)	Chylomicrons	Exclusively found in chylomicrons, derived from apoB-100 gene by RNA editing in intestinal epithelium; lacks the LDL receptor-binding domain of apo-B100
Apo-B100 (513,000)	VLDL, IDL and LDL	Major protein of LDL, binds to LDL receptor
Apo-CI (7,600)	Chylomicrons, VLDL, IDL and HDL	May activate LCAT
Apo-CII (8, 916)	Chylomicrons, VLDL, IDL and HDL	Activates lipoprotein lipase
Apo-CIII (8,750)	Chylomicrons, VLDL, IDL and HDL	Inhibits lipoprotein lipase
ApoE (34,000) 3 alleles: E2, E3, E4	Chylomicron remnants, VLDL, IDL and HDL	Binds to LDL receptor, Apo E_4 allele amplification is associated with increased risk of Alzheimer's disease

cholesterol and proteins. Therefore, the major fate of dietary fat is the transfer of triglycerides to the adipocytes and cholesterol to the liver. In the postabsorptive state, VLDL, produced by the liver from free fatty acids, is the major source of plasma triglycerides. Similar to chylomicrons, VLDL molecules interact with LPL to provide free fatty acids to the adipocytes and myocytes. The remaining molecule is called IDL. About 50% of the IDL is taken up by the liver. The remaining IDL is converted to LDL that functions in transferring endogenous and exogenous cholesterol to tissues. HDL is a complex macromolecule that is composed of lipids and apoproteins; apoproteins A-I is the most abundant HDL protein and is present on virtually all HDL particles. They are synthesized de novo in the liver and small intestine or directly in the blood, by free cholesterol and phospholipid released during the lipolysis of chylomicrons and VLDL, as primarily protein-rich disc-shaped particles (HDLn). These nascent HDL acquires free cholesterol from peripheral cells via a cellular transporter called ABC1. Free cholesterol is esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT). LCAT is synthesized in the liver and so named because it transfers a fatty acid from the C-2 position of lecithin to the C-3-OH of cholesterol, generating a cholesteryl ester and lysolecithin. The activity of LCAT requires interaction with apoA-I, which is found on the surface of HDL. As nascent

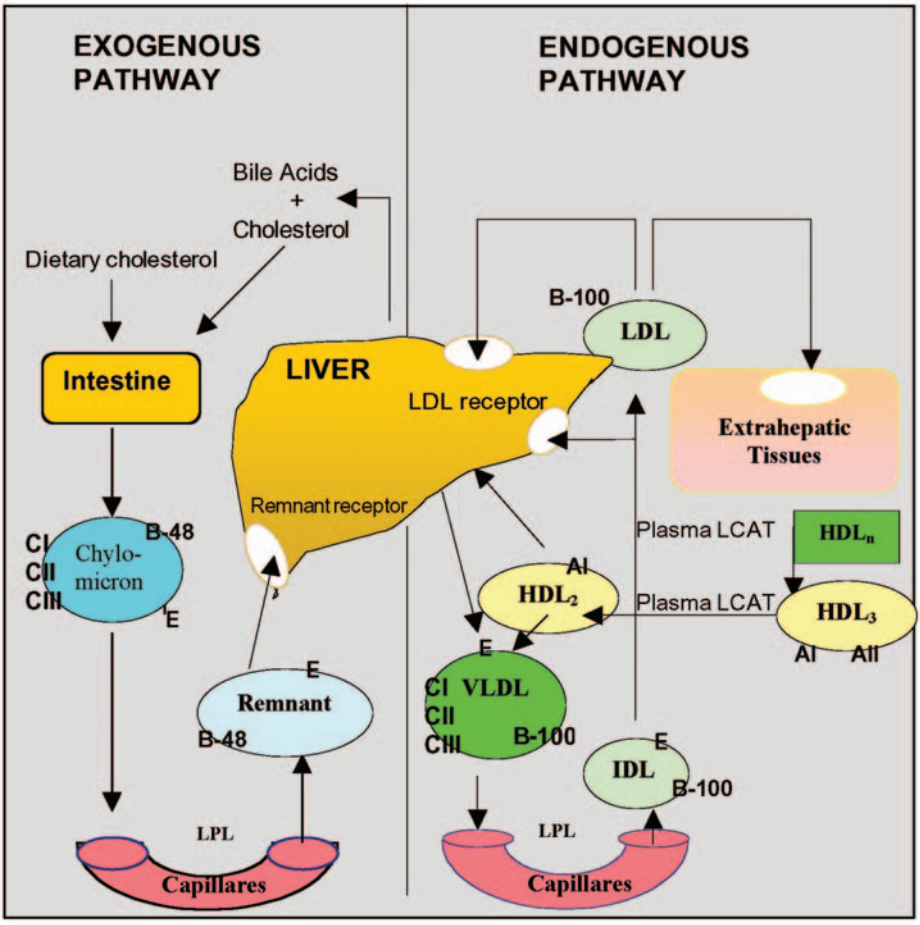

Fig. 4.1. Diagrammatic representation of tissues, sites of origin and the intravascular metabolism of the different lipoprotein classes.. A, B, C and E refer to the corresponding apoproteins. Chylomicrons transport dietary lipids via lymph into plasma and are degraded to remnant by extrahepatic lipoprotein lipase (LPL). Chylomicron remnant are then taken up by hepatic receptors which recognize the apoE on their surface. VLDL carry endogenously synthesize triglyceride from the liver into the plasma, where, like chylomicrons, they undergo partial degradation to VLDL remnant or IDL. The latter are then taken up by LDL receptor, which recognized both the apoE and the apoB100 which they contain (as distinct from the smaller apoB-48 present in chylomicrons, or are further degraded to LDL, which contains apoB100 but not apoE. LDL in turn undergoes catabolism mainly via LDL receptor. HDL has diverse origin, its lipids being also derived from free cholesterol and phospholipid released during the lipolysis of chylomicrons and VLDL as well as from free cholesterol effluxing from peripheral cells, whereas its major apoprotein, apoA-1 is synthesized both in the liver and small intestine. Nascent HDL particles initially form HDL_3 in plasma but eventually get converted to larger HDL_2 by the action of LCAT. This has the added effect of allowing the excess cellular cholesterol to be returned to the liver through the LDL-receptor pathway as well as the HDL-receptor pathway.

discoid HDL particles generate more cholesteryl ester, they evolve into spherical HDL particles (HDL_3 and HDL_2). In this manner the excess of cholesterol within the body is returned to the liver and excreted from the body (reverse cholesterol transport).

A simplified scheme illustrating lipoprotein metabolism is shown in Figure 4.1.

Table 4.3. List of diseases found in the Medline search for hypocholesterolemia and cancer

Adenocarcinoma
Adrenal adenoma
Large-bowel cancer
Colorectal adenoma
Glucagonoma
Hemoblastosis
Hodgkin's Disease
Hyperthyroidism
Acute leukemia
Acute lymphoblasticleukemia
Acute myeloid leukemia
Chronic myeloid leukemia
Chronic myclomonocytic leukemia
Chronic lymphoidleukemia
Endometrial cancer
Hairy cell leukemia
Lung cancer
Lymphoma
Lymphoproliferative diseases
Acute and chronic myceloproliferative diseases
Idiopathic myelofibrosis
Myclofibrosis
Multiple myeloma
Myeloma
Policytemia vera

Hypocholesterolemia and Human Tumors

Hypocholesterolemia was among the first abnormalities of cholesterol metabolism found in cancer patients. As early as 1974 Rose et al[1] reported that the initial levels of serum cholesterol were lower than expected in colon cancer patients. This same group did not find hypocholesterolemia in patients with other types of cancer such as stomach, pancreas, liver, bile ducts and rectum.[2]

Contradicting previous data by Stamler et al[3] in 1980, Kark et al[4] reported low cholesterol levels in lung cancer patients.

One years later, Dyer et al[5] did not find a stabilized association between serum cholesterol levels and overall risk of death from cancer. The Authors reported an inverse correlation between serum cholesterol level and cancer death for sarcoma, leukemia and Hodgkin's disease but not for lung cancer, colon-rectal cancer, cancer of oral cavity, pancreatic cancer, or all other cancer combined.

Although these first experimental evidences were contradictory and appeared to depend upon the specific tumor type analyzed, they provided support to the idea that alterations of circulating cholesterol also occur, in humans, during the course of tumoral progression.

In the past years, there have been several articles concerning the importance of serum cholesterol in cancer patients. A list of diseases found in the Medline search for hypocholesterolemia and cancer is reported in Table 4.3.

Questions about the role of cholesterol (particular blood cholesterol) in human cancer began to assume considerable scientific and public health importance by virtue of a series of epidemiological studies not specifically intending to measure cancer incidence and mortality, but designed to examine the association between cholesterol and cardiovascular diseases. Most

of these studies, showed that, at any age, low total cholesterol values were associated with a higher risk of death, particularly from cancer.[6-9]

These results produced a sure alarm in the public opinion mainly for the possibility that cholesterol-lowering drugs could increase the risk for cancer.

Since 1971 Pearce and Dayton[10] reported that lowering of serum cholesterol by a diet rich in polyunsaturated fatty acids resulted in a higher incidence of cancer death in men. In addition, an epidemiological study of 1978,[11] revealed that the beneficial effect against hypercholesterolemia of clofibrate, the most widely used lipid-lowering drug in Europe and in USA in the eighties, was counterbalanced by a probable higher risk of tumors of the gastrointestinal tract.

Additional evidence for a link between carcinogenicity and lipid-lowering drugs was provided by a review published in the prestigious Journal of the American Medical Association in 1996.[12] The authors found that all members of the two most popular classes of lipid-lowering drugs (the fibrates and the statins) cause cancer in rodents, in concentrations close to those prescribed to humans at maximum doses. Although the Authors also reported that "information are insufficient to come solid conclusions, and longer-term clinical trials and careful postmarketing surveillance are needed to determine whether cholesterol-lowering drugs cause cancer in humans"; they concluded: "the results of experiments in animals and in humans suggest that lipid-lowering drug treatment, especially with the fibrates and statins, should be avoid except in patients at high short-term risk of coronary heart disease."

This matter has serious healthy, ethical and financial implications and it is still a great object of debate. However, on the basis of the available findings at least four important point need to be better defined:

- Is hypocholesterolemia a significant risk factor for cancer or is it a secondary phenomenon of malignancy?
- How is the distribution of cholesterol in the different lipoprotein classes in cancer patient?
- Are changes in plasma cholesterol a peculiarity of cancer process or are they related overall to cellular growth?
- Is there a link between the intracellular cholesterol metabolism and circulating cholesterol during tumoral growth?

The following sections endeavor to reply to these questions.

Lipoprotein Metabolism in Hematologic Neoplasms

Since the 1980s, we began a series of clinical studies to verify whether alterations in cholesterol metabolism similar to those observed in experimental models and described in Chapter 3, were also associated with human tumors.

In 1991[13] we examined total and HDL cholesterol (HDL-C) in 66 cases of different human hematologic malignancies, characterized by a wide range in the extent of cell proliferation. In all tumoral types a significant decrease of HDL-C was observed, whereas total serum cholesterol was found to be lower only in chronic myelogenous leukemia, in acute non-lymphocytic leukemia and in Hodgkin's disease. The reduction in HDL-C levels, expressed as a percent of the total cholesterolemia, varied between 17.5% in non-Hodgkin's malignant lymphomas, and 4.4% in acute lymphoblastic leukemia, indicating an apparent inverse correlation between the levels of HDL-C and the severity of the clinical type of neoplasm.

To extend these previous findings, in 1993[14] we determined HDL-C, LDL, triglyceride, phospholipid, apoprotein AI (Apo AI) and apoprotein B-100 (Apo-B100) in 160 newly diagnosed patients (100 males and 60 females) with a variety of hematologic neoplasms. A consistent significant reduction of HDL-C and Apo-AI levels were observed in patients when compared to normal healthy population. No change in other lipid parameters were observed. When

patients were divided into four age groups (<50, 51-60, 61-70, >70 years), total cholesterol, LDL cholesterol (LDL-C), and Apo-B levels increased in both men and women patients aged 50-60 years, while a decrease was observed in patients > 61, with a trend similar to that observed in the general population. HDL-C and Apo-Al significantly decreased in male and female cancer patients within all age groups compared to aged matched values of normal subjects. However, the levels of HDL-C and Apo Al showed a trend to increase with age in men but not in women. In addition, the levels were significantly lower in men compared to women in patients aged <60 but not in those >60 years, indicating that sex hormone levels contribute to the difference in HDL observed in men vs women. These results demonstrated that patients with hematologic neoplasms are characterized by alterations of lipoprotein metabolism leading to a decrease in both HDL-C and in APO-AI, and that cancer did not influence lipid differences related to age and sex observed in the general population.

A significant decrease in the total cholesterol, HDL cholesterol and apo-A1, was also reported in 1997 by Aixala et al.[15] In this study, the lower values of the above parameters were observed in oncohematologic patients with higher lymphocyte count. More recently, Allampallam[16] obtained similar results in 108 patients with myelodysplastic syndrome (MDS). In this patients, total and HDL serum cholesterol were negatively related to biopsy cellularity and triglycerides negatively to labeling index.

These clinical studies, established the existence of a derangement on lipid metabolism, mainly of HDL, in certain types of leukemia. However, they did not directly address the question as to whether cholesterol alterations are the cause or the consequence of the disease.

In three different studies Baroni et al[17-19] investigated total serum cholesterol (TC), triglycerides, HDL-C (HDL_2 and HDL_3), LDL-C, VLDL-C, apoAl and B, in patients with newly diagnosed acute lymphocytic leukemia (ALL) before and after induction treatment. They found that the mean basal plasma levels of TC, HDL-C and its subfractions, LDL-C, and ApoA1 were significantly lower than the mean values observed in normal subjects, whereas TG and VLDL-C were significantly higher. After the induction treatment, a significant increase of HDL-C and Apo Al values was observed only in those patients that achieved a complete remission.

Similar findings were reported by us in 1994[20] in a study in which serum lipids and lipoprotein profiles were determined in 20 children affected by different types of malignancies (leukemias or lymphomas and solid tumors), both before any treatment and after remission of the disease following chemical or surgical therapy. At the time of diagnosis, children bearing tumors showed hypertriglyceridemia and reduced concentrations of HDL-C levels, the decrease being particularly prominent in patients with hematological tumors. Children bearing solid tumors displayed an increase of total cholesterol, while those with haematological cancer showed decreased phospholipid levels; LDL-C in neoplastic patients was not significantly different from control values. Clinical remission after therapy was accompanied by an increase of HDL-C compared to values observed at diagnosis. In contrast, post-treatment levels of triglycerides were higher than those observed before therapy.

Although based on a rather small number of patients, these results provided a clear support to the idea that some serum lipids, such as HDL-C and Apo Al, may be considered reliable markers of complete remission and that their variations may be a secondary phenomenon of malignancy.

One year later, Juliusson et al[21] analyzed serum lipids in 66 patients with symptomatic hairy cell leukemia (HCL) both before and repeatedly after treatment with cladribine, while more recently Halton and Coll[22] investigated the frequency and clinical significance of altered lipid profiles in children with acute lymphoblastic leukemia (ALL). In both studies, HDL cholesterol strongly decreased before chemotherapy, and returned to normal on completion of therapy.

Table 4.4. Alterations of serum lipids in oncohematologic patients

Years of Publication	TC	TG	HDL-C	LDL-C	Apo-AI	Apo-B	References
1991-1993-1995	=/↓	=/↑	↓	=	↓	=	13,20,23
1994-1996-1996	↓	↑	↓	=/↓	↓	=	17,18,19
1995	↓	↑	↓	↓	↓	-	21
1997	↓	-	↓	=	↓	=	15
1998	=	↑	↓	=	↓	=	22
2000	↓	-	↓	↓	-	-	16

Taken together these findings suggest that the decrease in HDL fraction, virtually present in all types of hematologic malignancies, represents a generalized phenomenon related to cancer, while other lipid parameters, being variable and dependent on the type of tumors, do not seem to reflect a general pattern associated with cancer. In addition they strongly support the idea that low HDL-C is not a cause but most probably reflect the metabolic consequence of cancer.

Table 4.4 summarizes the results on lipid parameters obtained in oncohematologic patients.

Lipoprotein Metabolism in Solid Tumors

The successive step was to estimate whether alterations in lipoprotein metabolism similar to those observed in oncohematologic patients were also present in patients with solid tumors.

In the following sections, we report the studies pertinent to this subject cited for type of tumors and years of publication.

Lung Tumors

In 1992[23] we determined, cholesterol distribution in tumor tissues and lipid composition in the plasma compartment in male patients affected by different histologic types of lung cancer.

An increase in cholesterol esters of about 3.5-fold was observed in lung tumor tissues when compared to the corresponding normal tissues. The alterations in intracellular cholesterol were associated to peculiar changes in cholesterol distribution in the plasma compartment. Serum HDL cholesterol levels were markedly lower in patients than in controls. No significant changes in other lipid parameters were observed in serum of these patients.

In this study all blood measurements were made preoperatively, and any difference found cannot, therefore, be attributed to the effects of surgery, anaesthesia, drug or recovery. In addition, no patient was started on chemotherapy prior to surgery. Although cigarette smoking has been reported to be a predisposing factor for lung cancer, and HDL-C levels are known to be decreased by cigarette smoking, it is unlikely that smoking is the cause of the about 30% reduction in HDL-C observed in cancer patients. Only a slight decrease in the levels of HDL-C was in fact observed in healthy smokers when compared to a non-smoker population.

In 1993 Umeki[24] investigated serum lipid concentrations including triglycerides, free fatty acids, phospholipids, LDL, total cholesterol, HDL-C, and lipoprotein fractions in 43 male patients with advanced nonresectable lung cancer and 37 age and sex-matched healthy male controls. The cancer patients as a group demonstrated significantly lower total and HDL cholesterol as compared with the healthy controls.

More recently, Siemianowicz and Coll[25] analyzed fasting serum level of HDL-C in 135 patients with newly diagnosed lung cancer and compared them to a control group of healthy men. All lung cancer patients, as well as subgroups of squamous cell and small cell lung cancer had statistically significantly lower HDL-C concentration than controls.

Gastrointestinal Tumors

Hypocholesterolemia related to risk of colorectal cancer has been evaluated in extensive surveys since the 1970s. However, studies on lipoprotein metabolism in gastrointestinal tumors are less numerous.

In 1992[26] Trichopoulou, in a case-control study probing the role of diet on the occurrence of colorectal cancer, collected sera from 100 cases and 100 controls in which they determined serum total cholesterol, HDL-C and triglycerides. The biochemical results were analyzed in conjunction with nutrient intakes and a dietary score that summarizes in a linear way the dietary contrast between high-risk (high protein, saturated fat and dietary cholesterol; low vegetable) and low-risk (low protein, saturated fat and cholesterol; high vegetable) patterns. Cases with colorectal cancer had significantly and substantially lower values of serum total cholesterol and particularly HDL-C, but these associations did not reflect dietary practices, since protein intake and, to a lesser extent, saturated fat and dietary cholesterol intake were higher among cases than among controls. In absolute terms, the dietary effect is more evident among persons with low total serum cholesterol and HDL-C than among those with high levels of these serum lipids. They concluded that a diet beneficial with respect to the risk of coronary heart disease is also likely to reduce the risk of colorectal cancer, even though low levels of serum total cholesterol and particularly HDL-C represent important independent correlates of clinically overt colorectal cancer.

In 1994,[27] cholesterol distribution in tumor tissues and lipid composition in the plasma compartment were also determined by us in patients with different types of gastrointestinal cancer, such as colon, rectum and stomach. Also in these type of tumors, malignant tissues contained increased amounts of cholesterol esters when compared with the corresponding normal tissues. Intracellular alterations of cholesterol were accompanied by specific changes of cholesterol in the plasma compartment: high-density lipoprotein (HDL) cholesterol was markedly reduced in the serum of patients with gastrointestinal cancer and the lipoprotein profiles showed a decrease in HDL_3 fraction, the main HDL subfraction in human serum. The decrease of HDL cholesterol was negatively associated with the clinical stage of the disease. No significant changes in either total or low-density lipoprotein cholesterol levels were observed in these types of tumors.

Among others, of interest it seems a study of 1993[28] of Bayerdorffer and Coll on the relation between serum lipoprotein levels and the frequency of colorectal adenomas, the benign precursors of colorectal cancer. The study included 822 of 1124 consecutive patients who underwent colonoscopy at their institution. Of the 822 patients, 194 had colorectal adenoma. Patients with colorectal adenomas had lower HDL cholesterol levels and higher LDL and VLDL cholesterol levels. This paper covers a particular importance since evidentiated the presence of low HDL-C also in benign tumors.

Breast and Gynecologic Tumors

These types of tumors may have particular interest since females in western societies have higher plasma levels of HDL-C than males. The difference in plasma lipids between the sexes is believed to contribute to differences in risk of heart disease.

An association between high HDL-C levels and the epidemiology of breast cancer risk has been found and HDL-C levels have been reported to be higher in subjects with mammo-

graphic dysplasia and a family history of breast cancer.[29] By contrast, depressed levels of HDL-C have been reported in women with clinically overt mammalian cancer.

As early as 1986, Bani et al[30] observed that plasma lipids, phospholipids, triglycerides, cholesterol and free fatty acids were all higher in blood obtained from breast cancer patients prior to surgery. HDL-C levels were significantly lower in all patients. These differences remained when the patient groups were sub-divided according to menopausal status.

In 1991, Knapp et al[31] analyzed fasting venous blood collected from 83 patients with breast cancer for triglycerides, total, HDL and LDL cholesterol. Patients with stage IV disease had significantly higher triglyceride concentrations and significantly lower concentrations of total and HDL cholesterol than did patients with less advanced disease or age-matched controls. In the same years, Kumar et al[32] compared cases of postmenopausal, untreated women with malignant and benign breast tumors, with their age, body weight, plasma lipid fractions and lipoproteins. Both benign and malignant patients had significantly decreased HDL-C levels compared with control subjects. There was a significant increase in body weight, total plasma lipids, total cholesterol, LDL-cholesterol, VLDL-cholesterol, phospholipids, triacylglycerols, and free fatty acids in malignant breast cancer.

In 1992, Araki and Yamamoto[33] evaluated serum levels of cholesterol and HDL-C in 144 patients with breast carcinoma, 64 with cervix carcinoma, 52 with benign breast diseases and 83 healthy subjects. Samples from patients with carcinoma of the breast and the cervix, were taken prior to radical surgery and at the time of sampling, none of them had received chemotherapy and/or total intravenous nutritional therapy. No significance was observed in the serum levels of cholesterol between the patients with carcinoma, benign diseases and healthy females. The serum levels of HDL-C, were lower among the patients with breast carcinoma than among the patients with cervix carcinoma and the latter among healthy females. The HDL-C levels exhibited a trend to decrease with the increase in malignancy.

In 1994, Kokoglu[34] in order to evaluate the changes in serum lipids and lipoproteins in early and advanced stages of the disease quantified serum triglycerides, total cholesterol, HDL, LDL, and VLDL cholesterol levels in Stage 1 and Stage IV breast cancer patients. When compared with data from age-matched healthy females, fasting serum triglycerides and VLDL cholesterol levels were found to be significantly increased and HDL-C significantly decreased in patients with breast cancer. Furthermore, a significant increase in triglycerides and VLDL cholesterol and a decrease in total, HDL and LDL cholesterol levels were demonstrated in patients with Stage IV disease when compare to those with Stage 1 breast cancer. No significant difference was found in total and LDL cholesterol between Stage 1 breast cancer patients and healthy controls. Another more recent study by Schreier[35] also shows an increase in triglycerides and a decrease in HDL-C, especially in HDL_2 subfraction, in patients with breast cancer.

An interesting study was conducted by Subbaiah et al in 1997.[36] These authors studied the plasma lipid composition and the activity of lecithin-cholesterol acyltransferase (LCAT), the enzyme responsible for the formation of most of EC in human plasma, in 12 women with breast cancer and 9 age-matched control women. The plasma EC concentration was found to be significantly decreased in cancer patients, whereas the FC concentration was unchanged, leading to an increased FC/EC ratio. The concentration of phosphatidylcholine, the acyl donor in the LCAT reaction, was reduced significantly, whereas all other phospholipids were unaffected. The cholesterol-esterifying activity of LCAT was significantly lower in cancer patients, whether assayed with endogenous substrates or with an exogenous substrate.

In the same year Gadomska et al[37] studied associations between levels of serum lipids, lipoproteins and ovarian cancer. The analysis demonstrated that also ovarian carcinoma is associated with a significant reduction of total cholesterol and its esters in serum, and in HDL fractions compared to controls.

Liver Cancer

Low HDL-C levels were also found in patients with primary and metastatic hepatic neoplasms as early as 1983 by Kanel and Coll.[38] and in 1992 by Ahaneku et al[39] in 15 patients with hepatocellular carcinoma.

In 1986 Hachem et al[40]compared serum apo-AI, A-II and B in hepatic metastases with other liver diseases: hepatomas and cirrhosis. Serum concentrations of lipids and apo- AI, A-II and B were determined in patients with hepatic metastases of colorectal cancer, with primary liver cancer and with cirrhosis. In all three liver diseases, the HDL fraction and apo-AI and A-II showed significantly low values, while Apo-B was only increased in hepatic metastases.

Studies Which Examined More Than One Type of Tumor Together

Many studies have been published reporting data on lipoprotein profiles in different human tumor combined.[41-43] Here we reported in detail the last one in chronological order. In 2000 Fiorenza et al[44] analyzed, total serum cholesterol, LDL-C, HDL-C and serum triglycerides in 530 patients with newly diagnosed cancer (97 with hematological malignancies, 92 with tumor of the lung, 108 of the upper digestive system, 103 of colon, 32 of breast, and 98 of the genitourinary system) and in 415 non-cancer subjects. Anthropometric (body mass index) and biochemical (serum albumin) indices of nutritional status were also determined in all subjects. Total cholesterol, LDL-C, HDL-C, serum albumin, and body mass index were significantly lower in cancer than in non cancer-subjects. The lowest values of total cholesterol, LDL-C and HDL-C were recorded in patients with hematological malignancies and the highest in patients with breast tumor. All the cancer groups, with the exception of women with breast cancer, showed significantly lower total cholesterol, LDL-C and HDL-C than age-and sex-matched non-cancer subjects. Multiple regression analysis with LDL-C, HDL-C, and triglycerides as dependent variables and sex, age, body mass index, albumin, and cancer (dummy variable) as independent variables, showed that cancer was independently associated with low levels of LDL-C and HDL-C and with high values of serum triglycerides. Total cholesterol, LDL-C, HDL-C, serum triglycerides, body mass index and serum albumin were significantly lower in patients with metastatic than in patients with non-metastatic solid tumor. The significant difference in LDL-C and serum triglycerides between patients with metastatic and non-metastatic cancer was lost when lipoprotein cholesterol and serum triglyceride levels were adjusted for nutritional variables.

Table 4.5 summarizes results related to studies on lipoprotein metabolism in solid tumors.

Serum Lipid Profiles in Non-Tumoral Human Proliferative Disease

In the light of the above information, very few doubts remain that low HDL cholesterol levels, are a common feature of both hematological and solid tumors. However, they raise the important question as to whether this abnormality is caused by the actual process of cancer or rather it is the result of the proliferative process per se.

In an attempt to answer this question, in 1992[45] we determined plasma lipid concentrations, in sera from G6PD-deficient children during hemolytic crisis induced by fava bean ingestion, at different times after admission. Reductions in total, LDL, and HDL cholesterol were found in association with the maximum of bone marrow hyperplasia. A return towards normal values occurred with regression of the hyperplastic bone marrow process. No changes in other lipid parameters were observed.

In 1991, Goldfarb et al[46] analyzed the composition of plasma lipoproteins in 67 patients with homozygous beta-thalassaemia and compared to healthy or heterozygous members of the same families and to patients with either sickle cell or iron deficiency anemia. Total, LDL and HDL cholesterol levels were low in patients with homozygous beta-thalassaemia and with sickle

Table 4.5. Alterations of serum lipids in patients with solid tumors

Years of Publication	TC	TG	HDL-C	LDL-C	Apo-AI	Apo-B	References
Lung cancer							
1992	=	=	↓	=	-	-	23
1993	↓	=	↓	=	-	-	24
2000	-	-	↓	-	-	-	25
Gastrointestinal cancer							
1992	↓	-	↓	-	-	-	27
1995	=	=	↓	=	-	-	28
Colorectal adenomas							
1993	-	↑	↓	↑	-	-	29
Breast cancer							
1986	↑	↑	↓	-	-	-	31
1991	↓	↑	↓	=	-	-	32
1991	↑	↑	↓	↑	-	-	33
1992	=	-	↓	-	-	-	34
1994	↓	↑	↓	↓	-	-	35
1999	-	↑	↓	-	-	-	36
Ovarian cancer							
1997	↓	-	↓	-		-	38
Liver cancer							
1983	-	-	↓	-	-	-	39
1986	-	-	↓	-	↓	=/↑	41
1992	↓	-	↓	-	-	-	42
All cancer							
2000	↓	↑	↓	↓	-	-	44

cell anemia. Plasma triglycerides did not differ among subjects. The low plasma and lipoprotein cholesterol was independent of age, transfusion requirements and splenectomy.

In this context, it is noteworthy to recall that low HDL-C has been also found in patients with benign tumors.

These results evidentiated the presence of alterations of lipoprotein metabolism similar of those observed in cancer patients, in humans with non-malignant proliferative processes, suggesting that low HDL-C levels may be related to cell proliferation *per se* rather than to tumoral growth in particular.

Cholesterol Metabolism in Tumoral Tissues

Since within the body the metabolic pathways of cellular and circulating cholesterol are closely linked, another important point is to establish whether a correlation exists between circulating and intracellular cholesterol metabolism in cancer patients.

A number of clinical studies assaying the intracellular cholesterol status in cancer patients support an affirmative answer.

As reported above, low levels of HDL-C in plasma were associated with an increase in cholesterol esters in tumor tissues in patients with lung as well as gastrointestinal cancer.[24,29] When cholesterol was evaluated in leukemic cells, despite the very low levels in free cholesterol content, an increase of cholesterol esters/total cholesterol ratio was observed. In these cells, we also found an increase in expression of LDL receptor, HMG-CoA-reductase and ACAT genes.[47]

Omsjo and Norum[48] (1985) observed that human endometrial cancers had 3-7-fold higher ACAT activity than normal secretory endometrium.

An increase in cholesterol ester synthesis due to an increased ACAT activity was also found by Jeng et al[49,50] in glioblastoma cells.

Kokoglu et al[51] and more recently Nygren et al[52] observed a consistent increased concentration of cholesterol esters in human brain tumors (glioma and meningioma) compared to normal brain tissue.

Finally, Gebhard et al,[52] reported in the clear cell form of renal cell carcinoma, or hypernefroma, which is the most common type of renal malignancy, that the clear appearance of tumor cells results from cellular storage of lipid and glycogen. The lipid most consistently stored in theses tumor cells is cholesterol, primarily in the ester form. Clear cell cancer tissue contained 8-fold more total cholesterol and 35-fold more esterified cholesterol than found in normal kidney. The cholesterol in clear cell tumors did not appear to be a result of excessive synthesis from acetate since HMGCoA-reductase activity was lower in cancer tissue than in normal kidney. By contrast, ACAT activity was drastically higher in tumor tissue than in normal kidney. They conclude that free cholesterol within the tumor cells, whether arising from synthesis or uptake, is preferentially channeled into storage as cholesterol ester via ACAT rather than being released from the cells to circulating HDL.

Conclusions

Although cholesterol metabolism is complex, and some of their function are still unclear, the evidence reviewed here demonstrates the extension of our animal results to humans.

The following is a list of principles which appear to be established or quite probable.

- Alterations of lipoprotein metabolism (mainly a decrease of HDL cholesterol) is present in the serum of all, or almost all, cancer patients.
- They are present in both sexes and at all age.
- They are also present in benign tumors as well as in subjects with non-malignant proliferative processes.
- In geographically and culturally diverse populations, the relation of lipids with cancer is similar.
- Lipid and lipoprotein disorders reported in cancer patients are reversible by effective treatment of the tumor
- Cholesterol esterification is dramatically overactivated in human hyperplastic tissues.

It is apparent from these data, that human proliferative processes, including neoplasms, are characterized by peculiar changes of cholesterol metabolism not only in the plasma compartment but also in the proliferating tissues. It is also apparent that alterations in serum lipoprotein profiles may be dependent on the altered cholesterol metabolism occurring in proliferating tissues. In the body, endogenous and exogenous pools of cholesterol are finely regulated and the fluxes of cholesterol are directed according to the functional demands of the cells. Because high density lipoproteins transport cholesterol from peripheral tissues to the liver, thereby acting as a scavenger to prevent excess accumulation and deposition of cholesterol in tissues, we speculate that during growth the observed decrease in HDL and the concomitant accumulation of cholesterol esters may be due to a reduction of cholesterol efflux from proliferating cells presumably to prevent loss of intracellular cholesterol which may be needed to sustain cell growth and division. In our laboratory we are currently studying the mechanism of how cholesterol esterification may interact with cell division leading to uncontrolled cell proliferation. This research can have substantial impact by contributing to the development of new strategies for the prevention of the progression of cancer process.

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Chapter 5

The Mobilization of Cholesterol Released at Sites of Tissue Injury

Robert Kisilevsky and Shui-Pang Tam

Regardless of the cause, sites of acute tissue injury and cell death accumulate large quantities of cell debris which include plasma membrane fragments,[1] rich in cholesterol.[2,3] Failure to mount a proper reactive cellular response to deal with such material, as may be seen in the central parts of large necrotic areas into which the peripheral inflammatory reaction fails to penetrate, eventually results in the release of this cholesterol and its accumulation as cholesterol crystals.[1] As part of the local reactive inflammatory events macrophages recruited to the site of injury ingest these membrane fragments for further processing and they thereby acquire a considerable cholesterol load. Thus, during acute tissue injury, and the consequent acute inflammation, a cholesterol removal mechanism focused on the macrophages is required to mobilize this cholesterol load either for excretion or re-use. In addition to the local inflammatory reaction provoked by the injured tissue there is a systemic response, part of which is directed precisely towards this cholesterol mobilization issue.

The systemic response [the acute phase response, reviewed in refs.4,5] relies, in part, upon cytokines (such as the various interleukins) released by inflammatory cells at the local site of injury. These cytokines serve as messengers alerting, and preparing, the organism as a whole to the general consequences of the local injury and inflammatory reaction (Fig. 5.1). They affect the central nervous system's control of body temperature leading to fever, prime the immune system for the receipt of, and response to, novel antigens, and induce the liver to synthesize a set of proteins (the acute phase proteins) whose concentration increases in the plasma and that have profound beneficial effects on the events occurring at the site of injury. These acute phase proteins include fibrinogen presumably to limit local bleeding at sites of injury; a series of proteinase inhibitors which limit the effects of active proteases to the site of injury by inactivating those which have been absorbed into the circulation;[6] serum amyloid P (SAP) which can complex with fragments of DNA and chromatin and accelerate their clearance;[7-10] and C-reactive protein (CRP) and serum amyloid A (SAA).

The plasma concentrations of these latter two acute phase proteins increase by 2-3 orders of magnitude within 24 h of acute tissue injury, a magnitude far greater than any of the other acute phase proteins.[11,12] Though each has been the subject of study for over 30 years their precise primary physiological functions remain to be elucidated. The present chapter will focus on SAA and its possible physiological role in mobilizing macrophages cholesterol that is ingested as a result of the phagocytosis of cell membranes at sites of tissue injury.

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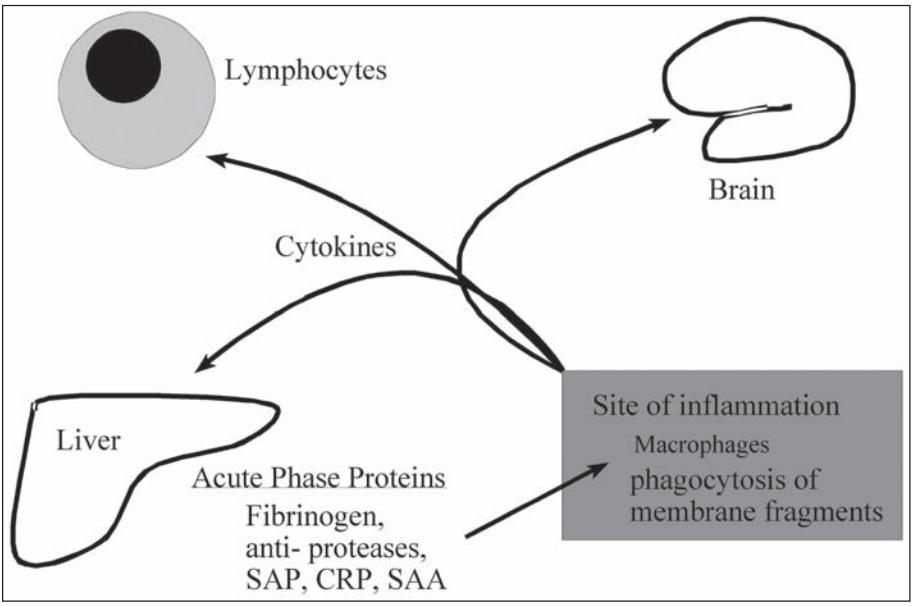

Fig. 5.1. A schematic presentation of the cytokine signaling process whereby the whole organism is alerted to the events occurring at a site of tissue injury. In addition to their effects on the brain and immune system the cytokines induce the synthesis of acute phase proteins by the liver which, in turn, exert an influence on the events taking place at the site of injury. SAP = serum amyloid P, CRP = C-reactive protein, SAA = serum amyloid A.

Serum Amyloid A (SAA)

SAA originally described the serum component that cross-reacted with antibodies to the amyloid peptide responsible for inflammation-associated amyloidosis (AA amyloidosis). Since its discovery in the mid-1970's it has become apparent that SAA is, not one, but, a family of four closely related proteins the genes for which have been conserved for well over 600 million years.[13,14] This evolutionary conservation, and SAA's physiological response to tissue injury, to be described below, indicates that SAA is involved in some important, probably beneficial, function associated with inflammation which has yet to be clarified.

Among the four SAA isoforms (1.1, 2.1, 3, and 4) 1.1 and 2.1 are the primary acute phase proteins. The details of their induction during inflammation are well understood and have been extensively reviewed.[4]. Briefly, during any inflammatory reaction, regardless of the cause of the tissue injury, inflammatory cells secrete cytokines such as IL-1, IL-6 and TNF (which in cooperation with steroids as co-stimulators, and nuclear trancription factors as downstream effectors of the cytokine response) induce the synthesis of acute phase proteins by the liver. Among these are SAA1.1 and 2.1, isoforms produced primarily by hepatocytes, and up to 2.5% of total liver protein synthesis may be devoted to their production during inflammation,[15] resulting in a dramatic increase their plasma concentration [1 μg/ml – 1 mg/ml, (a 500-1000 x increase)] within 15-20 h after an inflammatory stimulus. These isoforms are made in such large quantities presumably because they have important functions in inflammation. The precise functions are not known but most evidence indicates a role in cholesterol metabolism.[16-22] As demonstrated in vitro,[23] on emerging from hepatocytes the SAAs associate with HDL and displace apoA-I, markedly remodelling the apolipoprotein composition of HDL and forming a HDL/SAA complex.[24] This complex has a higher affinity for reticuloendothelial

cells, such as macrophages, than HDL alone,[17] probably binds to such cells through a receptor and is internalized into endosomes.[17,25,26] At pH 5 SAA separates from HDL (unpublished results) suggesting that this dissociation can occur at the surface of the cell and/or in the endosome.

What Is the Physiologic Function of Acute Phase SAA?

The primary physiologic function(s) of SAA1.1 and 2.1 is largely unknown, and the list of potential biologic roles for these acute phase forms keeps growing based primarily on in vitro studies. The main suggestions have been reviewed recently,[27] and are briefly discussed below. Included are;

1. an inhibition of antibody production as assessed in mixed lymphocyte cultures.[28] This possibility was raised and examined in the 1970's when it was thought that amyloid, regardless of where it was found, was due to a derangement of the immune system. SAA did possess inhibitory properties vis a vis antibody production in tissue culture. However, the original rationale for these studies has been largely superceded by a wealth of additional data which makes this role very unlikely;
2. chemotactic properties as assessed with T-cells, monocytes, neutrophils and mast cells.[29-32] The rationale for this possibility is the close temporal connection between inflammation and the induction of SAA. Nevertheless, physiologically, chemotaxis is dependent on changing concentrations of local molecules serving as signals to induce the local congregation of inflammatory cells. It is not clear how SAA, an abundant protein that circulates systemically, can exercise such a function. Furthermore, the chemotacic properties of SAA are not apparent when SAA is part of its natural carrier, HDL,[29] implying that SAA dissociates from HDL before SAA can exercise this function, a situation in which SAA is remarkably insoluble;
3. anti-tumor growth and migration properties as assessed in tissue culture.[33,34] Though demonstrable in tissue culture, this possibility is not consistent with the acute phase response which occurs in a host of clinical situations other than malignancies. Furthermore, varying degrees of inflammation are consistently associated with tumors and adequately explain the increases of SAA plasma concentration seen in patients carrying malignancies;
4. anti-platelet aggregation effects.[35] It is not clear why SAA would have to be an apolipoprotein of HDL to exercise such a function;
5. inhibition of oxidative bursts in neutrophils and monocytes.[36] This is an intriguing possibility and rests on the role that SAA would have in damping down an event in inflammation that may damage adjacent peripheral tissue. Assuming this activity as the primary one for SAA, there is however no clear reason why the delivery of SAA to inflammatory sites should require, or be through, HDL;
6. induction of collagenase synthesis.[37,38] Several investigators have noticed a sequence homology between domains of SAA3 and collagenase and reasoned that sites of inflammation would require stromal remodeling during the process of repair. Again, assuming this activity as the primary one for SAA, there is no clear reason why the delivery of SAA to inflammatory sites should be through HDL;
7. enhancement of non-pancreatic phospholipase A2 activity when using acute phase HDL as a substrate.[39] The increased sensitivity of phospholipid hydrolysis in the presence of acute phase HDL relative to normal HDL was attributed to SAA. A role for this process in atherogenesis was suggested. Such events in atherogenesis describe a pathological process, not a physiological one. This possibility is not consistent with the acute phase response and SAA production which occurs in a host of situation other than atherogenesis. Nevertheless, as discussed below, an influence on phospholipase A2 activity may be required in the context of phospholipid catabolism of ingested membranes;

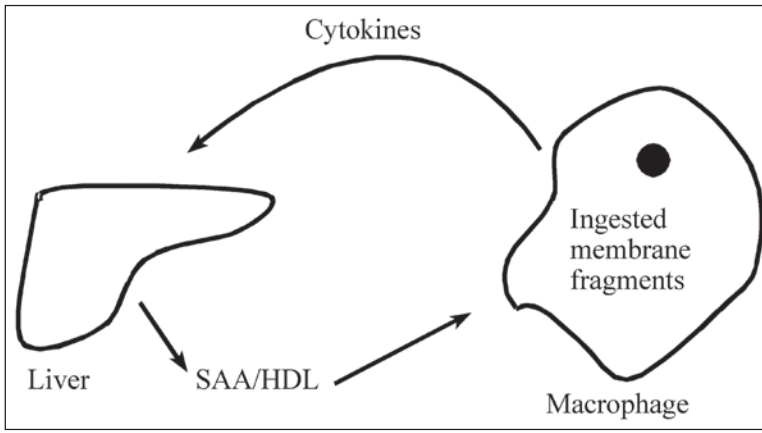

Fig. 5.2. A schematic representation of the process following macrophage activation during an inflammatory reaction and following the ingestion of cell membrane fragments. Macrophages secrete cytokines that induce the synthesis of SAA which associates with high density lipoprotein (HDL). SAA/HDL has a high affinity for activated macrophages and likely binds to them through a receptor mediated mechanism and is then endocytosed.

8. though the evidence is weak, SAA may inhibit HDL lecithin:cholesterol acyl transferase (LCAT) activity;[40] and
9. SAA may play a more direct role in cholesterol metabolism by binding to and/or influencing its mobilization (see below). With the exception of the latter two possibilities none of the aforementioned roles would imply a particular need for SAA1.1 or 2.1 to be an HDL apolipoprotein, the in vivo physiological state in which these two proteins find themselves during inflammation.

Whatever SAA's primary function, it should be consistent with several clearly established events which define the context in which SAA functions. The first is the dramatic up-regulation of isoforms 1.1 and 2.1 during inflammation, which suggests a role for SAA in this process. The second is the long history of evolutionary conservation of SAA's amino acid sequence, which suggests a beneficial role for SAA. And, the third is SAA's predominant association with HDL, suggesting that SAA may modulate HDL's established role in reverse cholesterol transport.[41,42] The working hypothesis which has guided our work is that one or other, and perhaps both, of SAA1.1 and 2.1 are involved in mediating removal of cholesterol from macrophages at sites of tissue injury and cell death.[16,20,21]

In addition to work which we will describe below the supporting evidence consistent with the working hypothesis includes:

1. a reduced ability of SAA containing HDL to accept cholesterol from LDL/VLDL [experimental work published in ref.20], ensuring that HDL, in its afferent route, arrives at macrophages with a large ability to accept cholesterol. It may be that SAA's inhibitory effect on LCAT,[40,43] plays a role at this level;
2. the higher affinity of HDL-SAA for macrophages when compared to HDL alone,[17,44] and the increased number of binding sites for HDL-SAA on macrophages obtained from animals with an acute inflammatory reaction,[17] both acting to direct HDL (the reverse cholesterol transporter) towards the cholesterol-laden macrophages, as illustrated in Figure 5.2;
3. macrophage bound radio-labelled HDL-SAA is effectively displaced by unlabelled HDL-SAA but not HDL,[17] suggesting the presence of an SAA receptor, which is also supported by several other lines of evidence;[45-47]

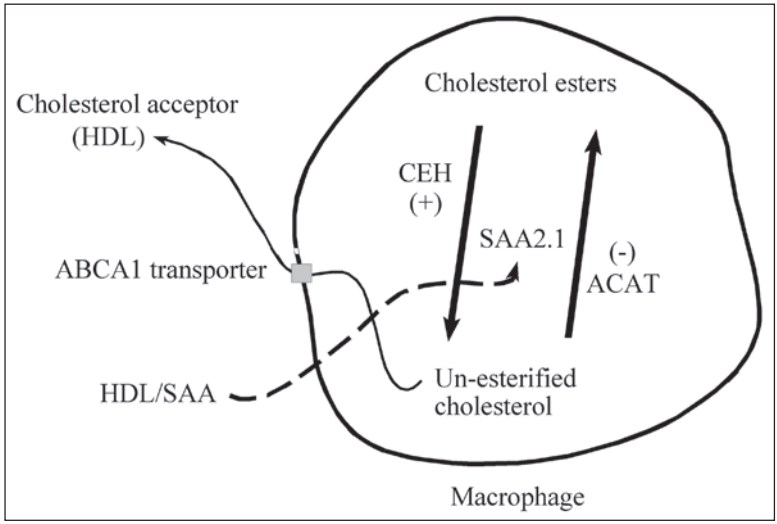

Fig. 5.3. A schematic representation of events at, and in, the macrophage following the uptake of HDL/SAA. SAA likely separates from the HDL in a low pH compartment such as the endosome. SAA2.1 exerts an inhibitory effect (-) on acyl CoA cholesterol acyl transferase (ACAT), and a stimulatory effect (+) on neutral cholesterol ester hydrolase (CEH). These effects drive cholesterol from its storage form (cholesterol esters) to its transportable form (un-esterified cholesterol) which may then be transported by the ABCA1 transporter to an extracellular acceptor, such as HDL.

4. within 5 min of incubation with macrophages HDL-SAA is associated with clathrin coated pits (consistent with the concept of receptor mediated endocytosis), a process which is dependent on cell surface heparan sulphate,[26] to which SAA binds effectively,[48] and 10 minutes later appears in endosomes;[25,26]
5. SAA enhances HDL uptake by macrophages;[44] and
6. SAA has an affinity for cholesterol.[18,19]

SAA2.1 (but not SAA1.1) inhibits macrophage acylCoA:cholesterol acyl transferase (ACAT) activity in post-nuclear homogenates, the inhibitory activity residing in the NH_2 -terminal domain.[22] Furthermore, SAA stimulates hepatic, macrophages, and purified pancreatic cholesterol esterase activity.[21] This latter effect is also due to SAA2.1, specifically its COOH-terminal region.[22] The complementary, but opposite, influences of SAA2.1 on macrophages cholesterol esterase and ACAT activities [the two cellular enzyme activities that regulate the balance between the storage form (esterified) and transportable form (un-esterified) of cholesterol] indicate that SAA2.1 would drive cholesterol primarily into the transportable form (Fig. 5.3). This is consistent with the hypothesis that this isoform plays a role in facilitating cholesterol removal from cholesterol laden macrophages at sites of tissue injury.

Nevertheless, conflicting data have been obtained when direct demonstrations of macrophage cholesterol efflux have been assessed with HDL in the presence and absence of SAA. Some studies have indicated enhanced efflux,[49-51] whereas others revealed little or no stimulatory effects.[44,46,52,53] Such differences may be a function of the cells being examined, as some studies involved peritoneal macrophages while others used fibroblasts or continually dividing macrophages cell lines. None of the studies have yet examined the effect of HDL containing SAA on cholesterol-laden macrophages, or those from inflamed individuals, the physiologic state in which these cells exist when encountering SAA.

Table 5.1. Effects of RBC cholesterol loading on macrophage ACAT activity and the subsequent effect of HDL, AP-HDL, T-AP-HDL or various protein- containing liposomes on this activity

Treatment	fmol ^{14}C-oleate/mg Cell Protein	% Inhibition Relative to RBC
None	1192 ± 131	-
RBC	6205 ± 675	-
RBC + HDL	5356 ± 903	13.7
RBC + AP-HDL	2351 ± 213	62.1
RBC + T-AP-HDL	4950 ± 674	20.2
RBC + PC	6153 ± 553	0
RBC + SAA1.1	6362 ± 398	0
RBC + SAA2.1	2578 ± 183	58.5
RBC + ApoA-I	5912 ± 432	4.7

Nearly confluent monolayers of J774 cells were incubated in the absence (none) or presence of RBC membrane fragments (RBC) and labeled with [^{14}C]-oleate. The ACAT activity was determined in cells without cholesterol loading and in cholesterol-laden cells cultured in medium plus HDL, AP-HDL, or T-AP-HDL (trypsin-treated AP-HDL), 50 μg/ml in each case, or protein-free liposomes (PC), or liposomes containing 2 μmoles of apoA-I, SAA1.1 or 2.1. Differences in cellular ACAT activity between control cells and cells incubated with RBC are significant, with $P< 0.001$. No significant difference is found when comparing RBC vs. either RBC + HDL or RBC + T-AP-HDL treatment. Differences between RBC and RBC +AP-HDL are significant, with $P<0.005$. Significant differences in cellular cholesteryl oleate radioactivity were observed when comparing any of PC, SAA1.1 or apoA-I to liposomes containing SAA2.1, with $P<0.005$. Values are the mean ±SEM of four determinations.

The Influence of SAA on Macrophage Cholesterol Metabolism during Inflammation

The Effect of Cell Membrane Loading on Macrophage ACAT Activity and the Subsequent Effect of HDL, AP-HDL, T-AP-HDL and Various Protein-Containing Liposomes on this Activity

As illustrated in Table 5.1 macrophages that ingest cell membrane material, in this case red blood cell (RBC) membranes which are 50% cholesterol by weight,[2] rapidly experience a marked increase (5 fold) in ACAT activity which drives cholesterol into its esterified form. When such cholesterol-laden macrophages are then exposed to HDL or AP-HDL the latter is approximately 5 times more effective in inhibiting this increase in ACAT activity than HDL. This inhibitory property is a function of the apolipoproteins of AP-HDL as trypsin treated AP-HDL (T-AP-HDL) loses the bulk of this inhibitory activity. Furthermore, among a variety of apolipoprotein-containing liposomes only SAA2.1-containing vesicles exhibit the ACAT inhibitory properties of AP-HDL suggesting that it is the 2.1 isoform in AP-HDL that is responsible for the ACAT inhibitory property of AP-HDL. Liposomes containing apo A-I, SAA1.1, or protein-free vesicles fail to inhibit macrophage ACAT activity, indicating clearly that the inhibitory effect is not a function of the lipid vesicle itself. The effect of AP-HDL on cell membrane laden macrophages is thus to inhibit the conversion of the ingested cholesterol into the esterified (i.e., storage) form.

Table 5.2. Rate of ^{14}cholesterol-ester hydrolysis in cholesterol-loaded macrophages exposed to HDL, AP-HDL, T-AP-HDL or various liposomes

Treatment	Cholesterol Ester Hydrolase (CEH) Activity fmol ^{14}C-oleate Cleaved /mg Cell Protein /h	Increase in CEH Activity Relative to No Treatment
None	99.6 ± 4.9	1.00
HDL	122.9 ± 6.3	1.23
T-AP-HDL	166.1 ± 8.6	1.67
AP-HDL	335.4 ± 18.1	3.37
PC	112.5 ± 5.6	1.13
SAA1.1	134.8 ± 6.9	1.35
SAA2.1	374.3 ± 19.4	3.76
Apo A-I	131.8 ± 6.6	1.32

Nearly confluent J774 cells were cholesterol loaded with RBC membrane fragments and labeled with [^{14}C]-oleate. The cells were then incubated for up to 24 h in the presence of 2 μg/ml of the ACAT inhibitor Sandoz 58-035 with 2 ml of DMEM containing 5% LPDS medium (no treatment), 50 μg/ml of HDL, 50 μg/ml T- AP-HDL, or 50 μg/ml AP-HDL; or 2ml of DMEM/LPDS supplemented with protein-free liposomes (PC), liposomes containing 2 μmoles of either apoA-I, SAA1.1 or SAA2.1. At 0, 2, 4, 8, 16, and 24 h time point, cellular lipids were extracted and analyzed for cholesteryl ester radioactivity. The rates of hydrolysis of cholesterol oleate were then determined over this time period. Results are the mean ± SEM of four determinations.

The Effect of Cell Membrane Loading on Macrophage Cholesteryl Ester Hydrolase Activity and the Subsequent Effect of HDL, AP-HDL, T-AP-HDL and Various Protein-Containing Liposomes on this Activity

J774 Macrophages pre-loaded with cell membranes in the presence of radio-labeled oleate rapidly transfer the oleate into radio-labelled cholesterol (Table 5.1). Using such cells we examined the effect of native HDL, AP-HDL and T-AP-HDL on cholesterol ester hydrolase (CEH) activity. This was done in the presence of Sandoz 58-035, an ACAT inhibitor, to prevent the re-esterification of liberated cholesterol and [^{14}C]-oleate. Incubations proceeded for different times following which the residual quantities of [^{14}C]-labeled cholesteryl oleate were measured. This allowed us to determine the rate of hydrolysis of cholesteryl ester. With re-esterification blocked, there were no significant difference in the rates of hydrolysis of [^{14}C]-labeled cholesteryl oleate in cells incubated without or with 50μg/ml HDL (Table 5.2). However, an equivalent amount of AP-HDL caused an approximately 3-4 fold increase in CEH activity. Mild trypsin treatment of AP-HDL reduced by half its ability to enhance CEH activity. As in the case of AP-HDL's effect on macrophage ACAT activity, the foregoing results indicate that the enhanced cholesterol esterase activity is a function of an AP-HDL apolipoprotein and not the lipid component. To determine which apolipoprotein contained this esterase enhancing property the foregoing experiment was repeated with protein-free liposomes or with liposomes containing 2 μmoles of either apoA-I, SAA1.1 or SAA2.1. Amongst these various liposomes, only those containing SAA2.1 resulted in a 3-4 fold increase in the rate of cholesteryl ester hydrolysis (Table 5.2). The physiological effect of AP-HDL on cell membrane laden macrophages is thus not only to inhibit the conversion of the ingested cholesterol into the esterified (i.e., storage) form, but to promote the conversion of cholesterol esters into un-esterified cholesterol, the transportable form. The combined effect of AP-HDL on cell membrane laden macrophages apparently is to shift the balance of the ingested cholesterol toward the exportable

Table 5.3. Effect of various acceptors on efflux of cholesterol from cholesterol-laden macrophages

EffluxMedium	[^{3}H] Cholesterol		
	Medium	Cell Free % of total	Cell Ester
DMEM	2.9 ± 0.1	82.8 ± 0.7	14.3 ± 0.6
HDL	23.8 ± 1.2	68.7 ± 1.4	7.5 ± 0.4
AP-HDL	32.6 ± 1.7	60.5 ± 2.3	6.9 ± 0.5
PC	17.5 ± 1.1	72.4 ± 1.8	10.1± 0.4
SAA1.1	18.6 ± 1.3	71.8 ± 2.1	9.6 ± 0.6
SAA2.1	51.4 ± 3.4	43.6 ± 2.7	5.0 ± 0.3
Apo A-I	33.4 ± 2.8	59.5 ± 2.4	7.1 ± 0.6

J774 cells were laden with cholesterol using RBC membrane fragments and simultaneously incubated for 3 h with DMEM/BSA containing 0.5 mCi/ml [^{3}H]-cholesterol followed by an overnight equilibration period. Cells were then incubated in medium containing 2 μg/ml Sandoz 58–035 in DMEM; or the same medium plus 50 μg/ml of HDL, or AP-HDL; or the same medium plus liposomes containing no protein (PC), liposomes containing equimolar amounts (2 μmoles) of either SAA1.1, SAA2.1 or apoA-I and incubated for 8h at 37°C. After incubation, the medium was collected and centrifuged at 10,000 g for 10 min, and radio-labeled cholesterol in the supernatant was determined. Cellular lipids were analyzed for remaining free and esterified [^{3}H]-cholesterol. Results are expressed as percent of total (cell plus medium) radioactivity in each well. In the case of the intact lipoprotein experiments the total [^{3}H]-cholesterol was (1.5–1.7) x 10^6 dpm/mg cell protein, and in the case of the liposome experiments it was (1.8–2.2) x 10^6 dpm/mg cell protein. Values are the mean ± SEM of four determinations.

form (Fig. 5.3). Export of cholesterol from such macrophages was therefore studied both in culture and in vivo.

Cholesterol Export Studies in Tissue Culture

As demonstrated above the effects of AP-HDL and SAA2.1 liposomes on ACAT and CEH activities in cholesterol-loaded macrophages suggests that the balance between esterified and un-esterified cholesterol in such treated cells is shifted in favor of the latter, the transportable form of cholesterol. The data in Table 5.3 compare native HDL and AP-HDL for their abilities to mobilize cholesterol from RBC cholesterol-laden J774 cells that had been labeled with [^{3}H]-cholesterol. [^{3}H]-Cholesterol efflux to medium containing 0.2% BSA was of the order of 3% of total counts. Cells cultured in the presence of equivalent amounts of HDL or AP-HDL, exported 23.8 ± 1.28% and 32.6 ± 1.7% of total cellular [^{3}H]sterol to the medium, respectively. In addition, during the first 4 h of incubation with AP-HDL the rate of cholesterol efflux was 2-fold faster than with HDL treatment (data not shown). Liposomes, protein-free, or containing either 2 μmoles SAA1.1, 2.1, or apoA-I also promoted cholesterol efflux in cholesterol-loaded J774 cells. Protein-free liposomes and those containing SAA1.1 withdrew approximately 18% of the cellular cholesterol when compared to DMEM/BSA alone. Cells cultured for 24 h in the presence of apoA-I or SAA2.1 liposomes exported 33.4 ± 2.8% and 51.4 ± 3.4% of total cholesterol counts, respectively. On a molar basis, apoA-I liposomes were 1.7 times more efficient than those containing SAA1.1. However, liposomes containing SAA2.1 were 1.5 and 2.8-fold more effective than apo A-I and SAA1.1 liposomes, respectively

Cells that were purposely pre-incubated with AP-HDL, washed free of AP-HDL, and then exposed to HDL now exported radio-labelled cholesterol to the HDL acceptor at a rate

Table 5.4. Cholesterol export in vivo

Time (hours)	[^{3}H] Cholesterol Released			
	Control	Inflamed	Control + DIDS	Inflamed + DIDS
	dpm/μl plasma			
1	6.9 ± 0.3	16.4 ± 1.2	8.0 ± 0.24	14.6 ± 0.7
6	17.5 ± 1.6	29.8 ± 1.9	14.3 ± 0.5	28.2 ± 1.6
24	8.8 ± 0.3	67.4 ± 10.2	10.8 ± 0.4	21.4 ± 1.6
30	9.5 ± 0.7	70.1 ± 12.8	11.4 ± 0.6	16.4 ± 1.0
38	7.3 ± 0.2	70.0 ± 9.5	9.1 ± 0.6	15.0 ± 1.5
48	7.3 ± 0.4	67.1 ± 5.6	8.3 ± 0.5	12.6 ± 0.8
72	6.2 ± 1.0	48.1 ± 7.4	5.0 ± 0.4	9.4 ± 0.6
96	4.6 ± 0.2	37.8 ± 6.5	4.3 ± 0.4	7.0 ± 0.6

J774 cells were cholesterol-loaded and labeled with [^{3}H]-cholesterol as described in Table 3, washed extensively and detached from the culture dishes. Five x 10^6 cells in 200 μl DMEM containing 5 x 10^5 dpm were injected into control or inflamed mice through the tail vein. At the indicated times, approximately 25 μl of blood was collected from the tail vein of each animal. Blood samples were centrifuged to separate the RBC from plasma (10-15 μl) and the [^{3}H]-cholesterol determined by scintillation spectrometry. To examine whether efflux of cholesterol from J774 cells to plasma is mediated by the ABCA1 transporter pathway, the [^{3}H]- cholesterol-laden cells were incubated overnight with 400 μM DIDS, an inhibitor of ABCA1, prior to injecting into the animals. Results are the mean ± SEM of 5 animals and are representative of two independent experiments.

and in quantity similar to those having AP-HDL as the acceptor (data not shown). This suggests that pre-treatment with AP-HDL resulted in readily available free cholesterol for efflux independent of which acceptor (HDL or AP-HDL) was present.

Cholesterol Export Studies in Vivo

To examine cholesterol export from cholesterol-laden J774 cells in vivo, five million [^{3}H]-cholesterol-loaded J774 cells, containing 5 x 10^5 dpm, were injected intravenously into non-inflamed and inflamed mice. Cholesterol export was determined over a 96 h period by measuring the appearance of [^{3}H]-cholesterol in plasma as described in Tables 5.4 and 5.5. The amount of radio-labeled cholesterol released to plasma was approximately 6 fold greater in mice with an acute phase response than in control animals. The [^{3}H]-cholesterol export peaked between 24-48 h after injection of cholesterol-laden cells in inflamed mice. To determine whether this export of cholesterol from J774 cells to plasma is mediated by the ATP binding cassette (ABCA1) transport pathway, we repeated the above procedure, but, prior to injection into mice we incubated the cells overnight with 400 μM 4,4-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), an inhibitor of the anion transport activities of ABCA1, and blocker of acceptor-mediated cholesterol efflux in human fibroblasts.[54] DIDS treatment almost completely blocked the enhanced cholesterol release from J774 cells in inflamed mice.

The following experiments were then preformed to determine whether the increase in cholesterol export in vivo in inflamed mice is due to a general non-specific aspect of the inflammatory response in vivo. Cholesterol-laden macrophages, labeled with [^{3}H]-cholesterol, were pre-incubated with either medium alone, HDL, or AP-HDL for 4h, followed by extensive washing and detachment from the culture dishes. Five millions cells from each treatment were then injected into the tail vein of un-inflamed animals. As shown in Tables 5.6 and 5.7, only the cells that have been pre-treated with AP-HDL release approximately 3-fold more

Table 5.5. Integrated plasma [^{3}H]-cholesterol released from J774 cells in vivo over 0-96 h when cells were administered to either un-inflamed or inflamed mice

Treatment	dpm/ml Plasma	Relative to Control	Percent Released
Control	67,970	1.00	13.6
Inflamed	406,520	5.98	81.3
Control+DIDS	71,550	1.05	14.3
Inflamed+DIDS	125,270	1.84	25.1

Legend as to Table 5.4.

[^{3}H]-cholesterol to plasma in control mice , a result not seen with HDL pre-treatment. The radioactivity peaks at 24 h as observed in Table 5.4 where non-AP-HDL treated cells were injected into animals with an acute inflammatory process. To determine which of the apolipoproteins of AP-HDL exert this influence the foregoing experiment was repeated with protein-free liposomes or with liposomes containing 2 μmoles of either apoA-I, SAA1.1 or SAA2.1. Amongst these various liposomes, only pre-treatment of cells with liposomes containing SAA2.1 resulted in a 3-fold increase in cholesterol efflux to plasma of un-inflamed animals.

The foregoing data using intact macrophages in tissue culture and in vivo, in conjunction with those published previously using post-nuclear homogenates,[22] indicate that SAA2.1 has a significant effect on the esterification of macrophage cholesterol and the ability to mobilize this

Table 5.6. Effect of pre-incubating cholesterol-laden macrophages with HDL, AP-HDL, or various liposomes on cholesterol efflux in un-inflamed mice

	Treatment						
Time (h)	None	HDL	AP-HDL	PC	ApoA-I	SAA1.1	SAA2.1
	[^{3}H] Cholesterol Released (dpm/ml plasma)						
2	17.4 ± 2.0	15.5 ± 0.5	14.1 ± 0.6	15.1 ± 1.8	18.2 ± 1.2	14.2 ± 1.1	19.3 ± 1.2
6	28.2 ± 1.8	28.7 ± 2.8	24.5 ± 1.6	25.3 ± 1.7	29.3 ± 1.4	27.1 ± 1.2	31.2 ± 1.8
10	18.4 ± 1.9	19.8 ± 2.1	56.8 ± 7.5	22.2 ± 1.9	20.1 ± 1.8	25.5 ± 1.4	45.3 ± 3.1
20	17.5 ± 2.1	17.8 ± 1.4	58.7 ± 8.8	18.4 ± 2.1	20.3 ± 2.1	19.1 ± 1.5	57.3 ± 3.2
24	13.8 ± 1.8	12.6 ± 1.5	50.8 ± 8.1	15.5 ± 1.8	18.1 ± 1.8	16.2 ± 1.7	64.5 ± 2.8
36	10.9 ± 1.2	11.0 ± 1.0	51.6 ± 8.5	11.8 ± 1.2	14.2 ± 1.7	15.3 ± 1.6	50.8 ± 2.7
48	8.9 ± 1.0	7.8 ± 0.4	33.2 ± 8.8	10.2 ± 1.0	13.5 ± 1.6	11.4 ± 1.2	51.2 ± 1.8
72	6.8 ± 1.0	6.2 ± 0.3	39.0 ± 6.8	9.1 ± 1.0	11.2 ± 1.3	8.1 ± 1.1	40.1 ± 1.7
96	5.1 ± 0.8	5.0 ± 0.6	30.2 ± 5.3	5.2 ± 1.1	8.2 ± 1.0	6.5 ± 1.0	33.4 ± 2.1

J774 cells were cholesterol-loaded and labeled with [^{3}H]-cholesterol as described in Table 5.3. The cells were then incubated further (for 4 h) with medium alone (no treatment) or medium containing either 50 μg/ml HDL or AP-HDL, or liposomes, protein -free (PC) or containing either 2 μmoles of apoA-I, SAA1.1, or SAA2.1. Cells were then washed extensively and detached from the culture dishes. Five millions cells in 200 μl DMEM from each preparation, containing 5 x 10^5 dpm, were injected into un-inflamed mice through the tail vein. At the indicated time intervals, export of labeled cholesterol from control J774 cells, cells pre-incubated with either HDL, AP-HDL , PC, liposomes containing either apoA-I, SAA1.1 or SAA2.1 to plasma were determined as described in Table 5.4. Results are the mean ± SEM of five animals and are representative of two independent experiments.

Table 5.7. Integrated plasma [^{3}H]-cholesterol released from J774 cells in vivo over 0-96 h when cells were pretreated with HDL, AP-HDL, or various liposomes prior to being administered to un-inflamed mice

Treatment	dpm/ml Plasma	Relative to Control	Percent Released
Control	127,000	1.00	25.4
HDL	124,400	0.98	24.9
AP-HDL	358,900	2.83	71.8
PC	132,800	1.05	26.6
Apo A-I	153,100	1.21	30.6
SAA1.1	143,400	1.13	28.7
SAA2.1	393,100	3.10	78.6

Legend as to Table 6

cholesterol in the presence of an appropriate cholesterol acceptor and transporter. The results are quite consistent with the concept that this isoform of SAA plays a role in mobilizing macrophages cholesterol acquired during the ingestion of cell membrane fragments at sites of tissue injury. To date, the role of SAA1.1 remains unclear. Nevertheless, a comparison of the amino-acid sequences of the active and in-active isoforms of SAA vis a vis cholesterol mobilization provides valuable information about the structure of SAA2.1 necessary for its biological activity. The ACAT inhibitory effect resides at the amino-terminal end of SAA2.1, in the first 16 residues (see below). In this region SAA1.1 differs from 2.1 only at position 6 and 7 where IG replaces VH. These substitutions are apparently sufficient to cause the loss of the SAA ACAT inhibitory properties. The CEH enhancing effect resides in the carboxy-terminal 80 residues. There are only 7 residue differences in this stretch of 80 amino-acids. Further work is in progress to refine the CEH enhancing domain more precisely.

Sequence Comparison of First 23 Residues of SAA1.1 and 2.1

SAA1.1 GFFSFIGEAFQGAGDMWRAYTDM
SAA2.1 GFFSFVHEAFQGAGDMWRAYTDM

SAA, Atherogenesis, Unstable Angina, and Prognosis of Myocardial Infarction

Because plasma SAA levels increase whenever tissue damage with consequent inflammation has occurred, it is not surprising that SAA is elevated in patients who have suffered myocardial infarctions.[55,56] More interesting are the observations that elevated SAA plasma levels are a poor prognostic indicator in individuals with unstable angina prior to objective evidence of myocardial damage.[57,58] Recent evidence,[59] as well as long standing histological observations of atherosclerotic plaques,[60] indicate that inflammation is an ongoing process within such lesions. This process itself, if sufficiently active, can therefore be the inducing stimulus for the elevation SAA plasma levels, and may independently increase the likelihood that vascular occlusive events ensue. It is in this context that SAA serves as a prognostic factor in relation to unstable angina, for it may indicate which patients have more active atherosclerotic vascular disease. Acute phase SAA may be the innocent bystander in these events, induced by the very lesions it has been alleged to cause. This correlation allows SAA to serve as a marker of potential outcome but this does not mean that elevated plasma SAA levels play a causative role in the process as has been proposed.

The association of SAA with HDL, and the presence of SAA and its mRNA at sites of atherosclerosis,[61] have, not surprisingly, suggested that SAA may play a role in atherogenesis. Although SAA may play a beneficial role in mobilizing cholesterol at sites of acute tissue injury, a current view proposes that SAA interferes with several properties of HDL and in so doing creates a particle which is pro-atherogenic.[62] The evidence includes an apparent loss of HDL's protective effects against LDL oxidation,[63] a reduction in LCAT activity following inflammation,[40,43] and a possible reduced cholesterol carrying capacity of HDL in the presence of SAA.[20,43] With the existing clinical correlation between elevated plasma SAA and poor prognosis of patients with unstable angina,[58] these observation leave the impression that increased plasma SAA may in some way be an etiologic factor in atherosclerotic vascular pathology.

The potential role of inflammation/infection in the pathogenesis of atherosclerotic vascular disease is now well recognized.[62,64-66] However, the fact that SAA induction occurs during any acute tissue injury (vascular damage included) will necessarily result in a correlation between SAA and active atherogenesis. As argued above this does not necessarily implicate SAA in a causative manner. The evolutionary conservation of SAA and its rapid induction during acute inflammation, regardless of etiology, would argue that SAA's principal function is one related to a beneficial role in inflammation (possibly cholesterol mobilization), and not specifically atherogenesis. Its possible role in atherosclerosis, if any, should be looked on as an aberration of its natural function rather than its primary role. Furthermore, not only are there changes in vascular adhesion factors during inflammation, but, HDL itself undergoes considerable apolipoprotein compositional changes during the acute phase response. HDL acquires not only SAA but loses a significant proportion of its other apolipoproteins such as A-I, A-II, apoE, and apoC.[21,67] To conclude that changes in HDL properties and function, and the relationship of inflammation and atherosclerotic vascular disease, are due specifically to SAA is premature. Events in inflammation may well be involved both in the pathogenesis of vascular disease and independently in the induction of SAA.

Acknowledgements

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Chapter 6

Cholesterol Esters and Cell Growth in Human Lymphocytes:
Possible Implication of P-gp Modulators

Francesca Sanna, Marirosa Putzolu and Barbara Batetta

Introduction

In the cells, cholesterol is present either as free cholesterol (FC) in the membrane or as cholesterol esters (CEs) in cytoplasm. FC and CEs are in a dynamic equilibrium being tightly controlled by FC concentrations in the endoplasmic reticulum (ER). Under steady state conditions, in fact, if more cholesterol is released into the cells, its excess is promptly transported from the plasma membrane to ER. In this site, FC down regulates both new cholesterol synthesis by HMG-CoA-reductase and LDL receptor synthesis, and upregulates acyl CoA-cholesterol acyltransferase (ACAT) that catalyze the formation of CEs.[1,2] This fine regulation, however, could be modified according to cellular needs, e.g., in response to mitogenic stimulus, suggesting that genes and proteins involved in the regulation of cholesterol transport could be determining factors for cell growth and essential for DNA synthesis in a variety of eukaryotic cell types. In this context, an increasing interest has been given to studies indicating that cholesterol, which is concentrate in specialized membrane platform (rafts or caveolae), could have a role in the regulation of a number of signal transduction pathways, including those involved in cell division and differentiation.[3,4]

Recent studies have indicated that a transmembrane glycoprotein called P-glycoprotein (P-gp) and encoded by MDR1 gene, originally identified for its ability to confer multiple resistance against unrelated cytotoxic drugs in tumor cells (MDR), is mainly localized in caveolae. It has been also suggested that it is involved in the intracellular cholesterol transport from the plasma membrane to the ER, the site of cholesterol esterification by ACAT.[5-9]

However, in spite that MDR1 gene and its product P-gp are recognized as playing a major role in intracellular cholesterol ester metabolism, it is still unclear why and when excess membrane cholesterol is preferentially shifted to the ER rather than being released to the appropriate extracellular acceptors (HDL).

Studies conducted in our laboratory have shown that cholesterol synthesis and esterification, ACAT and MDR1 gene expression are always positively correlated with the rate of cell growth in different human cell lines.[10-13] Collectively, these results support the hypothesis that both intracellular cholesterol esters and MDR-1 overexpression are linked in some way to the ability of cells to initiate DNA synthesis and the subsequent proliferative response. However the physiological significance of these events remains to be established.

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To better understand the mechanism by which MDR1 overexpression and cholesterol esterification may influence cell proliferation, we performed a series of analyses utilizing freshly isolated lymphocytes from healthy human donors, stimulated "in vitro" to move on a quiescent to a proliferative state. Freshly isolated human lymphocytes are, in fact, sluggish from a metabolic point a view, also with respect to cholesterol metabolism, exhibiting very low levels of free and esterified cholesterol and undetectable expression of ACAT and MDR-1. This offers a good model system to study possible modifications of cholesterol metabolism and MDR1–P-gp activity which are directly correlated to the induction of cell growth and division.

The results of theses studies will be the main object of this chapter.

Proposed Roles of P-gp in Cell Physiology

Multidrug resistance (MDR) is one mechanism that tumor cells use to escape death induced by chemiotherapic drugs. The classic form of MDR is related to the overexpression of the MDR-1 gene which encodes for a 170kDa glycoprotein termed P-glycoprotein (P-gp).[5,6]

P-gp, is considered to confer resistance to drug-sensitive cells by acting as a membrane-bound ATP-consuming drug efflux pump. Thus, ATP-dependent extrusion of the drugs out of the cytosol of the MDR cells, represents the currently most accepted working hypothesis for P-gp functioning.

However, the molecule is highly conserved throughout evolution, indicating alternative physiological functions for this protein. Really, in the last years multiple physiological functions have been proposed for P-gp, including roles in immunology and apoptosis as well as in regulating cell growth and differentiation.[14]

In this context, we and others obtained evidence that P-gp somehow plays a role in trafficking cholesterol from the inner plasma membrane to the ER. Since our previous studies have shown in "in vitro" models of both normal and tumoral cell proliferation, that cell growth rate is positively correlated to MDR1 and ACAT gene expression and to the rate of cholesterol esterification,[10-12 and chapter 7] we supposed that the translocation of key molecules (e.g., cholesterol) may be one likely way by which P-gp regulates a range of different physiological responses, i.e., those involved in cell proliferation.

However, the detailed nature of these relations remains to be determined. Studies on cells that express high levels of P-gp in the absence of any type of drug selection, will help to better define not only the physiological importance of the proposed P-gp functions but also the possible interrelationships between MDR1, cholesterol esters, and cell growth.

Lymphocytes, being easily accessible, readily inducibile to growth, and representative of many cell types, provide an ideal system in which further evaluating these important issues in vitro. As above mentioned, freshly isolated lymphocytes possess low levels of LDL receptors, low hydroxy-methyl-glutaryl coenzyme A-reductase, low cholesterol esters as well as low MDR1 expression, all of which can be increased by entry into the replicative cycle.[15-17]

These cells can be easily induced to proliferate with lymphocyte-specific mitogens, such as phytohemaglutinin (PHA) and concanavalin A, and to initiate a programmed sequence of biochemical events leading to induction of DNA synthesis.

Starting from these notions, we set up a series of in vitro experiments to evaluate and correlate the regulation of cholesterol trafficking and P-gp activity during lymphocyte growth, in an attempt to define a potential role for P-gp during cell cycle progression.

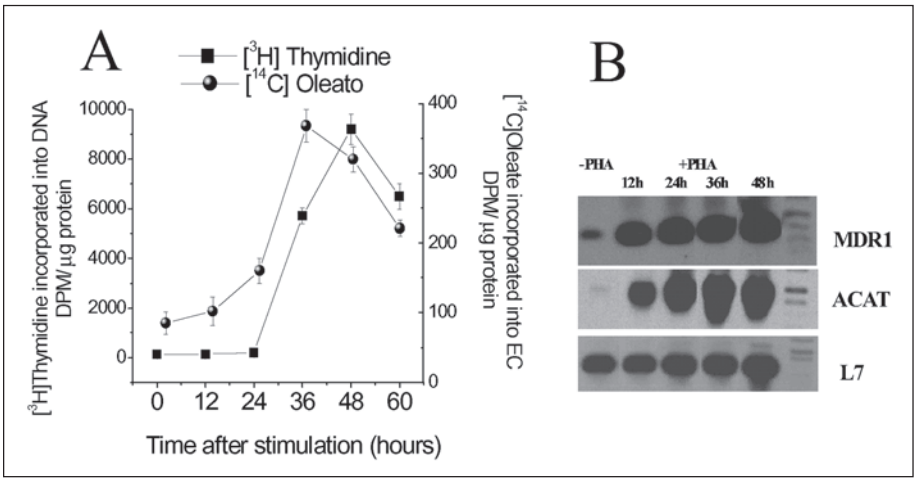

Fig. 6.1. Cholesterol esterification and expression of related genes during the growth of human peripheral lymphocytes. Freshly isolated peripheral human lymphocytes were incubated with PHA (10mg/ml) and harvested at 0 (unstimulated cells) and 12, 24, 36 and 48 hours after treatment. Panel A: [^{3}H]thymidine incorporation into DNA and ^{14}C-oleate incorporated into cholesterol esters. Panel B: representative autoradiograms of MDR1, ACAT, and L7 mRNA levels as determine by RT-PCR analysis.

Time-Dependent Changes in Cholesterol Ester Synthesis and MDR1 and ACAT Gene Expressions in Lymphocytes Stimulated to Growth by PHA

We initially analyzed time-dependent changes in cholesterol esterification pathway in freshly isolated lymphocytes stimulated to grow by PHA. As shown in Figure 6.1A, DNA synthesis began to increase 24 h after PHA stimulation, peaked at 48 h, and decreased thereafter. Changes in the ^{3}H-thymidine incorporation were preceded by similar changes in the ^{14}C-oleate incorporated into cholesterol esters, which began to increase at 12 h after stimulation, and then followed the same trend of timidine incorporation. In these experiments, changes in cholesterol esterification paralleled those of ACAT and MDR1 gene expression. In fact, as shown in Figure 6.1 panel B the mRNA levels of both genes, scarcely detectable in unstimulated cells, underwent a strong increase as early as 12 hours after lectin induction; this increase continued throughout all time points considered (24, 36 and 48 hours).

These results clearly demonstrate that in stimulated lymphocytes, cholesterol esterification pathway and MDR1 gene expression are activated before the beginning of DNA synthesis, supporting the concept that cholesterol esterification may have a role in the regulation of cell cycle progression of lymphocytes and that MDR1 gene product could contribute in some ways to this regulation.

P-gp Inhbition Suppresses Proliferation of PHA Stimulated Lymphocytes

In an attempt to gain more insight on the possible relationship between cell growth, cholesterol esterification and intrinsic P-gp activity, we next performed a series of analyses in PHA stimulated lymphocytes exposed to a P-gp reversing agent.

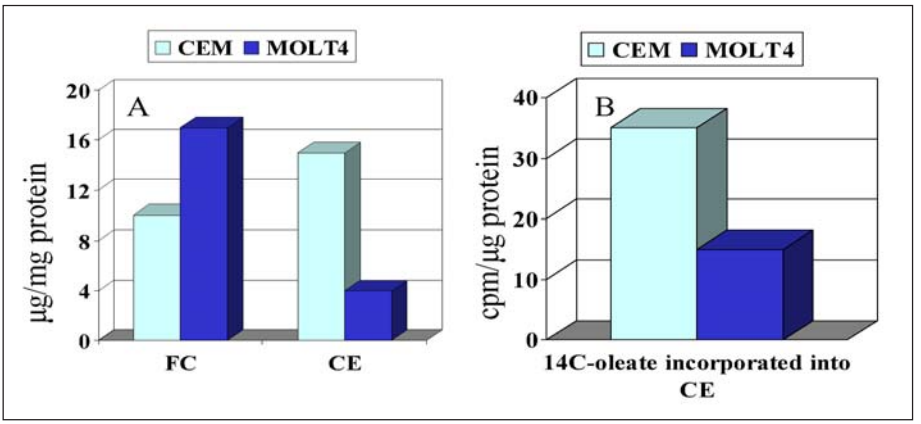

Fig. 6.2. Effect of progesterone (PG) on DNA synthesis and cholesterol esterification during the growth of human peripheral lymphocytes. Progesterone (20 µM) was added at the time of PHA stimulation. All cells were harvested after 48 hours of incubation. Panel A: [^{3}H]thymidine incorporation into DNA. Panel B: ^{14}C-oleate incorporated into cholesterol esters.

A wide variety of compounds have been reported to restore the drug-sensitivity status of MDR cells. These drugs, termed chemosensitizers or reversing agents, physically interact with P-gp inhibiting its function. Among these, progesterone appeared particularly suited for our purposes. Other than modulating P-gp activity, progesterone has been reported to act intracellularly at multiple levels: i) interfering with cholesterol transport from plasma membrane to ER, and ii) inhibiting the process of cholesterol esterification.[18-20] For these reasons, progesterone might be used as a tool to unravel the relationships between the multiple effects of this hormone on cholesterol trafficking and its action as P-gp modulator.

As expected, an inhibition of about 90% of cholesterol esterification in progesterone-treated lymphocytes was seen 48 h after PHA stimulation (Fig. 6.2B). Inhibition of cholesterol esterification was accompanied by a parallel reduction (90 %) of ^{3}H-thymidine incorporation (Fig. 6.2A). Cell viability by the trypan blu exclusion was more than 95% suggesting a specific growth arrest rather than cell death.

In PHA Stimulated Lymphocytes, MDR1 Gene Expression Is Not Correlated to a MDR Phenotype

MDR phenotype can be presented in either intrinsic (tumor cells express high levels of P-gp without any drug treatment) or acquired (high level of P-gp are expressed after several rounds of chemotherapy) form. However, several normal tissues constitutively express high levels of P-gp raising the question as to whether the functionality of P-gp is the same in MDR and non-MDR cells.

In order to evaluate whether the increased gene expression of MDR1 observed following lymphocytes stimulation was associated with an increased ability of cells to extrude drugs, we next determined P-gp activity by evaluating the rate of intracellular accumulation of labeled vinblastine.

As shown in Figure 6.3A, [^{3}H]-vinblastine influx did not significantly change during growth stimulation, maintaining the same activity in proliferating as in resting cells. On the other hand, when proliferating cells were treated with progesterone, its inhibitory effect on cell growth did not paralleled a similar effect on vinblastine intracellular accumulation.

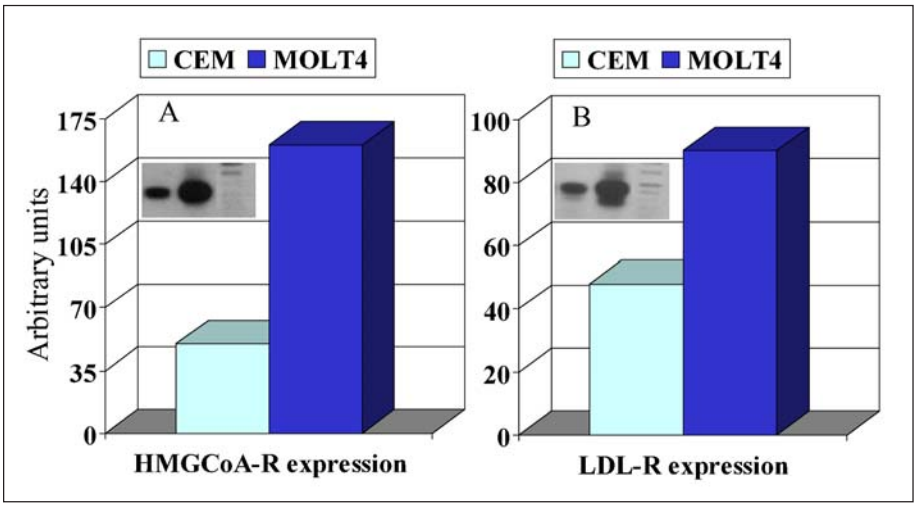

Fig. 6.3. [^{3}H]Vinblastine influx during the growth of human peripheral lymphocytes. Panel A: [^{3}H]Vinblastine influx at different time points after PHA stimulation. Panel B: [^{3}H]Vinblastine influx in PHA stimulated lymphocytes treated with progesterone (PG).

These results suggest that in non-MDR cells, P-gp may exert different functional roles other than being implied as drug efflux pump.

Progesterone Treatment Increased Raft-Cholesterol Content in PHA-Stimulated Lymphocytes

Our results clearly demonstrated that in lymphocytes MDR1 gene expression increases during proliferation independently from drug resistance-related activity, raising the question as to whether MDR1 gene expression is somehow involved in the proposed role of P-gp in transport of cholesterol from the plasma membrane to ER.

To explore this matter further, we evaluated the rate of cholesterol in the plasma membrane by treatment of the intact fixed cells with cholesterol oxidase. Cholesterol oxidase catalyzes the conversion of cholesterol accessible pool to cholestenone, that can be easily identified by thin layer chromatography. In fixed cells, the enzyme interacts only with cholesterol in the outer leaflet of the plasma membrane and it is generally believed that, in several cell types, the enzyme oxidize the sterol present in caveolae.[21] In lymphocytes, the specialized membrane sites corresponding to caveolae are rafts,[22] but studies investigating cholesterol movement in type of cells are absent. As shown in Figure 6.4 a significant increase in cholestenone was observed in 48 hours- PHA-stimulated lymphocytes treated with the P-gp modulator progesterone, indicating that inhibition of growth by progesterone is associated with an accumulation of cholesterol in membrane.

These results add further support for the concept that, during growth, P-gp may be involved in membrane cholesterol redistribution reported during growth of several cell types.

Conclusions

Overall, the results of these studies indicate that P-gp seems required for modulation of cholesterol content at the level of membrane, and thus that it may be involved in the regulation of cell growth and division. This hypothesis is in line with the close correlation observed be-

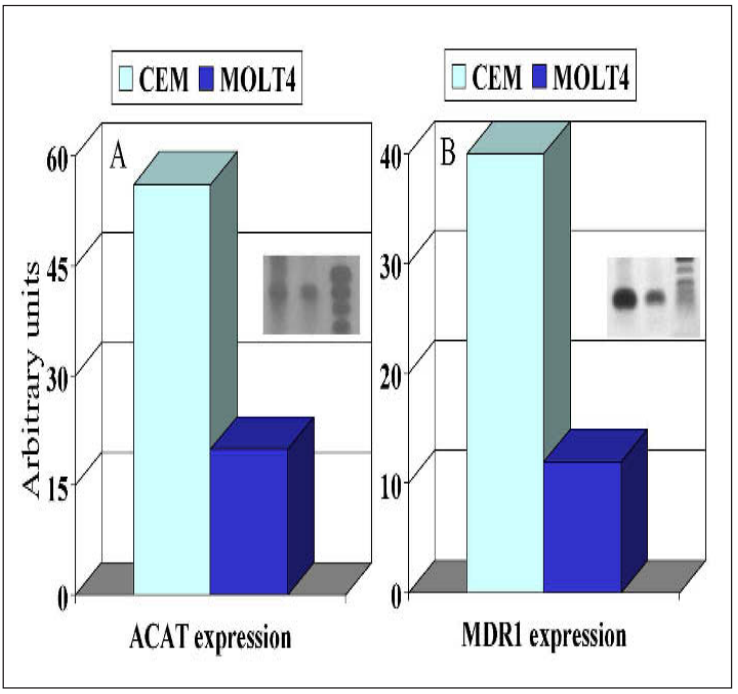

Fig. 6.4. Progesterone treatment increased cholesterol of plasma membrane. Following 48 hours PHA stimulation peripheral lymphocytes were washed, suspended in PBS and incubated 30 min at 18°C in presence of 7.4mBq/ml [^{3}H]cholesterol. After incubation cells were washed extensively and incubated for 1 hour in presence of progesterone, subsequently they were separated from medium and fixed with 1% glutaraldheide in PBS for at least 15 min. CN = cholestenone, FC = free cholesterol, PG =progesterone

tween expression of P-gp, regulation of cell cholesterol homeostasis and rate of growth in PHA-stimulated lymphocytes.

It has been well documented that in lymphocytes rafts are the sites where signaling molecules involved in cell division are concentrated.[22,23] These membrane sites play a crucial part in signal transduction by assembling inactive signaling complexes ready for rapid activation in response to growth factors. The list of signaling molecules apparently localized in rafts includes Src family kinase, nitric oxide synthases, epidermal growth factor receptor, platelet derived growth factor receptor, p21 Ras and most proteins involved in the MAPK cascade.

Recent studies in cholesterol-depleted cells demonstrate that a reduced cholesterol level in membranes is by itself a signal to activate pathways leading to cell division.[21]

Based on above and our results, we suppose that when resting lymphocytes are stimulated to divide, cholesterol, either endogenously synthesized in the ER or LDL-bound, move to the raft. This leads to an induction of MDR1 gene which codifies for a P-gp protein involved in the transport of free cholesterol (FC) to the ER. Here, cholesterol is esterified by ACAT, and stored as immediate resources for new membrane synthesis. The consequent reduction of cholesterol in membranes may trigger hyperactivation of signaling pathways involved in cell division, such as Ras-MAPK cascade, therefore contributing to accelerate lymphocyte cycle progression.

Even if preliminary, these results stimulate future investigations on other model systems since they may improve our knowledge on the molecular mechanisms operating in cell proliferation as well as on the relevance of cholesterol pathways and P-gp functions in the progression of important proliferative diseases such as atherosclerosis and cancer.

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Chapter 7

Cholesterol Esterification and MDR1-P-gp in Lymphoblastic Leukemia Cells:
Functional Relationships

Rosa Rita Bonatesta and Barbara Batetta

Introduction

Several studies have been published indicating a possible association between changes in cholesterol homeostasis and tumoral cell proliferation, including proliferation of leukemic cells. Leukemia cells show more low density lipoprotein (LDL) processing and higher hydroxy-methyl coenzyme A reductase (HMG-CoA-R) levels than normal blood cells. Moreover, cholesterol levels in leukemia cells often do not exhibit feedback repression in high-sterol media.[1,3] These results clearly indicate a direct relationship between cholesterol metabolism and the growth potential of leukemic cells. However, a few studies discriminated between the roles of free cholesterol (FC) and cholesterol ester (CE).

In this chapter we shall review recent results indicating the relevance of cholesterol esterification pathway in the growth of leukemic cells and the possible involvement of the multidrug p-glicoprotein (MDR-1-P-gp) transporter. In fact, studies which implicate this protein in the modulation of intracellular cholesterol transport are receiving increasing being cholesterol in membranes potentially involved in the regulation of signaling pathways which are important for cell growth and division of eukaryotic cells.

Altered Cholesterol Balance during Tumoral Growth

In the cells, cholesterol balance is a function of the amount taken up by cells, the amount made by the cell, and the amount removed by cholesterol acceptors like HDL. Based on the notion that activation of cell growth imposes additional need for cellular cholesterol to support membrane synthesis, a potential mechanism contributing to growth might involve altered cellular sterol balance and /or metabolism.

As reported in Chapter 6 of this book, in resting T lymphocytes cholesterol synthesis is virtually absent, the minimal amounts of required sterols being obtained from an external source (via LDL-receptor). Intracellular cholesterol esterification and efflux are also absent, in accordance with the low cell turnover present in this condition. As in all growing cells intracellular cholesterol metabolism consistently increases during lymphocyte growth and division. In the early 1970s Chen and Kanduch[4] reported that cholesterol utilized by mitogen-stimulated T lymphocytes was mainly synthesized endogenously, and was accompanied by a strong increase of gene expression and activity HMG-CoA-R, the key enzyme that converts HMG-CoA to mevalonic acid. However, initially, the remarkable increase of cholesterol synthesis reported

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during cell growth was confined to a sole structural role of cholesterol related to its requirement for new membrane biogenesis. In the late 70s researchers from Japan isolated, from certain microbes that require sterols, some metabolites inhibiting HMG-CoA-R.[5,6] The discovery of these metabolites (compactin and mevinolin), which are precursors of the statins, other than to improve the fight against atherosclerosis, gave also a new impulse to the knowledge of cholesterol metabolism allowing the discovery of several products deriving from the branched mevalonic acid pathway closely involved in cell homeostasis and growth.

Indeed, studies "in vitro" showed that only mevalonic acid supplement was able to revert the inhibitory effect on growth exerted by the HMG-CoA-R inhibitors compactin and/or mevinolin, the addition of cholesterol being totally ineffective. This provided the earliest evidence that non sterol products deriving from mevalonic acid were produced and involved in the regulation of cell growth,[7,8] and starting the era of prenylated proteins that are fascinating a large number of scientists (see Chapter 2).

Since then, a considerable time passed before intracellular cholesterol esterification was investigated in relation to growth. This was likely due to the technical limitations of the available procedures; in most of the early studies, in fact, cholesterol was extracted by digitonin that hydrolyzing cholesterol esters rendered they undetectable.

The relationship between cholesterol metabolism and cell growth has been extensively studied "in vivo" in our laboratory in different experimental models of tumors[chapter 3 and 9-11] as well as in various types of human neoplasms, including several hematologic and solid malignancies.[chapter 4 and 12-17] A constant finding of these studies was the unique pattern of cholesterol metabolism, namely:

i. increased cholesterol biosynthesis,
ii. intracellular accumulation of cholesterol esters, and
iii. corresponding decrease of HDL cholesterol in the plasma compartment.

When specifically investigated, a strong correlation between cholesterol esters accumulation, reduction of HDL-cholesterol levels, and growth rate, was found in the tumor models studied.

These results provided the first evidence that cholesterol esters might somehow be involved in the overall mechanisms governing cell proliferation.

Positive Correlation between Growth Rate and Cholesterol Esterification in CEM and MOLT4 Cell Lines

In order to get more insights on the underlying reasons why cholesterol esterification is increased in tumoral processes, we investigated the total cholesterol content (both free and esterified), and the cholesterol synthesis and esterification capacity of two different human leukemia cell lines (CEM and MOLT4) isolated from patients with acute lymphoblastic T cell leukemia. These cell lines were selected because, despite the fact that both derive from patients with the same type of leukemia, they show "in vitro" different growth characteristics.[18,19]

As shown in Figure 7.1A, the two cell lines showed significantly different doubling times, CEM cells growing at a faster rate than MOLT4. Similarly, the rate of ^{3}H-thymidine incorporation was consistently higher in CEM than in MOLT4 cells (Fig. 7.1B).

By contrast, cholesterol synthesis, as determined by ^{14}C-acetate incorporated into free cholesterol (FC), was significantly higher in MOLT4 compared to CEM cells (Fig. 7.1C) indicating that, at least in these cells, newly synthesized cholesterol did not appear to be directly correlated with growth rate.

When the cholesterol esterification potential was investigated by measuring ^{14}C cholesterol ester accumulation in cells exposed to medium containing ^{14}C-oleate (Fig. 7.2B), CEM cells showed a consistently higher cholesterol esterification capacity compared to MOLT4. The increased ability to esterify cholesterol in CEM cells was also reflected in a higher content of cholesterol esters in these cells compared to MOLT4 cells (Fig. 7.2A).

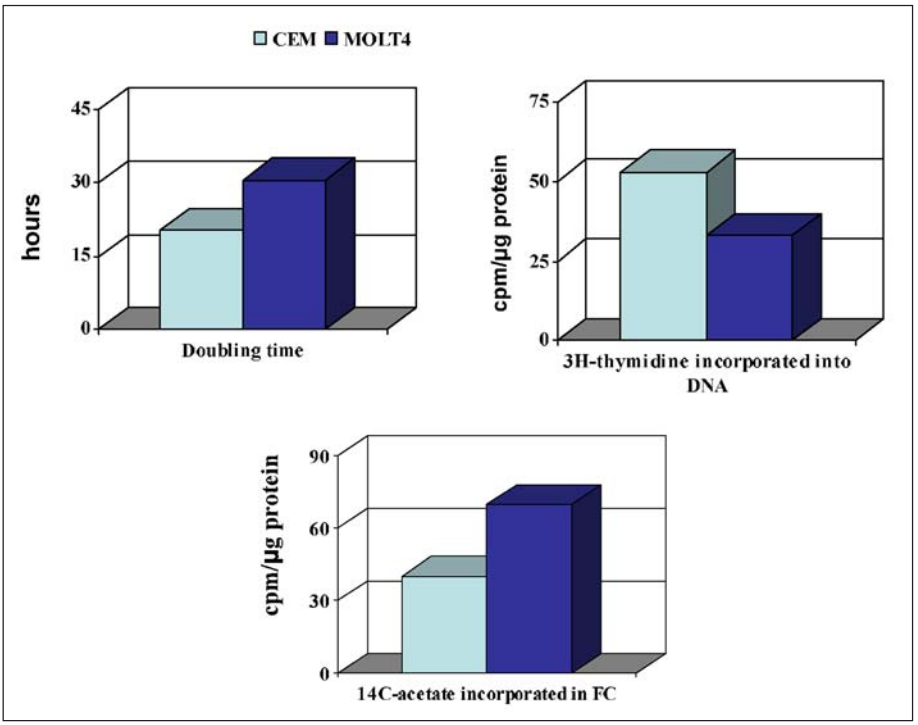

Fig. 7.1 Growth and cholesterol synthesis in CEM and MOLT4 cell lines. To determine DNA and cholesterol synthesis cells were incubated for 6 hours with ^{3}H-thymidine and ^{14}C-acetate, respectively.

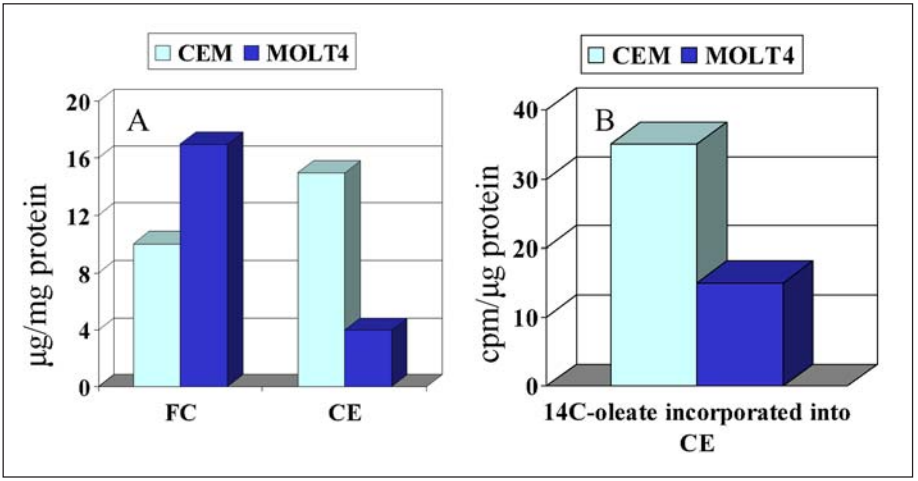

Fig. 7.2. Cholesterol content and esterification in CEM and MOLT4 cell lines.To determine cholesterol esterification cells were incubated for 6 hours with ^{14}C-oleate.

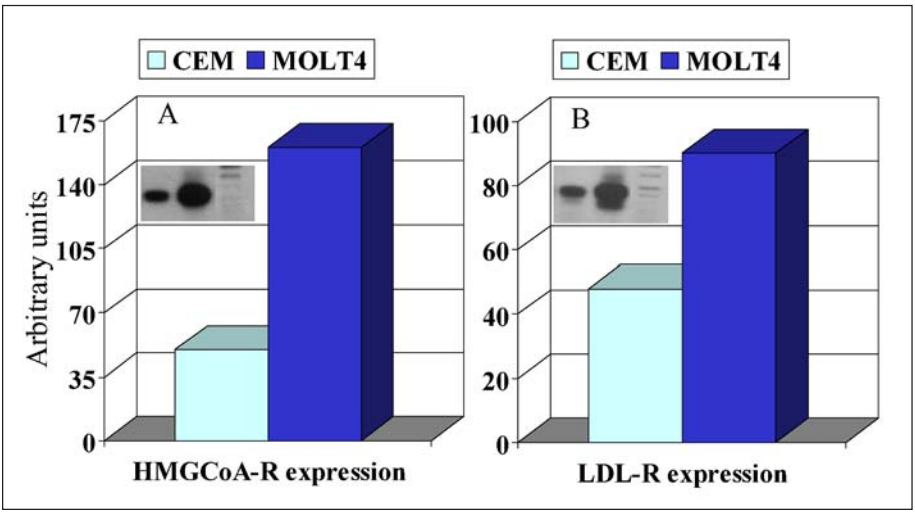

Fig. 7.3. Total RNA was isolated from cultured cells by the guanidine isothiocyanate phenolchloroform extraction method. During the PCR reaction the non radioactive label digoxigenin-11-dUTP (DIG) was incorporated. The relative levels of gene expression have been determined by comparing the PCR products of the target DNA and the L7 gene. Target gene/β-actin ratio was taken to represent the relative expression of the gene studied (arbitrary units) (for details see ref. 19).

It is worthy to note that when cholesterol efflux was evaluated in the two cell lines, the percentage of total cholesterol efflux into the medium was generally higher in MOLT4 than in CEM cells.

Altogether these results indicate that CEM have an increased ability to retain newly synthesized cholesterol inside the cells than MOLT4.

It is noteworthy that similar results were obtained in the murine leukemia L1210 cells that were chosen among others because of their particularly short doubling time (12h). As a matter of fact, in these fast growing cells, cholesterol esterification was consistently higher than in both CEM and MOLT4 cells.[20]

To further evaluate the possible relationship between cholesterol metabolism and tumoral growth, we subsequently determined in CEM and MOLT4 cells, the expression of the main genes implicated in the regulation of intracellular cholesterol homeostasis: HMGCoA-R, LDL receptor (LDL-R) and ACAT. Autoradiographs of PCR-amplified mRNA of HMGCoA reductase (Fig. 7.3), indicated that HMGCoA-R gene expression was higher in MOLT4 cells than in CEM cells. The differences of the two cell lines in the HMGCoA-R gene expression were obtained in three independent experiments, relative data of which are reported in Figure 7.3, panel A. Higher levels of LDL-R mRNA were also observed in MOLT4 cells compared to CEM cells (Fig. 7.3, panel B).

By contrast, ACAT mRNA levels were higher in CEM cells (Fig. 7.4 panel A). Quantitative densitometric determinations of the bands after normalization with β-actin confirmed that the mRNA levels were higher in CEM cells compared to MOLT4 (Fig. 7.4, panel A).

The results of the above studies can be summarized as follows:

i. CEM cells grow at a faster rate than MOLT4;
ii. CEM cells have higher baseline levels of cholesterol esterification as wells as higher levels of cholesterol ester content;
iii. MOLT4 cells display higher levels of cholesterol synthesis.

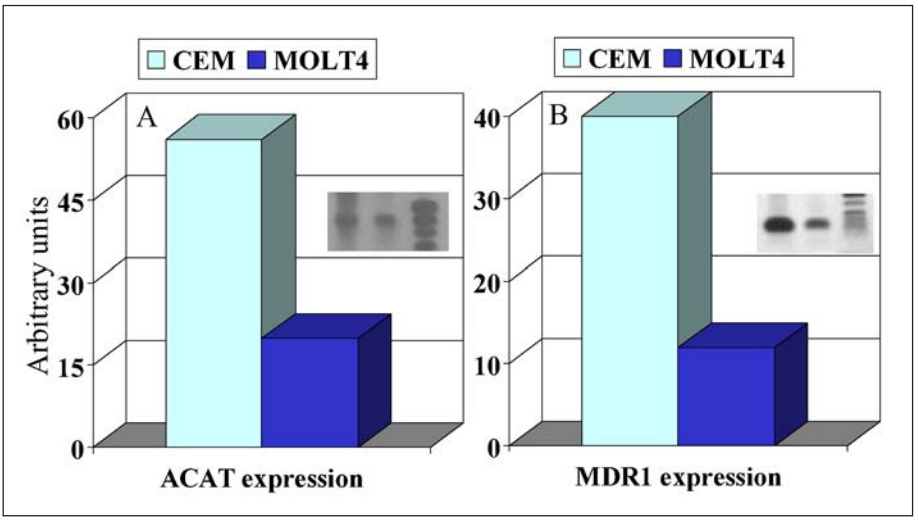

Fig. 7.4. Total RNA was isolated from cultured cells by the guanidine isothiocyanate phenolchloroform extraction method. During the PCR reaction the non radioactive label digoxigenin-11-dUTP (DIG) was incorporated. The relative levels of gene expression have been determined by comparing the PCR products of the target DNA and the L7 gene. Target gene/β-actin ratio was taken to represent the relative expression of the gene studied (arbitrary units) (for details see ref. 19).

Other than adding new insights on the possible role of cholesterol metabolism on tumoral growth, these data represent, to the best of our knowledge, the first strong evidence of an involvement of cholesterol esterification in the growth regulation of leukemia cells.

A Role for P-gp in the Lipid Transport of Leukemia Cells

P-glycoprotein (P-gp) is the product of the MDR-1 gene which was first identified in MDR (multidrug resistant) cancers as responsible for transporting across plasma membranes out of the cell a variety of antineoplastic agents, thus conferring the socalled MDR phenotype to cancer cells.[21-23] In the clinic, expression of P-gp is measured in cells from different leukemia patients, high levels of expression being considered as a major indicator of poor prognosis.[24-27]

Until recently, the biology of P-gp was investigated only with respect to its function as the major multidrug efflux pump, which hampers the success of chemotherapy, and with the aim to overcome the MDR status of leukemia by exploring the activity of several P-gp modulators.

However, a number of evidences are now indicating some relationships between P-gp and intracellular cholesterol trafficking:

1. P-gp has been related to the transport of cholesterol from plasma membrane to the ER, the site of cholesterol esterification by Acyl-CoA:cholesterol acyltransferase (ACAT). P-gp is the first protein reported to act as translocator of cholesterol from plasma membrane to the interior of the cell.[28-31] However, in spite of the fact that MDR1 gene, and its product P-gp, are recognized as playing a major role in intracellular cholesteryl ester metabolism, it is still unclear why and when membrane cholesterol is preferentially shifted to the ER rather than being released to the appropriate extra cellular acceptors (HDL).
2. P-gp expression is regulated by cholesterol levels: it has been demonstrated that in freshly isolated mononuclear cells cholesterol loading with acLDL strongly induces MDR-1 expression, and ii) cholesterol efflux mediated by HDL completely suppresses the expression of MDR1.[32]

3. P-gp is predominantly localized in the cholesterol-rich membrane microdomains, referred to as "lipid rafts".[33]

Despite all the above indications, the involvement of P-gp in cholesterol transport in leukemia cells has not been unequivocally demonstrated, yet.

For this reason, we then investigated whether a link might exist between P-gp and cholesterol metabolism and trafficking, in CEM and MOLT4 leukemia cell lines.

The results obtained under stationary culture conditions (Fig. 7.4B), showed that CEM cells expressed high levels MDR1 mRNA in contrast to MOLT4, that instead revealed the presence of very low levels of MDR1 mRNA.

Despite of the different levels of MDR1 gene expression between CEM and MOLT4 cell lines, no such differences between them was found when P-gp activity was evaluated by determining intracellular accumulation of [H^3]vinblastine. Both cell lines showed, in fact, similar levels of intracellular labeled vinblastine providing a strong support to the concept that, in tumor cells, high levels of P-gp expression not necessarily imply P-gp functioning as a drug efflux pump. Moreover, this finding allows to speculate that, in CEM cells, the high expression of MDR1 might be involved in the intracellular sterol trafficking processes. In these leukemia cells, P-gp may be part of a lipid transport system that counterbalances excessive influx and synthesis of cholesterol, which in turn may modulate cell growth and division.

A number of experimental evidences seems to support these conclusions.

1. A plausible role of cholesterol esterification and MDR1 in the control of cell cycle G1/S transition has been demonstrated during proliferation of VSMC.[34,35]
2. The active involvement of MDR1 in reverse cholesterol transport suggests its potential implication in the process of cholesterol ester accumulation in proliferating cells.[36]
3. A strong induction of MDR1 expression is observed in response to growth stimulation in different types of cells.[19,34,35]
4. Cholesterol in caveolae has been involved in the regulation of signaling pathways (i.e., MAPK cascade) which are important for cell growth and division of eucaryotic cells.[37-40]

Caveolae can be considered as specialized platforms for transduction signaling whose affinity is modified by lipid, particularly FC, in a site-specific manner in response to physiological stimuli. Studies with cholesterol-depleted cells demonstrated that a reduced level of cholesterol in caveolae is, by itself, a signal to activate pathways leading to cell division.[41]

Taken together, these findings raise the possibility that MDR-1 might specifically influence caveolae-dependent signaling by regulating, at least in part, the amount of cholesterol available for caveolae formation, in this way reinforcing the possibility that MDR1, cholesterol, and cell proliferation may be closely linked.

The cholesterol redistribution within plasma membrane induced by P-gp, recently reported by Garrigues,[42] may affect the intracellular cholesterol trafficking that involves both the endogenous biosynthesis and esterification of cholesterol in the ER, the import of exogenous cholesterol from the LDL by endocytosis, and the export of cholesterol to the HDL.

A model for the effects of cholesterol esterification and P-gp on the growth of CEM and MOLT4 cell lines is given in Figure 7.5.

Conclusions

The studies here summarized indicate a close functional relationship between cholesterol esterification, MDR1 gene expression, and rate of cell growth, in both human and murine non-MDR leukemia cells. Although these studies do not define which P-gp isoform is required for which P-gp function, they support the existence of different functional P-gp isoforms acting in MDR and in non-MDR neoplasms. Clearly, the field is expanding and further research is needed to define the physiologic importance of the proposed additional P-gp functions. As for now, however, the evidences given with the present studies indicate that CE and P-gp may

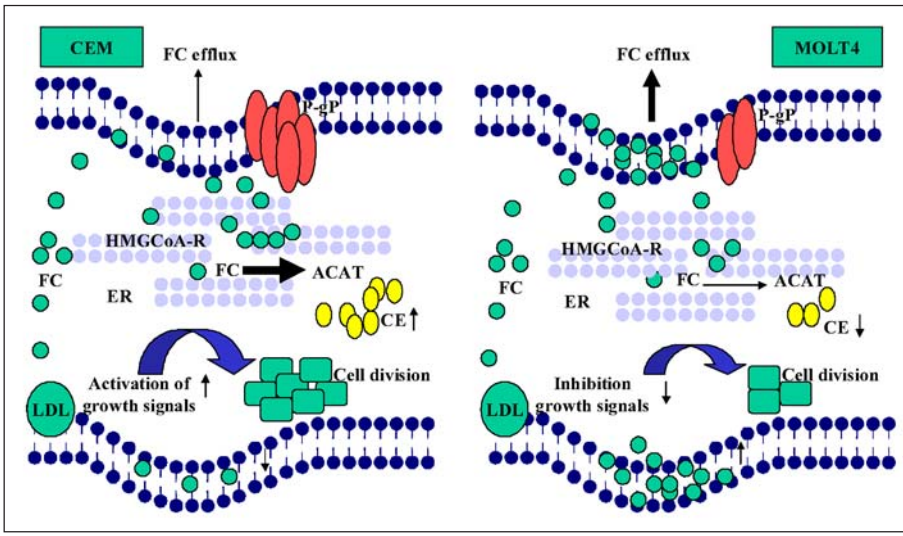

Fig. 7.5. Possible mechanism of action of P-gp in CEM and MOLT4 cell lines. Either LDL-cholesterol or endogenously synthesized cholesterol in the ER move to the caveolae from which is rapidly distributed within the plasma membranes. The excess of free cholesterol (FC), in presence of high P-gp levels (CEM cells) is transported to the ER, esterified by ACAT, and accumulated in the cytoplasm as lipid droplets (CE). This results in a depletion of cholesterol in caveolae that may trigger hyperactivation of signalling pathways involved in cell division, therefore contributing to accelerate tumoral growth. By contrast, when P-gp levels are low (MOLT4), less cholesterol is transported to ER and esterified by ACAT. This results in an accumulation of cholesterol in the caveolae with consequent inhibition of signal transduction pathways and lower rate of proliferation.

represent important elements in the control of cellular growth. Moreover, the finding that P-gp in tumoral high-P-gp-expressing-cells may not function as an active drug efflux pump, should be considered by oncologists at first diagnosis of leukemia since it implies that detection of high expression levels of this glycoprotein not necessarily indicate unresponsiveness to chemotherapy, and thus a poor prognosis. Finally, the likely involvement of the P-gp expressed in non-MDR cells in the regulation of cell proliferation via modulation of intracellular CE, might also lead to a novel pharmacological approach to the therapy of cancer.

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Chapter 8

MDR-1, Cell Growth and Cholesterol Esterification

Alessandra Pani and Sandra Dessì

Multidrug Resistance

The phenomenon of multidrug resistance (MDR) represents one of the major limitations to the efficacy of chemotherapy for human cancer, whereby tumor cells simultaneously possess intrinsic or acquired cross-resistance to diverse chemotherapeutic agents.[1-3] Clinical oncologists were the first to observe that cancers treated with multiple different anticancer drugs developed cross-resistance to many other cytotoxic agents to which they had never been exposed, effectively eliminating the possibility of curing these tumors with chemotherapy.

In most instances, cells grown in tissue culture from such multidrug-resistant tumors show patterns of resistance in vitro equivalent to those observed in patients. This observation suggested that MDR can be the result of heritable changes in cancer cells. Molecular investigations in MDR led in fact to the identification of genes encoding for several proteins associated with MDR, including MDR-1 encoded P-glycoprotein (P-gp),[4] the multidrug resistance associated protein (MRP1),[5] the lung resistance protein (LRP),[6] and, more recently, the breast cancer resistance protein (BCRP).[7] These transmembrane proteins cause MDR either by decreasing the intracellular levels of drugs or by redistributing intracellular accumulation of drugs away from target organelles. Generally, these proteins are expressed at varying degrees in different neoplasms associated with poor prognosis: gastrointestinal, hepatobiliary and renal cancers are largely unresponsive to chemotherapy and have a high degree of intrinsic MDR, whereas leukemia, lymphomas, ovarian and breast cancers often respond to treatment initially, but acquire resistance during the course of the cycles with chemotherapy.[8-12]

In this chapter we will review update literature on the biology and function(s) of the most studied drug efflux pump P-gp, focusing on data correlating the expression of this highly conserved glycoprotein with cell growth and cholesterol esterification potential.

P-gp and MDR

The best-studied mechanism of MDR is that due to overexpression of P-gp, an energy-dependent multidrug efflux pump, known as the multidrug transporter.[13,14] P-gp is a 170-kDa phosphorylated and glycosylated plasma membrane protein belonging to the ATP-binding cassette superfamily of transport proteins which was first described by Juliano and Ling in 1976.[14]

Humans have two MDR genes, MDR1 and MDR3 (also called MDR2), and rodents have three: mdr1a (also called mdr3), mdr1b (also called mdr1), and mdr2. Only the MDR1 gene (responsible for P-gp synthesis) in humans and the mdr1b and mdr1a genes in rodents

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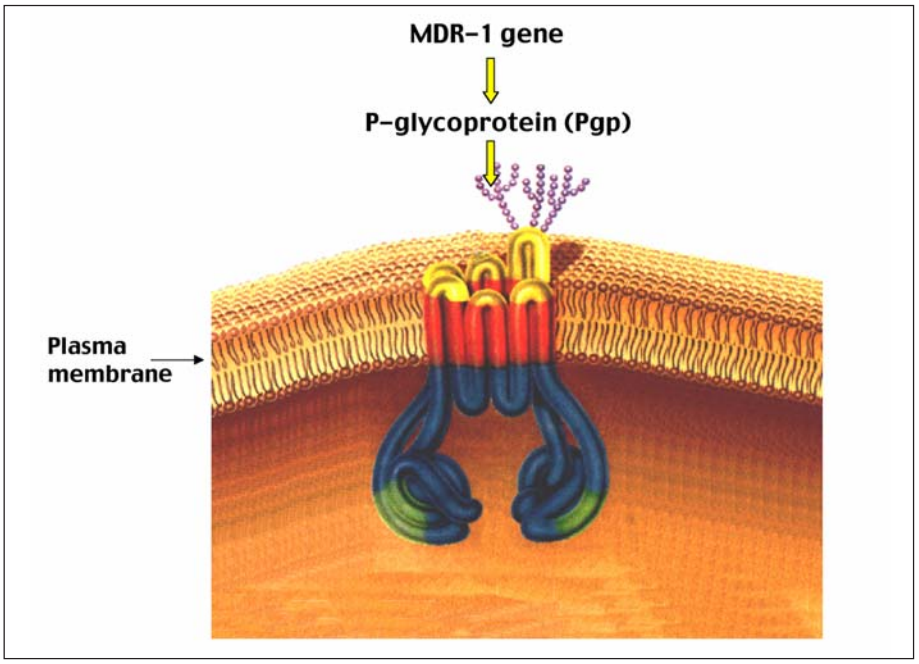

Fig. 8.1. Schematic representation of the MDR-1 encoded P-gp.

appear to be involved in drug transport and development of drug resistance.[15] The human MDR3 and murine mdr2 genes encode a glycoprotein which does not seem to have a role in drug transport, but rather in the secretion of phosphatidylcholine into bile, likely being a phospholipid transport protein.[16-18]

MDR1 P-gp is a transmembrane protein which is 1280 amino acids long and consists of two homologous halves of 610 amino acids joined by a flexible linker region of 60 amino acids (Fig. 8.1). Each half has an N-terminal hydrophobic domain containing six transmembrane (TM) domains followed by a hydrophilic domain containing a nucleotide binding site. The nucleotide binding sites can bind ATP and its analogues and both sites are essential since inactivation of either site inhibits substrate-stimulated ATPase activity.[19-21]

The majority of published data suggest that P-gp acts as a transmembrane pump which removes drugs from the cell membrane and cytoplasm (Fig. 8.2). ATP hydrolysis provides the energy for active drug transport, which can occur against steep concentration gradients. P-gp-dependent drug transport activity may depend on the level of expression of the MDR1 gene as well as on the functionality of the MDR1-encoded P-gp.

To date there is no consensus on the number, nature and interrelationships of the drug and modulator binding sites because data derived from a series of experimental studies addressing this issue have been ambiguous.[22 and references herein] However, since the substrates for P-gp are largely hydrophobic, access to the substrate binding site on the protein is likely to be from the lipid bilayer, rather than from the aqueous phase. Accordingly, transport and binding studies suggest that P-gp interacts with substrates in the inner leaflet of the lipid bilayer of the plasma membrane and acts by flipping substrates to the outer leaflet of the bilayer.[22-24] This could also explain the unusual broad specificity of P-gp as the main determinant of specificity would be the ability to intercalate into the lipid bilayer, for which the amphipathy of the substrate must

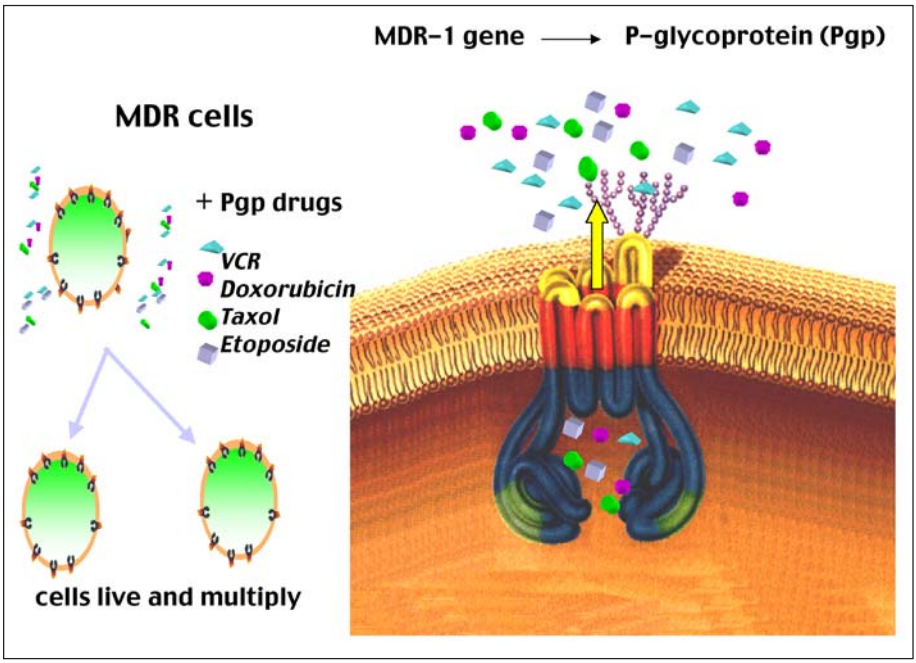

Fig. 8.2. Mode of action of the drug efflux pump P-gp. MDR drugs are actively extruded from the cell interior allowing cell survival and division.

be suitable. Thus, hydrophobicity or amphipathy within a certain range of the drug molecule is possibly crucial for drug-P-gp interaction.

P-gp Substrates and Antagonists

The MDR-related anticancer drugs are derived from plants or microrganisms, are structurallly dissimilar, and have different intracellular targets. What these drugs have in common is the relatively low molecular weight and the way they enter cells; e.g., passive diffusion due to their preferential solubility in lipids at physiological pH.

Molecules interacting with P-gp may be classified as P-gp substrates and P-gp antagonists or inhibitors (Table 8.1). The latters seem to bind P-gp more tightly and, failing to be transported, prevent the transport of other compounds, whereas substrates, i.e., molecules transported by P-gp, do not block the transport of other substrates.[25]

P-gp substrates have very diverse structures and as mentioned only share the properties of being hydrophobic amphipathic molecules (molecules having two sides with characteristically different properties) that are not negatively charged, and with a size in the range of 200-1800 Da. They include cancer drugs (i.e., doxorubicin, daunorubicin, vinblastine, vincristine, actinomycin D, taxol, teniposide, etoposide); steroids (i.e., aldosterone, hydrocortisone, cortisol, corticosterone, dexamethasone); HIV protease inhibitors (i.e., amprenavir, indinavir, nelfinavir, ritonavir, saquinavir); diverse anti-infective agents (i.e erythromycin, rifampicin, ivermectin); the fluorescent dye rhodamine-123.[25-32]

P-gp inhibitors include the immunosuppressant cyclosporin A and its non-immunosuppressive analogue PSC833 (valspodar); the calcium channel blocker verapamil; the progesterone antagonist mifepristone (RU486); the anti-estrogen tamoxifen; the antibiotic erythromycin; and

Table 8.1. Molecules interacting with P-gp classified as substrates and inhibitors

P-gp Substrates	P-gp Inhibitors
Vinca alkaloids e.g., vincristine	Ca channel blockers e.g., verapamil
Anthracyclines e.g., doxorubicin	Steroid hormones e.g., progesterone
Epipodophyllotoxins e.g., etoposide	Immunosuppressants e.g., cyclosporine A
Antibiotics e.g., actinomycin D	Antibiotics e.g., hydrophobic cephalosporins
Others e.g., taxol, topotecan, colchicine, puromycin	Others e.g., hydrophobic anphypathic drugs and analogs

See text for details.

the antifungal ketoconazole, among others.[15,25,26,33,34] P-gp inhibitors are also generally referred to as P-gp chemosensitizers or P-gp modulators since in vitro they are able to prevent the efflux of P-gp substrates from cells, thus causing reversal of the MDR phenotype (Fig. 8.3A, B).[33,34] The possibility of using P-gp inhibitors in the clinic to chemosensitize MDR tumors to the antineoplastic drugs, is presently under investigation (See chapter 11).

P-gp Tissue Localization and Physiologic Function(s)

Detectable levels of P-gp are found in liver, pancreas, kidney, and, in higher amounts, in colon and jejunum, and in the adrenal gland.[35] P-gp is also found in the epithelium of the choroid plexus of the brain (which forms the blood-cerebrospinal fluid barrier or CSF) as well as in the endothelium of blood capillaries of the brain (the blood-brain barrier or BBB).[36,37] P-gp has been also detected in normal bone marrow in hematopoietic stem cells and in peripheral blood mononuclear cells (PBMCs), mature macrophages, natural killer (NK) cells, antigen-presenting dendritic cells (DCs) and T- and B-lymphocytes.[26,38,39]

The physiological function of P-gp in the absence of therapeutics or toxins is not totally understood. From its tissue distribution, it may be inferred that P-gp has a general role in eliminating toxins and toxic metabolites from the body,[40-42] and in transport of certain cytokines.[43,44]

Expression of P-gp on the luminal surfaces of the epithelial cells of the small and large intestine, biliary ductules, and proximal tubules of the kidney suggest a role in decreasing the absorption from the gut and/or the excretion of endogenous and exogenous hydrophobic amphipathic toxins.[41]

Expression in the capillary endothelial cells of the brain, nerves, testis and placenta suggest a barrier function of P-gp to keep toxins out of the nervous system, gonads and fetus. Many relatively hydrophobic drugs that were expected to diffuse easily across lipid membranes did not readily enter the brain. P-gp has been shown to prevent or decrease the entry of certain drugs into the brain and to contribute to the BBB.[26,37,41,45] Studies on mdr1 knock out (KO) mice (mice lacking the MDR1 genes) showed that P-gp does not affect viability, fertility and a range of biochemical and immunological parameters and confirmed its role in the BBB.[46] Mdr1 knock out mice do have delayed kinetics and clearance of vinblastine and accumulate

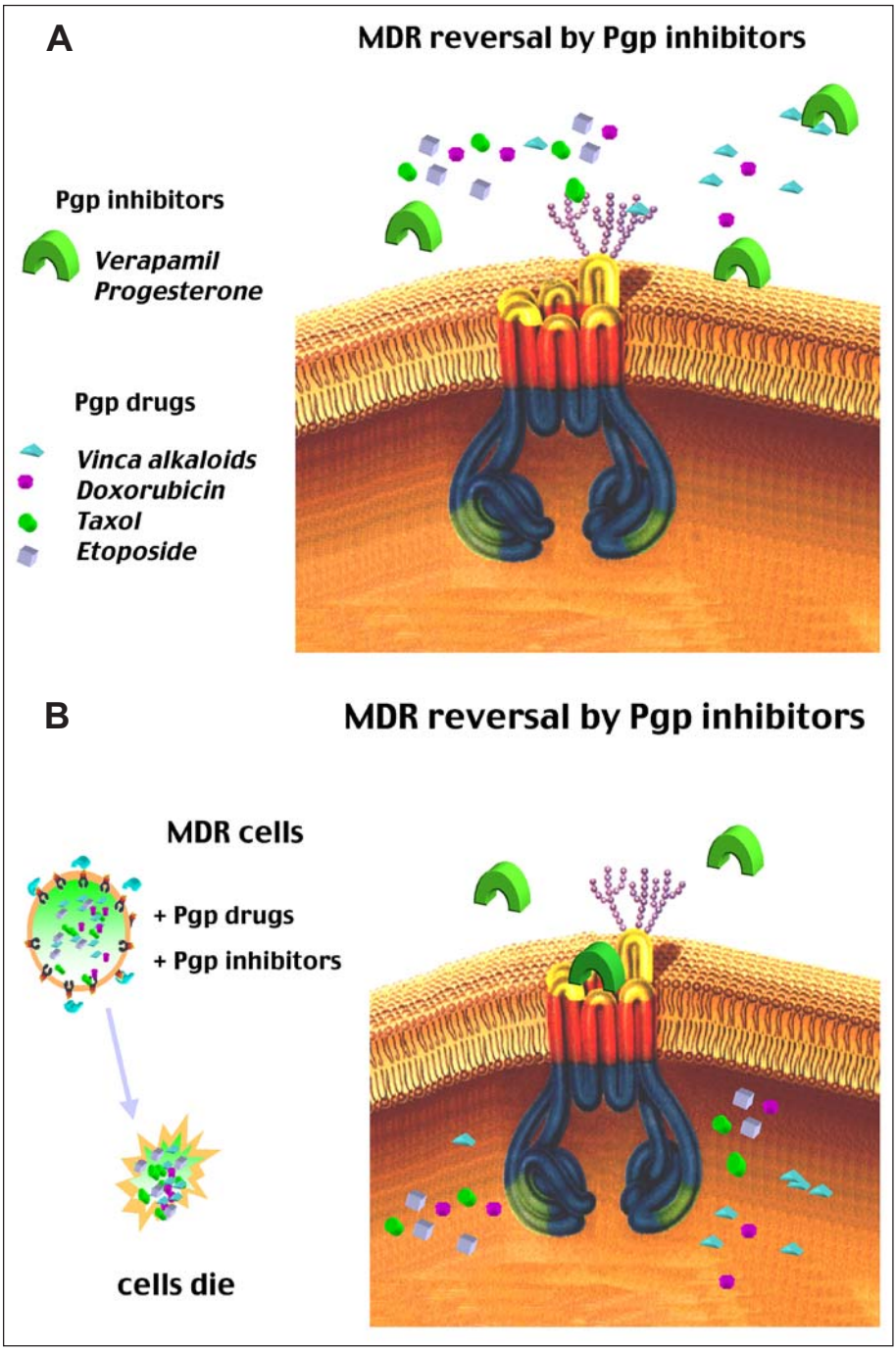

Fig. 8.3. MDR reversal by P-gp inhibitors. Panels A and B represent the mechanism by which P-gp chemosensitizers prevent efflux of P-gp substrates from cells leading to reversal of the MDR phenotype.

high levels of various MDR drugs in their brain, testis, ovary and adrenal gland compared to wild type (WT) mice.[27,36,41,45,47]

Due to its presence in hematopoietic stem cells, P-gp probably also contributes significantly to the removal of drugs and toxins from the bone marrow.[42,48] Pluripotent hematopoietic stem cells from P-gp knock out mice demonstrated markedly decreased rates of rhodamine 123 efflux compared with WT cells confirming the substantial contribution of P-gp to the extrusion of drugs from the bone marrow.

While it is irrefutable that P-gp can efflux xenobiotics out of cells, other biological functions of this protein have been suggested. Recent reports indicate that P-gp may be involved in the regulation of apoptosis,[49] chloride channel activity,[50] immune cell function,[43,44] phospholipid transport,[51] viral infection,[38,52,53] and cholesterol intracellular trafficking and esterification.[54-56 and references herein cited] The physiologic relevance of all these proposed additional functions for P-gp is subject of great debate among the scientific comunity since they may imply a role for P-gp in the overall mechanism(s) of regulation of cell proliferation and survival.

P-gp in Cell Death and Cell Growth

Given that there is not always a linear correlation between extent of drug efflux and resistance to cell death, P-gp may also prevent MDR cells from death by some additional mechanisms.[57]

The molecular events leading to programmed cell death (apoptosis) imply a quite well defined cascade of events involving as key proteins the cysteine-aspases, also known as caspases. It has been shown that cells induced to overexpress P-gp, either by drug-selection or by retroviral gene transduction with MDR1 cDNA, are resistant to all the caspase-dependent apoptotic stimuli (i.e., FasL, TNF, UV irradiation), whereas they retain full susceptibility to the induced programmed-cell-death by caspase-independent events (i.e., target-cell killing by CTL and NK cells).[58,59]

Although the mechanism of the anti-apoptotic activity of P-gp remains to be established, a number of hypothesis have been advanced. Given that P-gp may also efflux large proteins like interleukin-2,[43,44] it might be possible that a key caspase, or another caspase-related mediator of apoptosis, is being removed from P-gp overexpressing cells.[58] Alternatively, being P-gp an ATPase with constitutive ATPase activity,[60-62] it might affect apoptosis by altering intracellular adenosine triphosphate (ATP) level which, in turn, is critical for activation of caspase.[63] A third hypothesis implies the intracellular alkalinization consequent to the Na- and Cl-dependent pathway induced by P-gp,[64] which may place the cell in a state of caspase-inactivity. In addition to its drug efflux activity, P-gp seems in fact to function as a ion channel,[50] and expression of P-gp results in alkalinization of the cytosol as a consequence of an increase in pH_i and altered membrane potential (Vm).[64] In support of this hypothesis is that apoptosis induced by Fas ligation, UV irradiation, serum starvation, or even chemotherapeutic drugs, is indeed preceded by intracellular acidification.[65,66]

Besides events leading to programmed cell death, P-gp appears also involved in those regulating cell proliferation. A positive correlation between P-gp expression and doubling time of cells have been found in our laboratories in a number of human and murine leukemia-derived cell lines.[67] We also found that the extent of P-gp expression and rate of cell growth correlate with the cholesterol esterification potential of the cells (see below), thus suggesting a role for both P-gp and cholesterol esters in the mechanisms for regulation of the cell cycle.[67,68]

P-gp Involvement in Intracellular Cholesterol Trafficking

Cholesterol is an essential structural element of cellular membranes as well as a precursor for the synthesis of steroid hormones, bile acids, and lipoproteins.[69] Mammalian cells obtain cholesterol by internalization of low density lipoproteins (LDL) or by neosynthesis in the en-

doplasmic reticulum (ER). Excess cellular cholesterol, which is toxic to the cell, is mainly regulated by intracellular deposition in the form of cholesteryl esters by acyl-CoA:cholesterol acyl transferase (ACAT), a resident ER enzyme. Cholesterol esters can be hydrolyzed to free cholesterol by esterases within either the cytosol or the lysosomal system.[70] This regenerated free cholesterol can then reach other subcellular structures or be recycled back to plasma membrane, thus completing the socalled cholesterol/cholesterol ester cycle.[71,72] Rates of cholesterol biosynthesis, LDL internalization, and cholesterol esterification are exquisitely sensitive to cellular levels of free cholesterol (e.g., the rate of cholesterol esterification is limited by the availability of cholesterol substrate in the ER).

Just as cellular levels of cholesterol are tightly controlled and regulated, so is its compartimentation. At steady state, 70-80% of cellular cholesterol is in the plasma membrane. However, cholesterol is not uniformly distributed among cell membranes or within membranes, but is instead localized into cholesterol-rich and cholesterol-poor domains.[70,73-76] Cholesterol-rich domains can be identified in glycolipid rafts also known as cholesterol-rich detergent-insoluble glycosphingolipid-enriched complexes. Glycolipid rafts are present in membranes of all cell types and in the caveolae that represent invaginations of plasma membrane sections compositionally related to rafts and coated by the cholesterol-binding proteins caveolins.[76-78] Lipid rafts and caveolae are involved in a number of protein transport processes, including transcytosis, potocytosis, and other clathrin-independent endocytic processes.[77,79] In addition, they are enriched in signaling molecules, and certain receptor, transducer, and effector proteins are recruited onto caveolae upon cell surface receptor activation implicating these structures as platforms for assembly and launching of multimolecular signaling cascades.[80-83]

Increasing evidences point to an important role of cholesterol in both structure and function of lipid rafts and their specialized forms caveolae. Cholesterol-binding drugs have been reported to disassemble caveolae which also appear significantly reduced in cholesterol-depleted cells.[83-85] Cholesterol content greatly affects both production and infectivity of the retrovirus HIV, which has been shown to hijack lipids rafts at multiple stages of its replication cycle.[86] These results follow many others showing that, in general, cholesterol-rich glycosphingolipid-rich domains serve as entry and exit sites for both pathogens and toxins (i.e., enterobacteria, influenza virus, measles virus, cholera toxin), and that caveolae, in particular, are required for the conversion of the nonpathogenic prion protein to the pathogenic scrapie isoform.[86 and references herein]

Although for the most part residing within the plasma membrane, cholesterol is not a static membrane constituent but it constantly moves between the cell interior and surface.[70,87] This movement appears a regulated process that shuttles cholesterol to the cell interior and backward to surface according to cell needs. The membrane compartments involved in cholesterol inward and outward flux are the cholestereol-rich domains, lipid rafts and caveolae,[88] and evidences have been presented that caveolin-1 is involved in transporting newly synthesized cholesterol from ER to caveolae.[89] On the other hand, cholesterol trafficking from plasma membrane to the ER has been reported to be mediated or facilitated by the MDR1 encoded P-gp[90-92] (Fig. 8.4).

An increasing number of data suggest a functional link between P-gp and cellular cholesterol. P-gp is predominantly localized in lipid rafts and caveolae[93] and its expression is regulated by the cholesterol levels.[94] When P-gp is reconstituted in proteoliposomes with cholesterol (MDR liposomes), it exibits a higher basal ATPase activity than in the absence of cholesterol,[95-97] confirming the presence of an endogenous substrate. This is also supported by a recent study[98] demonstrating that P-gp actively mediates a membrane cholesterol redistribution from the cytosolic leaflet to the exoplasmic leaflet. Accordingly, P-gp may mediate cholesterol enrichment of the exoplasmic leaflet of plasma membrane at the level of rafts and caveolae, and cholesterol itself may represent the endogenous substrate of P-gp.

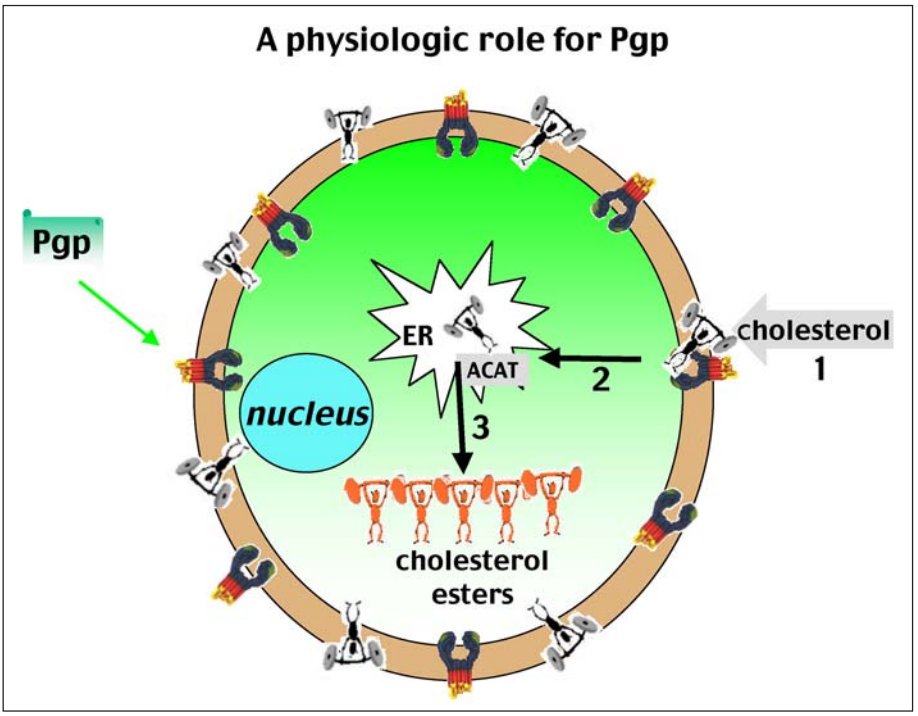

Fig. 8.4. A potential role for P-gp in intracellular cholesterol trafficking. Membrane cholesterol (1) is transported by P-gp (2) to ER where it is converted to cholesterol esters by ACAT (3).

In cells overexpressing P-gp (MDR phenotype), a marked changes in membrane lipid composition is observed.[99] Most notably, elevated levels of cholesterol, phospholipase-D, glycosphingolipids (e.g., glucosylceramide), and sphingomyelin have been reported. These lipids are enriched in caveolae and in lipid rafts. Lavie et al[99] demonstrated that in MDR tumor cells there is a dramatic increase in the number of caveolae and in the level of the essential structural constituent of caveolae, the caveolin-1. Other studies confirmed that in MDR cells overexpressing P-gp the amount of caveolin-1 is incresed and showed that, compared to parental cells, it appears more concentrated in the plasma membrane than in the cytosol.[100]

Recent investigations showed that overexpression of MDR1 reduces the susceptibility of CD4+ cells to HIV-1, likely by affecting viral fusion as well as downstream events.[101] Although the mechanism of inhibition of HIV infection is still not clear, the data indicate that the block cannot be explained by a major down-regulation or gross rearrangement of CD4, or coreceptor CXCR4, on the surface of MDR1-expressing cells. Experiments performed with an ATP binding site mutant of MDR1 indicate that ATPase acitivity of P-gp is not required for interference with HIV-infection. On the other hand, binding of MDR1-reversing agents (i.e., PSC833, quinidine) to the hydrophobic substrate binding site(s) of P-gp interferes in part with the ability of P-gp to block HIV-1 infection.[101] According to a recent report[102] the expression of the cholesterol binding protein caveolin-1 (and -2) correlates with the inhibition of HIV-1 production from cells that are ordinarily highly permissive to its infection. The inhibitory effect is specific, involves severely impaired expression of HIV-1 proteins, and seems mediated by the hydrophobic, membrane-associated domain of caveolins.

Altogether, these new insights have changed the view of cholesterol from simply being a membrane building block to a molecule that may exert, either directly or indirectly through the action of other factors, complex effects on overall cellular processes.

P-gp, Cell Growth and Cholesterol Esterification

As outlined above, several independent lines of evidence support the hypothesis that P-gp is involved in the intracellular cholesterol trafficking. A number of studies have correlated P-gp expression with regulation of endogenous biosynthesis and esterification of cholesterol in the ER, import of exogenous cholesterol from LDL by endocytosis, and export of cholesterol to the HDL.[88,103,104] In transfected intestinal cells producing P-gp, a marked increase in the uptake of exogenous cholesterol has been observed,[92] and in the presence of P-gp inhibitors, a decreased flux of cholesterol from the plasma membrane to the ER has been reported in P-gp overexpressing cells.[105] Moreover, by using the P-gp inhibitors verapamil, progesterone or other steroid hormones, which physically interact with P-gp to inhibit its activity, it has been demonstrated a positive correlation between MDR1 activity and cholesterol esterification in a number of cultured human cell lines.[54,67] Within a given cell type, greater expression of P-gp correlated with increased esterification of plasma membrane cholesterol, thus suggesting that P-gp increases esterification of the cholesterol derived from plasma membrane by facilitating the movement of cholesterol from the plasma membrane to the ER[54,55] (Fig. 8.4).

Despite of all the above evidences, however, the role of P-gp in the process of cholesterol esterification is still subject of debate, at least with respect to the origin of the cholesterol undergoing the conversion to cholesterol esters. Issandu and Grand-Perret[106] have recently observed in HepG2 cells and in the MDR1-overexpressing cells MCF7/ADR, that neither of the two P-gp inhibitors used in the study (i.e., progesterone and GF120918) affected the esterification of the cholesterol derived from the uptake of cholesterol-rich lipoprotein.

Another molecular mechanism which appears to link cholesterol esterification potential to P-gp expression is regulation of cell proliferation. Cholesterol metabolism had been studied extensively in our laboratories in different models of cell proliferation as well as in several types of human neoplasms.[107-112] A constant finding of these studies had been the higher esterification of cholesterol in all proliferating tissues, either normal or pathologic (Fig. 8.4). More recently, in in vitro studies on leukemia derived cell lines (i.e., MOLT4, CCRF-CEM, L1210), which had different doubling times, we demonstrated that the rate of cell growth correlates with the cholesterol esterification capacity of the cells as well as with the expression of ACAT and MDR1 genes[67,68] (Fig. 8.5A). Worth of noting is that neither the absolute level of cholesterol neosynthesis nor that of cholesterol uptake appeared to positively correlate with the differences in the cell growth rates of the different cell lines. In another report,[113] we have also showed that the levels of cholesterol esters increase with age in arteries prone to atherosclerosis and become predominant in advanced atherosclerotic lesions. The mRNA levels of ACAT and MDR1 showed the same correlation with age and reached the highest levels in atherosclerotic specimens, thus suggesting that P-gp may be involved in the accumulation of intracellular cholesterol esters in the atherosclerotic lesions which are the result of an abnormal proliferative process.[113 and Chapter 9]

These findings supported the hypothesis that P-gp might be involved in the regulation of cell proliferation by modulating the intracellular levels of cholesterol esters. To investigate this matter further, cell lines displaying documented differences in the expression and function of P-gp, i.e., cells with a MDR phenotype versus cells with a drug-sensitive phenotype, appeared most suitable. To this end, we compared cell growth rate, cholesterol metabolism, MDR1 gene expression, P-gp activity, and drug-resistance pattern in cell lines with differences in expression and possibly function of the MDR1 gene product P-gp[114] (Fig. 8.5 B). The cell lines used in this study were the human nasopharyngeal carcinoma KB cells (drug-sensitive parental cells)

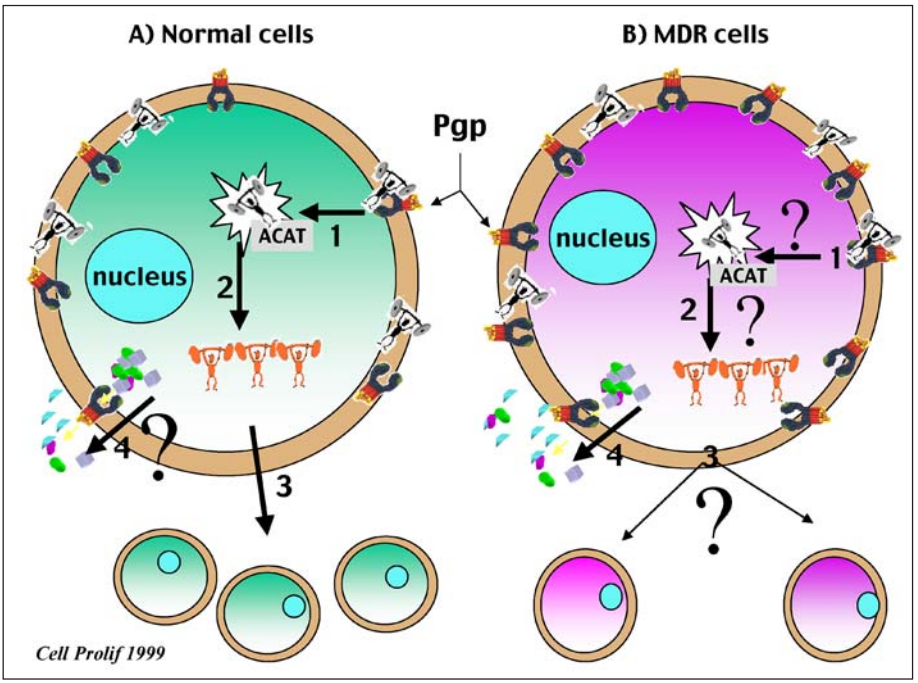

Fig. 8.5. Different functionality of P-gp in normal (A) versus MDR (B) cells. A) In non-MDR cells the amount of P-gp (1) positively correlates with the extent of cholesterol esterification (2) and with the rate of cell proliferation (3). These data support the hypothesis that in normal cells P-gp functionality is not related with drug efflux function (4?). B) In MDR cells P-gp (1) seems not involved in the process of cholesterol esterification (2) and, consequently, with the rate of cell proliferation (3) functioning mainly as drug efflux pump (4).

and two KB derived MDR-cell clones: KB^{V20C} (cells selected in the presence of increasing vincristine concentrations) and KB^{MDR} (cells engineered with the human MDR1 gene and maintained in culture in the presence of doxorubicin).

Several lines of evidence indicated that the MDR phenotype of the two MDR KB cell lines used in the study was mediated by increased levels of functional P-gp:

i. relative to parental KB cells, both KB^{MDR} and KB^{V20C} showed increased resistance to the MDR drugs vincristine, doxorubicin and etoposide, but not to non-MDR drugs cis-platinum and camptothecin;
ii. they had 10-fold lower levels of intracellular labeled vinblastine that can be increased up to that of parental KB cells levels by verapamil and progesterone;
iii. both MDR cell lines over-expressed MDR1 mRNA and P-gp protein relative to parental KB cells.

In agreement with our previous findings,[67,68] we found that the rate of cell growth correlated with the rate of cholesterol esterification also in the KB cell lines, irrespective of their drug-phenotype.[114] KB^{MDR} cells, which have a doubling time shorter than parental KB cells (20 hours vs 24 hours) (Fig. 8.6), showed higher rates of esterification of cholesterol, derived from either membranes, or neosynthesis, or total esterification (Fig. 8.7A). On the contrary, KB^{V20C} cells that have a consistently longer doubling time (30 hours) than the other two cell lines (Fig. 8.6), showed the lowest cholesterol esterification capacity (Fig. 8.7A). Progesterone and verapamil inhibited both cholesterol esterification (Fig. 8.7A) and cell growth (not shown).

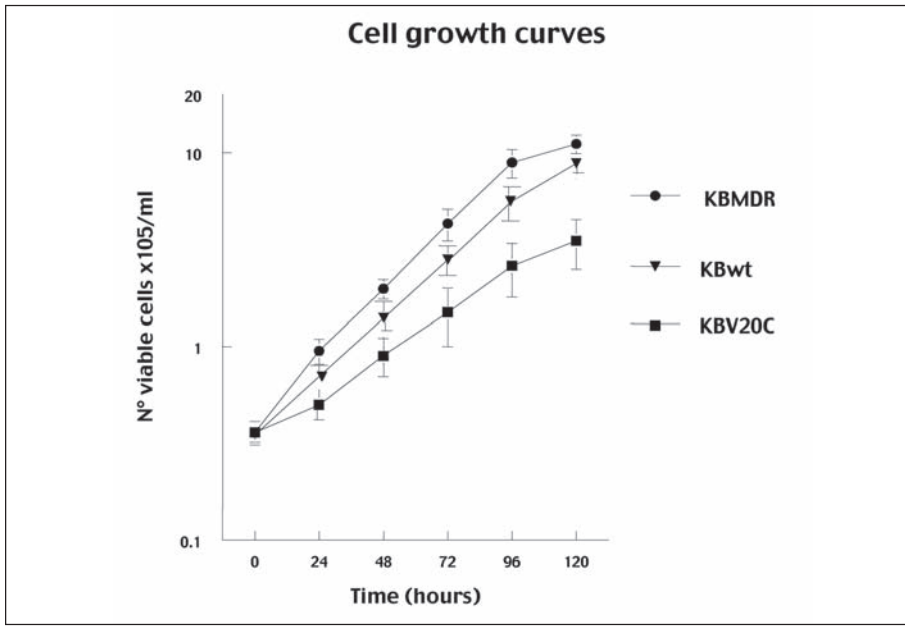

Fig. 8.6. Cell growth curves of KB cells (wild type) and of two KB MDR subclones (KB MDR, KB cells transfected with human MDR-1 gene; KB V20C, KB cells selected in the presence of increasing VCR concentrations).

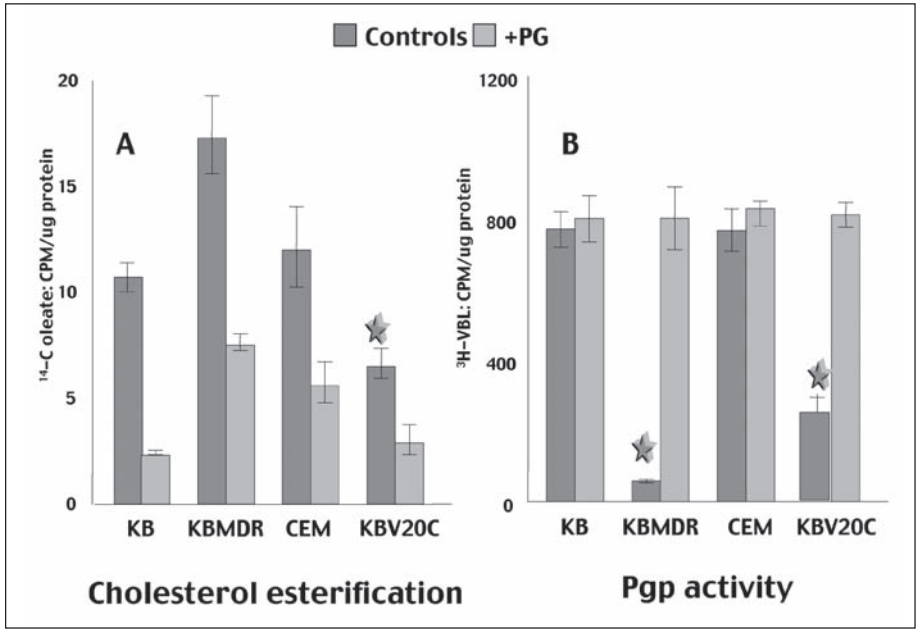

Fig. 8.7. Effect of progesterone on cholesterol esterification (A) and P-gp activity (B) in normal (KB; CEM) and MDR (KB MDR; KB V20C) cells.

However, unlikely that functioning in CEM and MOLT4 cell lines,[67,68] the P-gp present in drug-resistant cells, seemed not involved in the process of cholesterol esterification.[114] The increased levels of both MDR1 gene expression and functional P-gp in the two MDR cell lines, paralleled with the increased capacity to esterify cholesterol only in the KB^{MDR} cells. In KB^{V20C} cells, instead, the over-expression of both MDR1 gene and its product P-gp was associated to a reduced cholesterol esterification capacity (Fig. 8.7A).

Moreover, in both parental and MDR KB cell lines, the process of cholesterol esterification seems independent from activity of functional P-gp (Fig. 8.7A, B). In fact, the inhibitory effect of P-gp inhibitors on cholesterol esterification did not always correlate with their effect on P-gp activity. For instance, while inhibiting cholesterol esterification in all KB cell lines, neither verapamil nor progesterone determined an increase of the intracellular ^{3}H-vinblastine in the parental KB cells, despite of the constitutively high levels of MDR1 mRNA in these cells. That the observed effects of progesterone and verapamil on cholesterol esterification are independent on P-gp expression levels, appears also supported by the data obtained in PBLs.[114] These cells, that remain P-gp negative after mitogen stimulation, and are not affected by progesterone and verapamil in their ability to accumulate intracellular vinblastine, are strongly inhibited in their cholesterol esterification capacity by both P-gp inhibitors.

That functional P-gp in MDR cells is not involved in the transport/esterification process of cholesterol remains, however, to be unequivocally demonstrated. With respect to the data obtained in the KB cell lines, it has to be taken into consideration that the KB^{MDR} cells might represent a better model than KB^{V20C} cells to study the role of MDR1 P-gp in both cholesterol esterification and cell proliferation. In fact, KB^{MDR} cells were obtained following infection of KB cells with a retroviral vector carrying the human MDR1 gene. Therefore, they likely differ from the parental KB cell line only at the level of expression of this gene. On the contrary, KB^{V20C} cells were obtained by stepwise drug selection of resistant mutants in the drug-sensitive population and the MDR phenotype might be associated to genotypic modifications additional to that in the MDR1 gene, which may in turn influence the overall metabolism and cell cycle of the cells.

The different functional behaviour of P-gp with respect to cholesterol transport and esterification in normal vs MDR cells, may be also a consequence of drug selection of cells bearing P-gp with amino acid sequence modifications leading to changes in its substrate specificity. Mutations leading to amino acid substitutions had been reported to alter the drug-substrate spectrum of P-gp as well as the effectiveness of drug transport and the sensitivity of P-gp toward inhibition with specific inhibitory substances.[115] Thus, sequence modifications may also determine variation in the specificity of P-gp for its potential endogenous substrate, cholesterol. We can then reasonably suggest that the overall MDR1 P-gp activity depends on the following: a) level of expression of the MDR1 gene that controls the amount of P-gp protein synthesized, and b) functionality of the MDR1 encoded P-gp that determines which substrate(s) is recognized and transported with what effectiveness.

Conclusions

All the data herein summarized clearly point to multiple activities and functions of both cholesterol and P-gp in eukaryotic cells, some of which also appear interconnected with each other. Cholesterol now stands as a molecule essential to the cells not only for its function on membrane biogenesis and support, but also for its participation to many diverse and complex cellular processes. Among these, several data indicate that cholesterol esters, and the genes correlated with cholesterol esterification, play a role in the regulation of cell growth rate. As we have recently suggested,[116] one possibility is that cholesterol esters influence the balance of the growth regulator factors in those portions of cell membranes, the caveolae, where the signalling

events take place. In the overall process, the drug efflux pump P-gp responsible for the MDR phenomenon, may have an important part by modulating the intracellular cholesterol esters levels. In fact, if cholesterol truly represents the endogenous substrate of the physiologic P-gp, then this protein would mediate the transport of cholesterol to the ER where it is converted to cholesterol esters. Although a number of aspects still remain to be explained, and even if some involved players may still be missing, this fascinating hypothesis would also open the way to a different approach for the control of the neoplastic proliferation. In fact, as it is discussed in Chapter 11 of this book, the neoplastic cells, by virtue of their renewed ability to proliferate, might offer to chemotherapy a novel Achille's heel in both P-gp activity and cholesterol esterification.

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Chapter 9

Involvement of Cholesterol Ester Cycle in the Progression of Atherosclerosis

Maria Franca Mulas and Sandra Dessì

Atherosclerosis and its associated clinical entities, coronary artery, cerebrovascular, and peripheral vascular disease, is the major cause of death in industrialized countries. Hypercholesterolemia, cigarette smoking and hypertension are considered the principal risk factors for atherosclerosis and thus the best potential targets for pharmacological management of cardiovascular disease. However, despite changes in lifestyle and the use of new pharmacological approaches to lower plasma cholesterol concentrations, cardiovascular disease continues to be the principal cause of death in the United States, Europe, and much of Asia.

Until recently, atherosclerosis was thought as a degenerative, slowly progressive disease, predominantly affecting the elderly, and causing symptoms through mechanical obstruction to blood flow. However, recent researches into the cellular, biochemical and molecular events underlying atherosclerosis had led to a revision of these ideas.[1] It now understood that atherosclerosis is a dynamic process and its lesions represent a series of highly specific cellular and molecular responses to various forms of injurious stimuli to the arterial wall that can be best described, in aggregate, as a chronic inflammatory disease.[2] Thus studies illustrating the basic mechanisms of the pathogenesis of atherosclerosis apart from to enable us to understand how current therapies for atherosclerosis may act, should also aid the discovery of new therapeutic targets that would stimulate development of novel treatments. It appears that in the future the prevention of cardiovascular disease will involve not only risk factor correction, but also direct pharmacological control of processes occurring in the arterial wall. Such new treatments could further reduce the considerable burden of morbidity and mortality due to this modern scourge, and reduce reliance on costly technologies that address the symptoms rather than the cause of atherosclerosis.

Cell migration and proliferation, together with lipid deposition, are now recognized as crucial players in the atherosclerotic process. Thus, an understanding of the factors that induce and link such events may be important for the prevention and treatment of this disease.

This chapter, after an introduction to some general features of the process, will focus on the possible involvement of cholesterol ester cycle in the genesis of the atherosclerotic lesions.

Structure of the Normal Arteries

Arteries are living, dynamic vessels not mere pipes carrying fluid like the drain of the kitchen sink. The normal artery wall consists of three layers: the intima, the media, and the adventitia (Fig. 9.1).

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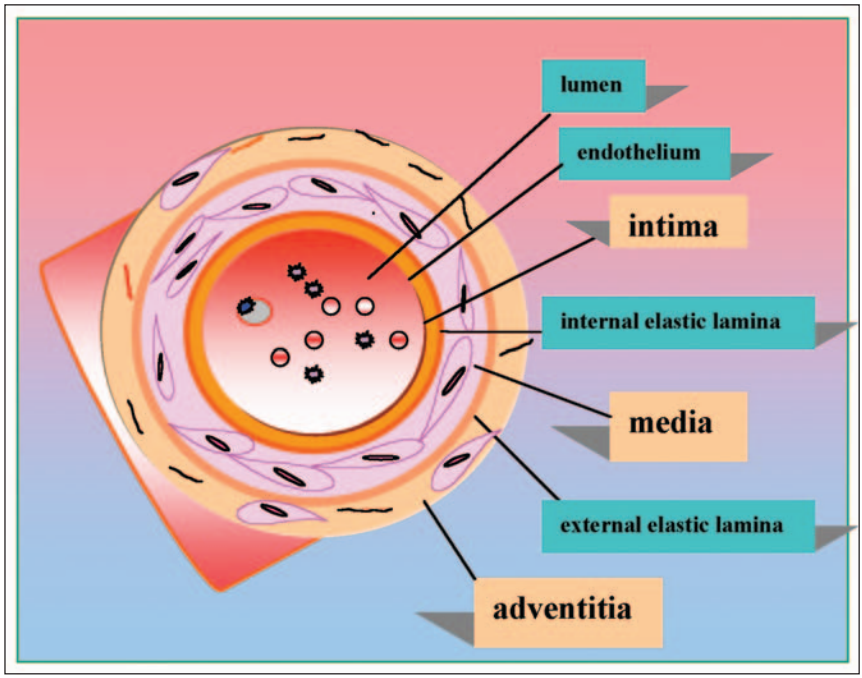

Fig. 9.1. Normal arterial wall. The normal artery wall consists of three layers: the intima, the media, and the adventitia.

The innermost layer (intima) is made up of three smaller layers: an elastic layer (internal elastic lamina) and a basement membrane layer that anchors the third layer, the endothelium. The atherosclerotic process involves this layer of artery.

The media consists of only one cell type, the vascular smooth muscle cell (VSMC), arranged in either a single layer or multiple lamellae. These cells are surrounded by small amounts of collagen and elastic fibers, which they elaborate, and usually take the pattern of diagonal concentric spirals through the vessel wall. The VSMC appears to be the major connective tissue-forming cell of the artery wall, producing collagen, elastic fibers, and proteoglycans. In this sense it is analogous to the fibroblast in skin, the osteoblast in bone, and chondroblast in cartilage. The media is bounded on the luminal side by the internal elastic lamina and on the abluminal side by a less continuous sheet of elastic tissue, the external elastic lamina.

The adventitia is the outermost layer of the artery, which is delimited on the luminal aspect by the external elastic lamina. This external coat consists of a loose interwoven admixture of collagen bundles, elastic fibers, smooth-muscle cells, and fibroblasts. This layer also contains the vasa vasorum and nerves.

Lesions of Atherosclerosis

Atherosclerosis is a complex pathophysiological process characterized by various progressive lesions commonly classified as:

1. early lesions (initial lesions and fatty streaks),
2. intermediate lesions,
3. fibrous plaques, and
3. advanced, complicated lesions

Initial Lesions

Initial lesions (fatty streaks) are focal, small and non obstructive and consist of lipid deposition in intimal macrophages (macrophage foam cells), and represent the first changes that have been found to evolve into lesions associated with clinical disease.

Intermediate Lesions

Intermediate lesions other than macrophage foam cells also contain a large accumulation of lipid filled smooth muscle cells and fibrous tissue in focal areas of the intima. The lipid is mainly cholesterol oleate and is mainly intracellular.

Fibrous Plaques

Fibrous plaques are palpably elevated areas of the intimal thickening mainly consisting of a cap containing large numbers of smooth muscle cells, macrophages, and fibrous tissue covered by a layer of endotelium, and a central core of extracellular lipid and necrotic cell debris.

Advanced Complicated Lesion

The advanced, complicated lesion is a calcified fibrous plaque and is considered the usual cause of mechanical obstruction to blood flow (stenosis).

Pathogenesis of Atherosclerosis

Among the many hypotheses developed to explain atherosclerosis the most commonly accepted is the response-to injury, first proposed by Ross and Glomset[3] in 1973 which emphasizes the importance of the endothelial damage as the initiating in atherogenesis.

According to this hypothesis, the endothelial cells lining the intima are exposed to repeated or continuing insults to their integrity. Examples of these insults include metabolic injury, as in chronic hypercholesterolemia, homocysteinemia or cigarette smoking, mechanical stress associated with hypertension, immunologic injury as may be seen after cardiac or renal transplantation, infectious microorganisms such as herpesviruses or Chlamydia pneumoniae, and combinations of these or other factors.

If damaged or dysfuncted, endothelial cells express adhesion molecules on their surface able to attract and capture inflammatory cells, particularly monocytes. Once bound to the endothelium, the monocytes migrate into the subendothelial space where they differentiate into macrophages. This process qualifies atherosclerosis as an inflammatory disease, and the recruitment of inflammatory cells has become an area of interest with regard to possible intervention.[2]

Injured endothelium could also allow entry or retention of low density lipoprotein (LDL) that accumulates in the intima and become oxidized (oxLDL) in the microenvironment of the subendothelial space. OxLDLs are then recognized by specific receptors present in the membrane of macrophage, known as “scavenger receptors”, and ingested by macrophages that become lipid-engorged or “foamy”. Hence, the term “foam cells” has been coined to describe the major cell type within fatty streaks as well as more advanced lesions.

If the offending agents are not effectively neutralized or removed, this response can be amplified and in the site of injury, endothelial cells and macrophages can elaborate chemoattractant proteins resulting in increased numbers of macrophages and T lymphocytes, which both emigrate from the blood and multiply within the lesion. Activation of these cells leads to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors. This could stimulated migration of VSMCs from their usual location in the media into the intima where they become intermixed with the area of inflammation to form an intermediate lesion. If

this response continue, VSMCs can proliferate, accumulate lipid becoming foam cells, deposit a connective tissue matrix and form a fibrous cap over the lipid and inflammatory cells (fibrolipidic plaque). As the lesion progresses and the intima becomes thicker, blood flow over the sites will be altered and will potentially place the lining endothelial cells at even greater risk for further injury with eventually focal necrosis and or apoptosis and deposition of extracellular cholesterol esters (necrotic core). This lead to inexorable cycles of intracellular and extracellular lipid accumulation, migration and proliferation of inflammatory cells, and formation of fibrous tissue culminating in the complicated lesion (for a review see ref. 2 and Fig. 9.2).

Plaque Rupture

Until recently atherosclerosis was thought as a degenerative disease causing symptoms through mechanical obstruction to blood flow. However, recent observations indicating that arterial stenoses per se rarely cause myocardial infarctions, had led to a revision of how atherosclerosis causes acute cardiac events and thus to the concept of lesion "activation", a process by which a quiescent atherosclerotic plaque (stable plaque) becomes susceptible to rupture and thrombosis (unstable plaque).[1]

Unstable plaque contains numerous inflammatory cells and few VSMCs. Inflammatory cells, particularly macrophages, secrete and activate matrix metalloproteinases (MMP), which break down extracellular matrix components, resulting in destruction of the collagenous matrix of the fibrous cap. If this is not balanced by repair, the cap thins and becomes increasing vulnerable to rupture.[4,5]

Several studies have reported a relation between the cholesterol content of plaques and their tendency to rupture, suggesting that progressive accumulation of lipids may destabilize plaques, leading to thinning, weakening, and ultimately destruction of the fibrous cap, with a subsequent rupture at points of high stress. In most cases, the fissure occurs at the junction of the plaque and normal intima (shoulder regions), a site often containing lipid-laden cells.[6] Richardson et al[7] examined coronary atherosclerotic plaques in patients who had died from coronary thrombosis and found that 87 percent of the fissured plaques contained a pool of extracellular cholesterol esters. Postmortem histologic studies and computer modeling have suggested that lipid-laden plaques are most likely to rupture when exposed to an increased shear rate, acute changes in coronary-artery pressure, or mechanical deflection of the artery during each heart contraction.[8,9]

In this regard, the chemical form of cholesterol within the lesion may have considerable importance. Cholesterol ester, usually liquid at body temperature, is relatively soft and thus a large pools of cholesterol esters can predispose plaques to rupture because the wall stress concentrates on the "shoulders" of the lesions on either side of the lesion surrounding the lipid core. Free cholesterol, in contrast, tends to form crystals, shown by biomechanical modeling to yield a "harder" plaque, less susceptible to fracture and hence to thrombosis.[10-12] Thus, cholesterol ester modifying therapy, by limiting the cholesterol ester pool of an atherosclerotic plaque, may reduce plaque thinning and the incidence of subsequent rupture.

Roles of Cell Proliferation and Cholesterol Ester Accumulation in the Pathogenesis of Atherosclerosis

Among the events involved in the pathogenesis of atherosclerosis, excessive cell proliferation together with cholesterol esters that accumulate in lesions at every phase of the disease are considered two of the most important factor contributing to atherosclerosis, having an important role not only in the early atherogenesis and in the progression of advanced lesions, but also in the induction of clinical manifestations that are the leading cause of mortality and morbidity.

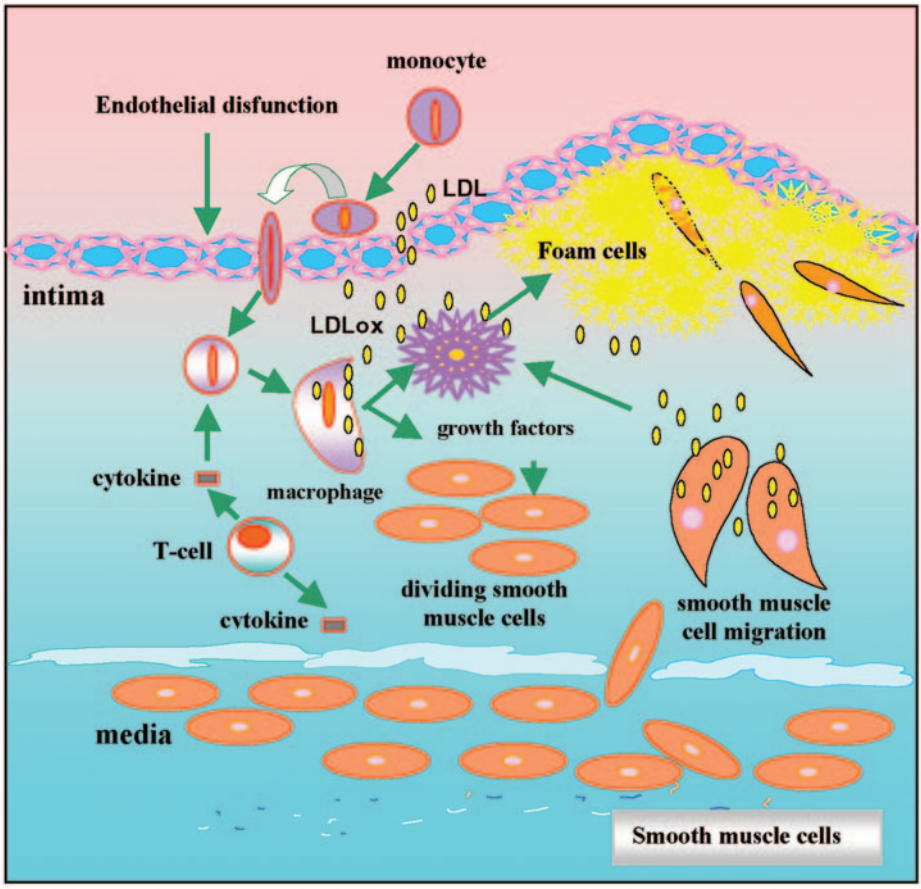

Fig. 9.2. Pathogenesis of atherosclerosis. Following injury, endothelial cells express adhesion molecules on their surface facing the lumen of the blood vessel. These attract and capture inflammatory cells, particularly monocytes, which migrate into the intima and differentiate into macrophages. Macrophages express receptors known as the 'scavenger receptor' on their surface, which allows them to ingest oxidized lipid. These macrophages engorged with lipid are called 'macrophage foam cells'. The foam cells and T-cells produce a variety of cytokines and inflammatory mediators, many of which are chemoattractant for the underlying vascular smooth muscle cells (VSMCs). Thus VSMCs emigrate from their usual location in the media into the intima where they form a fibrous cap over the lipid and inflammatory cells. In so doing they adopt a reparative role in contrast to their normal contractile role in the media. As the VSMC is the only cell in the vascular wall capable of performing this healing process, it follows that the change from contractile to synthetic or repair phenotype is not detrimental, but is in fact vital for the development of the fibrous cap and therefore plaque stability.

Proliferating Cells in the Artery Wall

The cell types of the artery wall, the endothelial cell, the VSMC and the monocyte-macrophage, are major players in the events involved in initiation and evolution of the atherosclerotic plaque. Other important participants are platelets and lymphocytes, which have modulating influences on VSMC, endothelial cells and macrophage behavior.

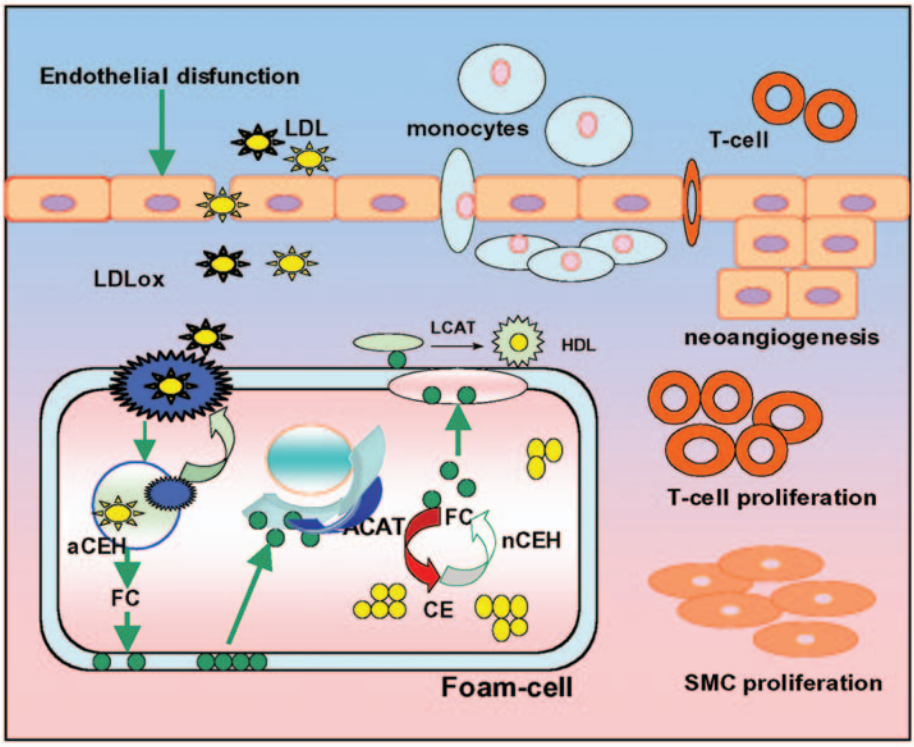

Fig. 9.3. Cholesterol esterification and cell proliferation during atherogenesis. The progression of atherosclerosis is characterized by two main events: cholesterol esterification and cell proliferation. As indicated in the figure, the role of cholesterol esterification in the progression of the lesions is not limited to mere accumulation of cholesterol esters inside the cells, but the whole cholesterol esterification pathway is involved. On the other hand, cell proliferation is not limited to smooth-muscle cells (SMC) as initially reported, but also replication of monocyte-derived macrophages, T-cells and endothelial cells is probably of equal importance for the expansion of atherosclerotic lesions.

It has been thought for quite a long time that atherosclerotic plaque are a sort of tumor, in that the cells composing them proliferate abnormally.

Originally, the only cell type thought to proliferate was VSMC. Today several lines of evidence indicate that also macrophages and macrophage-derived foam cells may proliferate in vitro as well as in situ in atherosclerotic lesions. Moreover, many studies have demonstrated that also other types of cells besides macrophages and smooth muscle cells can proliferate within atherosclerotic lesions.

The presence of neovascularization within human atherosclerotic lesions has been recognized for some time indicating that also proliferation of endothelial cells may have a potential role in promoting the progression of the disease. Finally active proliferation of CD3-positive T cells was also evidentiated in human atherosclerotic plaques (for a review see ref. 2 and Fig. 9.3). These cells secrete interferon-gamma (INF-γ), that not only can enhance macrophage foam cell formation but also can inhibit collagen synthesis by VSMC, thus contributing to plaque instability.

In summary, although the amount of each cell type differ according to the stage of lesions, all cellular components are always present and proliferating.

Cholesterol Ester Accumulation

The importance of cholesterol ester accumulation in the induction of atherosclerotic process was recognized early in this century. However, it is still unclear the mechanism by which cholesterol esters accumulate in cells leading to foam cell formation, and whether cholesterol esters are simply "innocent bystanders" or play a direct causal role in the progression of the disease.

The fact that feeding cholesterol to animals elevates plasma cholesterol levels and simultaneously induces atherosclerosis, led to the widely accepted conclusion that plasma cholesterol carried in lipoproteins (mainly LDL) is the main source of cholesterol esters that accumulates in foam cells. This idea is reinforced by numerous studies showing a correlation of plasma cholesterol levels and low density lipoprotein (LDL) with the development of coronary artery disease.[13]

The idea is that, elevated LDL levels damage endothelial cell layer and penetrate into the arterial intima. The accumulation of LDL in the arterial wall initiates monocytes and smooth muscle cell migration and transforms them into cholesterol-load foam cells.

The formation of the advanced atherosclerotic plaque's dangerous lipid core is thought to be the result of cholesterol released from dying foam cells. This lipid core expands and contributes to rupture of the plaque.

Although, the above hypothesis is the most commonly accepted among investigators for explaining lipid accumulation in atherosclerosis, some problems have not been yet resolved.

While LDL is believed to be the major source of cholesterol that accumulates in atherosclerotic plaques and in foam cells, it has so far proved impossible to transform monocytes into foam cells by incubating them with LDL in vitro. From this came the belief that LDL must be modified in some way to promote its uptake by vascular cells. Oxidation of LDL is one modification that has attracted the attention of researchers and has led to the oxidation hypothesis of foam cell formation. This hypothesis suggests that oxidation of LDL alters LDL such that it is recognized and taken up by macrophage receptors (scavanger receptors) converting the macrophage into a foam cell.[14-16]

There is now much evidence indicating that oxidized LDL (Ox-LDL) is present in atherosclerotic lesions in vivo.[17]

It was presumed that Ox-LDL mainly occurred locally in the arterial wall after entrance of normal LDL,[18] however, more recent studies have suggested that very small amounts of Ox-LDL are also present in plasma (see review from Nielsen[19]). These changes could have occurred elsewhere, or during a previous transient passage through the artery wall. Such minimally modified LDL might then be "primed" for more rapid oxidative modification on a subsequent entry into the intima. Therefore, Ox-LDL in the arterial wall can be derived both from normal LDL oxidized locally in the arterial intima and from Ox-LDL in plasma.[19]

Another paradox is that if cholesterol esters in foam cells are derived by LDL or other lipoprotein particles and the lipid core of atherosclerotic plaques is a consequence of released cell cholesteryl ester-containing lipid droplets, both intracellular and extracellular cholesterol in atherosclerotic plaque must resemble lipoproteins. However, it should be noted that much (if not most) of the cholesterol in foam cells does not resemble any blood lipoprotein. The fatty acyl chain of cholesterol esters in foam cells is in fact oleate, while LDL cholesteryl esters is predominately linoleate. By contrast, extracellular lipid particles, in the necrotic core, have cholesteryl esters with predominately linoleate similar to the fatty acid profile of LDL cholesteryl esters.

Cholesterol Ester Cycle

There is currently strong evidence that Ox-LDL in the arterial wall are taken up into atherosclerotic cells by scavenger receptors (class A or CD36) which reside in coated

(clathrin-associated) pits of cell membranes, The term 'scavenger receptor' was coined to describe the activity of macrophages which mediates the uptake of modified forms of LDL in cell culture. Other cell types such as endothelial cells and smooth muscle cells have been shown to have different receptors for modified LDLs and the scavenger receptor family has grown to include cell surface receptors which mediate cholesterol transport by 'scavenging' cholesterol from HDL (see next paragraph).

But what are the endocytic pathways by which cells accumulate their cholesterol to become foam cells?

Brown and Goldstein[20,21] first discovered that the cholesteryl esters in the modified LDL are not directly accumulated in the cytoplasm as lipid droplets, but that they are first hydrolyzed in lysosomes to free cholesterol and then re-esterified prior to intracellular storage as lipid-droplets. Numerous studies indicated that after Ox-LDL internalization, cholesterol esters present in the core of lipoprotein, undergo hydrolytic conversion to free cholesterol by acid cholesterol hydrolase (aCEH) in lysosomes. The free cholesterol released into cytosolic space moves to the plasma membrane, where it is utilized for membrane turnover. The excess of membrane cholesterol, rather than to be excreted from the cells, is transferred to endoplasmic reticulum, where it is trapped in a continual cycle of esterification catalyzed by the enzyme acyl-CoA:cholesterol acytransferase (ACAT) and its subsequent release catalyzed by a neutral cholesterol hydrolase (nCEH). This is called the "cholesteryl ester cycle".

Thus the level of intracellular CE is governed by a balance between its synthesis and degradation. Whether a foam cell will increase or decrease its stores of CE depends on the relative activity on nCEH and ACAT. These findings nonetheless point to a potentially important role of cholesterol esterification pathway in accelerating arterial lesion formation. Since the ACAT enzyme prefers oleate to linoleate during cellular esterification of cholesterol, and availability of oleate in the cells modulates how much cholesteryl ester is synthesized, the fatty acid profile of foam cell-derived cholesteryl esters is predominantly oleate.[22] This explains why lipid droplet cholesteryl ester in lesion foam cells shows a fatty acid profile different from LDL cholesteryl ester.

Cholesterol Efflux

Another important point in understanding foam cell formation is the question of how cholesterol in vascular cells is retained by the cells rather than being released into the plasma compartment.

Reverse cholesterol transport, defined as the flux of cholesterol from peripheral tissues to the liver, where it is excreted in the form of bile salts, is an important mechanism in the removal of cholesterol from sites of lipid deposition. HDLs are specialized lipoprotein cholesterol acceptors that circulate in the blood. When incubated with macrophages they induce cholesterol efflux by stimulating translocation of cholesterol from intracellular membranes to the plasma membrane. Then, the HDL acquires excess plasma membrane cholesterol. This also occurs with lipid-free amphipathic apolipoproteins of HDL such as apoAI that associate with macrophage phospholipid and form nascent high density lipoprotein particles.[23-27] The potential importance of such a process in humans is supported by several lines of evidence. Epidemiological studies show an association between HDL cholesterol levels and a reduction in cardiovascular events that is independent of LDL cholesterol levels. In addition, data from the angiographic lipid-lowering trials suggest that an increase in HDL cholesterol levels is an independent predictor of the regression or slowed progression of lesions.

The receptor mediating HDL binding has now been identified as scavenger receptor class B type 1 (SR-B1).[28] It binds HDL with high affinity and mediated bi-directional flux of cholesterol across the plasma membrane. SR-B1 can bind HDL reversibly and mediate cholesterol efflux and cholesteryl ester uptake, depending on cell type.

Overexpression of SR-B1 in liver increases cholesterol ester uptake and reverse cholesterol transport, and decreases susceptibility to atherosclerosis. By contrast, inhibition of its activity in vascular cells, decreases cholesterol efflux, favors cholesterol esters accumulation and accelerates the onset of atherosclerosis.[29,30]

Of interest, in a recent study, Han et al[31] showed that exposure of macrophages to Ox-LDL decreased SR-B1 expression in a dose-and time-dependent manner. These results imply that in addition to its effect in inducing foam cell formation in macrophages through increased uptake of oxidized lipids, Ox-LDL may also enhance foam cell formation by altering SR-B1-mediated efflux, which would act in synergic manner to promote lipid retention within macrophage.

Possibility of a Link between Cholesterol Esterification and Cell Proliferation during Atherogenesis

Cholesterol esterification, via ACAT, occurs according to the availability of cholesterol substrate in endoplasmic reticulum (ER). Although nascent cholesterol in the ER is most efficiently used for esterification by virtue of its proximity to ACAT, only a small percentage is esterified directly. Virtually all free cholesterol esterified in vivo derives from plasma membrane suggesting that a control membrane-associated mechanism might be involved in the delivery of cell surface sterol to ER.[32-34] Recently, a model has been proposed whereby a p-glycoprotein encoded by (multidrug resistance) MDR1 gene (MDR1 P-gp), better known for its ability to catalyse ATP-dependent efflux of cytotoxic agents from tumor cells when overexpressed (see also chapters 6,7,8,11), is also required for transport of free cholesterol from the plasma membrane to the ER where it is esterified by ACAT.[35,36] Another protein that interacts with cholesterol trafficking is caveolin-1. It is believed to take part in the transport of cholesterol from the ER to the caveolae membrane [37,38] and to mediate the efflux of excess free cholesterol from the cells.[39,40]

Starting from these notions, we begun a series of studies tending to demonstrate a possible mechanistic link between cholesterol esters and ACAT, MDR1 and caveolin-1 gene expression during the course of atherosclerotic process.

Thus, we evaluated cholesterol ester content, ACAT, MDR1 and caveolin-1 gene expression in the majority of human arteries prone and resistant to atherosclerosis as well as in human vein walls which usually do not present atherosclerotic lesions.[41-44]

Healthy segments of carotid common artery, abdominal aorta, iliac artery, superficial femoral artery (prone arteries), profunda femoral artery, and internal mammary artery (resistant arteries) have been obtained from cadaveric donors ranging in age from 16 to 48 years. It is worthy of note that the donors were all multiorgan donors and did not present symptomatic and/or macroscopic atherosclerotic lesions.

Samples of human atherosclerotic lesions of internal carotid artery, abdominal aorta, iliac artery, superficial femoral artery, were obtained from patients, ranging in age from 37 to 78 years, undergoing surgical intervention for occlusive carotid disease, aortic aneurysm or thrombosis, severe chronic leg ischemia. Histologically all specimens presented advanced atherosclerotic lesions. In addition, tissue samples of saphenous vein were obtained from patients undergoing saphenous vein stripping.

Mapping of the arterial tree showed an increase in cholesteryl ester content as well as in ACAT and MDR1 gene expression in the arteries prone to atherosclerosis. The expression was maximal in atherosclerosis-prone arteries from symptomatic patients, who underwent vascular surgery of the abdominal aorta, carotid artery, and superficial femoral artery .

On the contrary, cholesteryl ester accumulation in veins of the lower limb was very light and the amount of cholesteryl esters was not related to the age of subjects who underwent saphenous vein stripping. A low expression of the genes correlated to cholesterol metabolism such as ACAT and MDR1 genes has also been observed in the saphenous veins.

These studies indicate that expression of MDR1 is positively correlated with that of ACAT gene, and both with the intracellular levels of cholesteryl esters, suggesting a possible involvement of MDR1 in the evolution of atherosclerotic lesions. We hypothesize that, during atherogenesis, the increased internalization of Ox-LDL via scavenger receptors by atherosclerotic cells (macrophages and smooth muscle cells), may cause an accumulation of free cholesterol in the plasma membrane deriving from hydrolytic conversion of cholesterol esters-LDL in lysosomes. Subsequently, in order to avoid plasma membrane toxicity, MDR1 gene is stimulated to produce P-gp which transports the excess membrane free cholesterol to the ER; here it is immediately esterified by ACAT, resulting in a cytoplasmic accumulation of cholesterol esters with consequent formation of foam cells.

Another interesting finding of our studies was that caveolin-1 was highly expressed in vein walls and in arteries resistant to atherosclerosis, while very low mRNA levels were observed in the arteries prone to atherosclerosis and particularly in the atherosclerotic arteries.

It has been shown that caveolin-1 is required for the translocation of cholesterol from the ER to the plasma membrane and is involved in cholesterol efflux.[39,40]

Although the molecular mechanism involved in free cholesterol efflux by caveolin-1 is not yet known, it has been postulated that this protein may be capable of mediating or facilitating the export of free cholesterol via the SR-B1 HDL receptor.

Since veins and resistant arteries express higher level of caveolin-1, we supposed that this protein, by limiting cholesterol ester accumulation inside the cells, may prevent the early stages of atherosclerotic process in these vessels.

In addition to its role in cholesterol binding and transport, recent evidence suggests that caveolin-1 may participate in signal transduction-related events as well.[45-48]

It has been suggested that caveolin-1 is able to bind various signaling molecules involved in cell division, such as MAPK cascade, via a 20 aminoacyl residue domain, termed the "caveolin scaffolding domain". This bind results in an inhibition of the activity of the signaling molecules suggesting that one functional consequence of the upregulation of caveolin may be a slower rate of proliferation.

These findings raise the possibility of a mechanistic link between cholesterol ester cycle and proliferation of vascular cells.

Figure 9.4 describes a series of hypothetical events by which cholesterol esterification pathway in itself could account for the progression of the atherosclerotic lesions.

Conclusions

Recent years have seen advances in our understanding of the biologic properties of the individual cells of artery wall, and of the modifications in cholesterol esterification pathway that may be relevant in atherogenesis. These advances have increased our sophistication with respect to the intimate cellular processes in the artery wall. We are now in a position to put forward specific, well-formulated hypotheses about the events leading to the atherosclerotic lesions. The proliferation of VSMCs, endothelial cells, macrophages and possibly lymphocytes; the increased uptake and esterification of cholesterol and the consequent accumulation of cholesterol esters in the surrounding matrix and the associated cells; the decreased efflux of cholesterol from vascular cells, seems to emerge as hallmark characteristics for the formation and progression of the process.

In addition the postulated schema shown in Figure 9. 4 would predict that a mechanistic link may exist between changes in cholesterol esterification and proliferation of vascular cells.

Currently, we are using a combination of biochemical and molecular approaches to demonstrate if and possibly how cholesterol esterification may influence progression of VSMC cell cycle. These studies will extend our understanding of the mechanisms controlling vascular cell growth and might identify targets for future drug development for treating certain cardiovascular diseases.

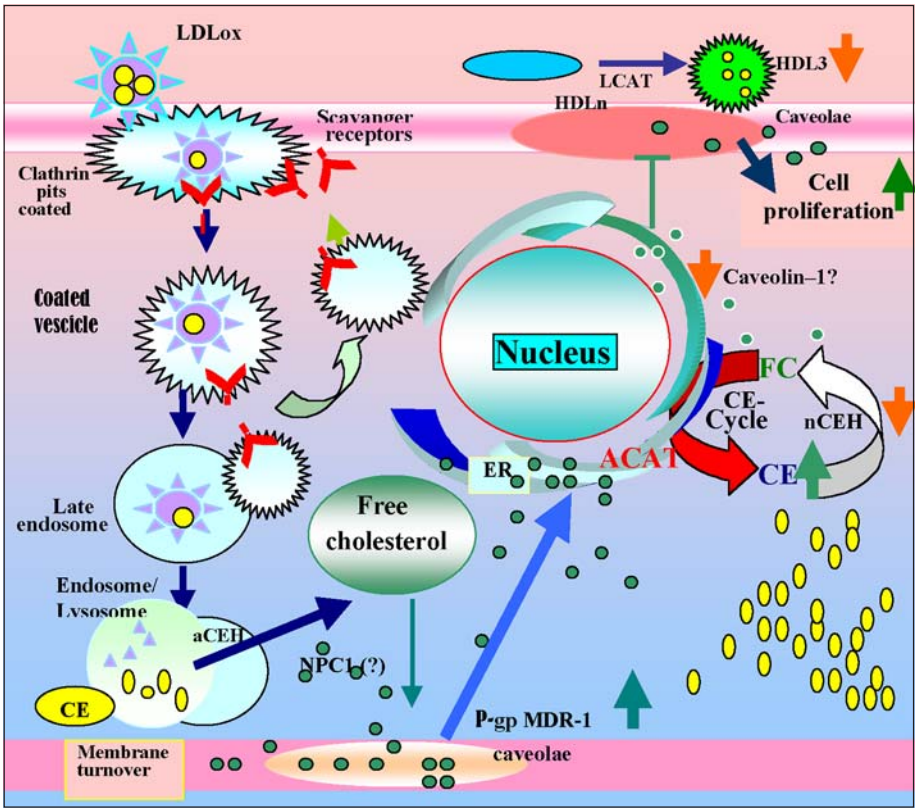

Fig. 9.4. Hypothetical sequence of events by which cholesterol ester metabolism could in itself lead to the progression of atherosclerotic lesions.

- LDL, that accumulates due to increased entry or retention in the vascular wall become oxidized in the microenvironment of the subendothelial space. LDLox is then taken up into vascular cells by scavenger receptors. After internalization, LDLox undergo hydrolytic conversion to free cholesterol by acid cholesterol hydrolase (aCEH) in lysosomes.
- Most of the LDL-bound cholesterol released from the lysosome rapidly emerges at cell surface caveolae, from where it may be used by the cells for the synthesis of new membrane and/or for the normal membrane turnover.
- The excess of membrane cholesterol is then transported to ER, at least in part, by the membrane protein (P-gp) encoded by MDR1, as suggested by the high expression of this protein in atherosclerotic lesions.
- In contrast to the LDL receptors, the number of scavenger receptors on a cell doesn't change with changes in the cell's cholesterol in the ER. So lots of cholesterol can enter the cell and more cholesterol is transported in the ER. This leads to ACAT activation, which in turn esterifies cholesterol. Cholesterol esters are then accumulated in cytoplasm as lipid droplets, and foam cells are formed.
- The accumulation of cholesterol esters makes cholesterol less available for the transport to the caveolae with consequent decrease in cholesterol efflux.
- The decrease in the expression of caveolin-1 and/or of free cholesterol in caveolae may trigger proliferation of vascular cells leading to progression of atherosclerotic process.

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Chapter 10

Role of Cholesterol Esterification in the Modulation of Vascular Smooth Muscle Cell (VSMC) Cycle

Maria Franca Mulas and Sandra Dessì

Abnormal proliferation of vascular smooth muscle cells (VSMC) is an important process for plaque formation in primary atherosclerosis and may be an early event in atherogenesis, as well as in other vascular occlusive diseases, such as restenosis associated with angioplasty. Because of both the public health importance and economic impact of these pathological processes, over the last decade several significant progress has been achieved elucidating the regulatory factors and the molecular mechanisms governing the control of VSMC cycle in the vessel wall. The aim was to develop novel therapies for the treatment of vascular proliferative diseases in human patients.

However, a plethora of mitogenic stimuli and second messengers[1-10] have been found to initiate VSMC proliferation rendering improbable that therapeutic intervention directed against any single initiating stimulus would have broad applicability. Thus a control point responsible for integrating many or all mitogenic stimuli would represent a more promising drug target.

Together with VSMC proliferation, cholesterol ester accumulation in foam cells, is now recognized as the major phenomenon occurring within arterial wall in response to injury. However, mechanistic links between these two events are as yet poorly elucidated.

As discussed in other chapters, our laboratory has recently demonstrated in a number of cell cultures that the rate of cell proliferation is positively correlated with cholesterol esterification and with ACAT and MDR1 mRNA levels and negatively with caveolin-1 expression,[11-14] suggesting that cholesterol esterification may have a role in regulating the rate of cell growth and division, and that MDR1-P-gp and caveolin–1 may contribute to this regulation by modulating, in an opposite manner, the availability of cholesterol substrate in the ER, which is a major determinant of ACAT activity.

In this chapter we review evidences indicating that cholesterol esterification and correlated genes (MDR1, ACAT and caveolin-1) may be a mediator of VSMC proliferation and thus that this pathway may have a functional role in the progression of vascular lesions.

Human Vascular Proliferative Diseases

In the adult organism, vascular smooth muscle cells (VSMCs) residing within the medial layer of elastic arteries are in a state of quiescence, however they can undergo phenotypic modulation and reenter the cell cycle in response to several physiological and pathological stimuli. Abnormal VSMC proliferation characterizes a number of human diseases loosely called

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"human vascular proliferative diseases". These include atherosclerosis, vessel renarrowing after successful angioplasty (restenosis), and graft atherosclerosis after coronary transplantation.[8] Because of the impact on the public health and economy of these pathologies, the pathways by which growth factors, their receptors and signal transduction allow VSMC proliferation, has been a large focus of biologists, and the ability of VSMC to proliferate in culture has been used extensively to measure pathological stimulation of cells in vivo.[15]

Atherogenic risk factors such as hypertension, hypercholesterolemic diet, and diabetes all enhanced proliferative rates of VSMC in vitro.[16-24] In addition, VSMCs obtained from aortic explants of atherosclerotic rabbits grew more rapidly, had greater protein content, and incorporated more ^{3}H-thymidine during both the log-phase of growth and at confluence than did control VSMC[25]. An increased growth rates was also found in VSMC from diabetic animals[24] as well as in those obtained from human patients with atherosclerosis[26]. These findings strongly suggest that induction of VSMC proliferation is an important part of vascular diseases. Therefore, a thorough understanding of the basic regulatory mechanisms of proliferation of VSMC might be important to the ultimate goal of prevention of vascular diseases.

VSMC Proliferation

Proliferation of VSMC starts with a phenotypic switch from 'contractile' to 'synthetic' and comprises three distinct activities: migration from the media to the intima, increased proliferation and inappropriate extracellular matrix synthesis. This phenotypic switch is characterized by the loss of normal regulatory control and anchorage independence of proliferation which together suggest a relationship to oncogenic transformation.

A myriad of mitogenic stimuli, including the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor type 1 (IGF-1) are known to initiate VSMC proliferation[1-10] (Fig. 10.1). These growth factors (GF) act by binding to cell surface GF receptors, which in turn initiates a cascade of events culminating in the regulation of transcription, protein synthesis, lipid and glycogen metabolism, and cytoskeletal rearrangements.

Each type of GF binds to the extracellular domain of its own specific receptor and conversely will not bind to receptors for other growth factors. This results in the activation of the tyrosine kinase domain at the other end of the receptor that is present in the cytoplasm. The phosphorylated tyrosine residues of the activated receptors, produce binding sites for proteins with SH2 domains. GRB2 is one of these proteins. GRB2, with SOS bound to it, then binds to the receptor complex causing the activation of SOS. The binding to SOS, via the linker GRB-2 promotes displacement of GDP by GTP on the low molecular weight G-protein Ras, resulting in activation. Activation of Ras then initiates a cascade of protein kinases including the protooncogene serine kinase Raf-1, and the dual specificity kinase MEK-1, culminating in the activation of ERK family members p42/44 MAP kinase. All these pathways are operative in proliferating VSMC stimulated by mitogens.[27-33]

Apparently, MAP kinase is dispersed throughout the cell prior to stimulation after which it translocates to the nucleus and phosphorylates certain transcription factors involved in activating genes necessary for proliferation.[34] One of these transcription factors, AP-1, consists of dimers of c-fos and c-jun. Although still controversial, MAP kinase may positively regulate the ultimate activation of c-fos[31,32], which, in turn, may trigger gene expression of the D-type cyclins, important integrators of early events of the VSMC cycle. Thus, the synthesis of D-type cyclins seems to be one of the main end points of the RAS/RAF/MAPK pathway. Although cell that lack cyclin D1 can proliferate indicating that this molecule is not strictly necessary for G1, it is also evident that overexpression of cyclin D1 facilitates passage through G1 and promotes cell cycle progression (Fig. 10.1).

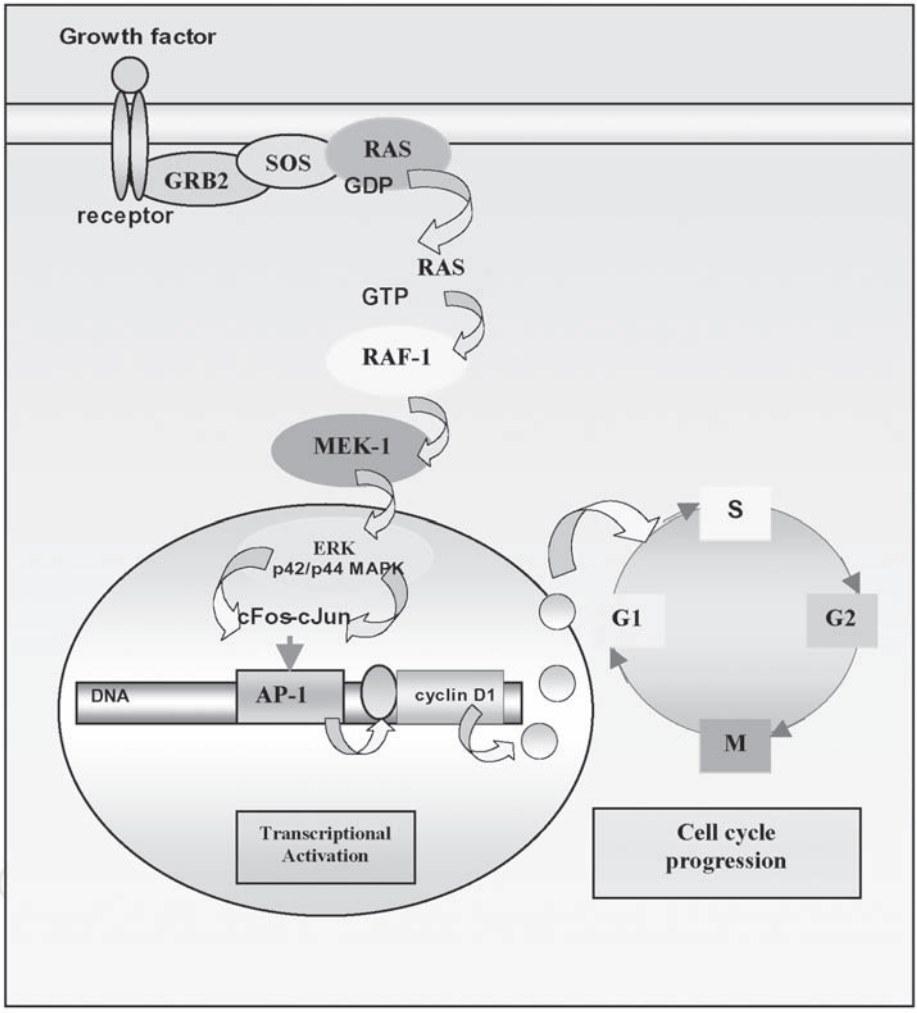

Fig. 10.1. Signal transduction in VSMC. Activation of MAPK induces expression of proto-oncogenes c-fos and c-jun, which form transcription factors such as AP-1: Functional AP-1 further initiates the transcription of cyclins cell cycle-regulatory genes, and cyclins stimulate cell proliferation.

Cell Cycle Regulation of Vascular Muscle Cell Proliferation

When cells acquire availability of sufficient amounts of D-type cyclins, they become independent of mitogenic stimulus, and advance through the various phases of the cell cycle. The basic cell cycle is divided into four phases, G1 (and G0), S, G2, and M. The G1 (Gap 1) phase is characterized by gene expression and protein synthesis. This is really the only part of the cell cycle regulated primarily by extracellular stimuli (like mitogens and adhesion). This phase enables the cell to grow and to produce all the necessary proteins for DNA synthesis that primes the cell to enter the next phase: S (Synthesis) phase. During the S phase, the cell replicates its DNA. This allows the cell to divide into two daughter cells, each with a complete copy of DNA. But, before the cell can do this, it needs to enter the third phase of the cell cycle: the

G2 (Gap 2) phase. During the G2 phase, the cell again undergoes growth and protein synthesis priming it to be able to divide. Once this is complete, the cell finally enters the fourth and final phase of the cell cycle: the M (Mitosis) phase. During the M phase, the cell splits apart (called cytokinesis) into two daughter cells, thus completing the cycle.

To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one (Fig. 2). During early G1, cells integrate external information that is derived from mitogenic stimuli and nutrient availability to prepare themselves for passing through the late G1: D-type cyclins are combined with cyclin dependent kinases 4 and 6 (CDK4/6–cyclin-D), this complex resulting in the phosphorylation of the retinoblastoma (RB) gene product. The hyperphosphorylation of RB in late G1 phase releases E2F transcription factors. These participate in the generation of molecules required for the cell cycle progression, such as cyclin E, A and B which link to CDK2. CDK2/cyclin E complex is critical for normal progression through the G-1 checkpoint (R, restriction point) in the S phase in which DNA synthesis occurs and there is a commitment to mitosis. Expression of CDK2 and cyclin E was detected in human VSMCs within atherosclerotic and restenotic tissue,[19-20] suggesting that induction of positive cell-cycle control genes is a hallmark of vascular proliferative diseases. Finally, cyclin A and cyclin B combined to CDK 2 are required for the progression toward G2 and M phases, respectively.[35,36]

CDK activity is negatively regulated by specific cell cycle regulators, termed cyclin-dependent kinase inhibitors (CKIs). Of them two families have been identified recently: the CIP/KIP family (p21, p27 and p57) binds to and inactivates CDK2-containing holoenzymes, while members of the INK4 family (p15, p16, p18, and p19) are specific for CDK4- and CDK6-containing holoenzymes.[9]

Figure 10.2 illustrates the main events occurring during VSMC cell cycle progression.

Molecular Regulation of Proliferation and Potential Therapeutic Applications

As outlined above, progression of VSMC into cell cycle is regulated by a plethora of mitogenic stimuli which intersect with various sets of signaling cascades, cyclins, cyclin dependant kinases and transcription factors. This make improbable that therapeutic intervention directed against any single initiating stimulus would have broad applicability. Instead, a downstream control point responsible for integrating many or all mitogenic stimuli would represent a more promising drug target.

Together with signal transduction, progression of cells into cell cycle is accompanied by profound and substantial biochemical changes, of interest in this regard appears to be changes in cholesterol metabolism and in intracellular cholesterol trafficking. Consequently, the study of regulation of VSMC growth in culture associated with that of cholesterol trafficking may be used to identify new targets for future drug development for treating certain vascular proliferative diseases.

VSMC Cycle and Cholesterol Esterification Pathway

Foam cells formation is now recognized as one of the major phenomena occurring in response to vessel injury. The cells types that display this character are largely of macrophage origin in the early lesions, but as the atherosclerotic process progresses, smooth muscle foam cells are also observed. The predominant lipid in foam cells is esterified cholesterol, and work in this laboratory has focused on understanding if a possible link may exist between cholesterol esterification and VSMC proliferation during the progression of atherogenesis.

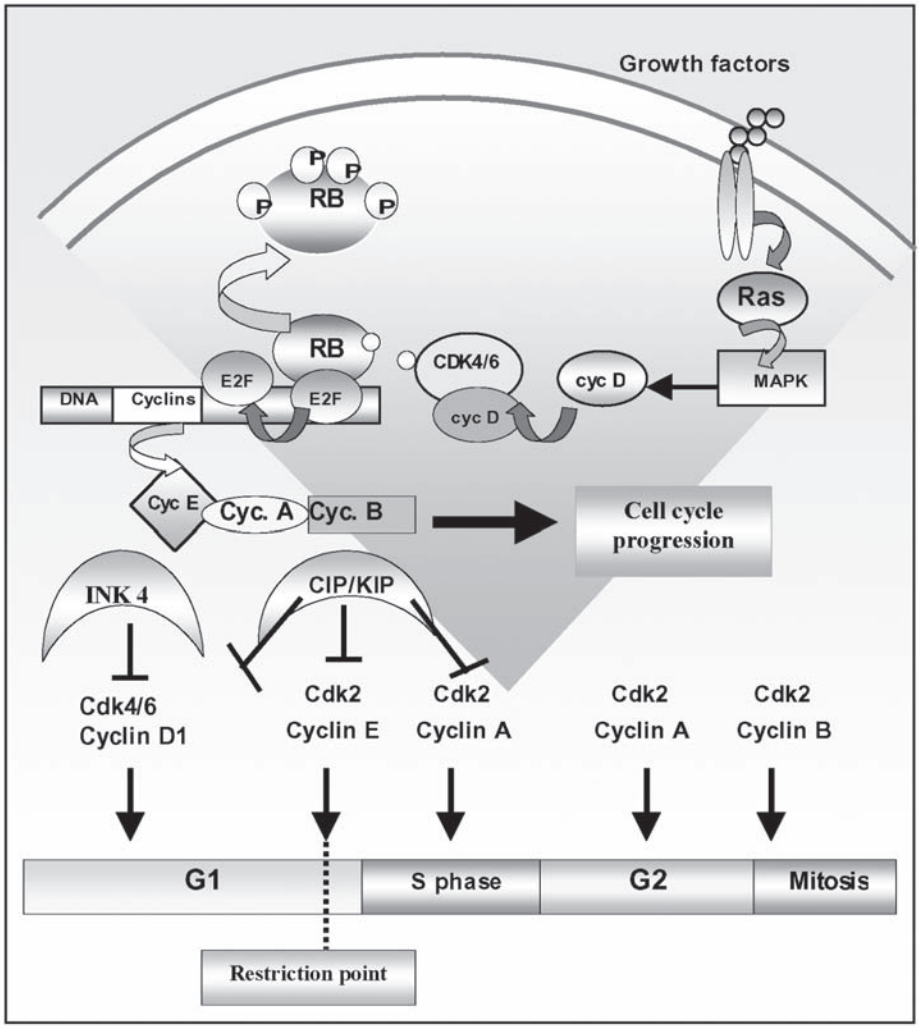

Fig. 10.2. The VSMC cycle. The cell-division cycle is usually divided into four distinct phases: G1 (gap1) is a growth phase that occurs before S (synthesis) phase (the stage of DNA replication). This is followed by a second gap phase, G2, which precedes M (mitosis) phase, during which chromosome segregation and cell division occurs. Growth factors exert their effect during the G1 phase. The restriction (R) point defines a critical time in late G1 after which a cell is committed to undergo DNA replication and is no longer sensitive to growth-factor signalling.

Our studies showed that ^{3}H-thymidine incorporation in VSMC induced by serum to proliferation was preceded by an increased ability of the cells to esterify cholesterol coupled with a higher expression of genes involved in cholesterol esterification, such as ACAT and MDR1 and a lower expression of caveolin-1. In addition, when VSMC were cultured in presence of inhibitors of cholesterol esterification, a direct and specific inhibitory effect on the proliferation of VSMCs was observed: these compounds causing a decrease in ^{3}H-thymidine incorporation as well as an accumulation of cells at G1 phase of the cell cycle associated with a reduction of cyclin D1 expression.[37]

These data provide a plausible link between cholesterol esterification and the control of the G1/S transition of cell cycle supporting the hypothesis that cholesterol esterification may accelerate the progression of the vascular proliferative diseases upregulating signal transduction pathways involved in VSMC cycle progression.

This concept is consistent with studies by Hjjar et al[38] which found an increase in ACAT activity and cholesterol esterification in VSMC stimulated with the potent mitogen, bFGF. These investigators also documented that the synthesis of bFGF, was altered during cholesteryl ester accumulation in VSMCs. VSMCs enriched with cholesterol ester or treated with 25-hydroxycholesterol, an inductor of ACAT activity, exhibited a strong increase of both bFGF in cellular lysate and mRNA level for bFGF as compared to control cells.[39]

Possible Mechanisms by Which Cholesterol Esterification May Regulate VSMC Proliferation

Until now, cholesterol esterification is considered to be instrumental in the pathology of atherogenesis. However whether and presumably how elevated cholesterol esters can trigger vascular disease is presently unknown.

To gain some information on this matter, we performed a series of experiments in which the expression of genes involved in cholesterol homeostasis (ACAT, MDR1 and caveolin-1) was evaluated in VSMC treated with two potent inhibitors of cholesterol esterification (Sandoz 58-035, and progesterone. These two compounds were chosen since they exert their inhibitory effects differently: Sandoz through a direct inhibition on the ACAT, while progesterone, an amphiphilic agent known to modulate MDR1 activity, by inhibiting the transport of cholesterol substrate from the plasma membrane to the ER, the site of cholesterol esterification by ACAT.

As shown in Figure 10.3 A and B treatment with both inhibitors downregulated the expression of the MDR1 and ACAT mRNAs in serum-stimulated VSMC. Down regulation appeared 12 h after serum stimulation, exhibiting its maximal effect at 18 h. In contrast, caveolin-1 levels were up-regulated by inhibitors, the effect being evident as early as 12 hours after VSMC serum stimulation. Changes in caveolin-1 protein abundance was also evidenced at 6 h and 12 h in treated VSMC (Fig. 10.3C).

Caveolin 1 is the principal structural component of caveolae which are cholesterol and sphingolipid rich specific membrane microdomains, and it has been implied to function in the transport of cholesterol within the cell. It has been suggested that caveolin-1 transports cholesterol from the ER to the plasmamembrane and mediates the efflux of free cholesterol derived either from the novo synthesis or low density lipoprotein. Although caveolin-1 and caveolae were originally implicated in cellular cholesterol transport processes, recent evidence suggests that they may participate in signal transduction-related events as well.[40,41] Relative to the rest of the plasma membrane, caveolae membrane fractions are enriched in Src-family kinases, the epidermal growth factor receptor, platelet-derived growth factor receptor, p21 Ras, and most proteins involved in the MAPK cascade, all of which are able to trigger proliferation of many cell types including VSMC.[42,43] Interestingly, the caveolin-1 scaffolding domain (residues 82-101) has been shown to directly interact with various signaling molecules through a common motif found within these proteins. These interactions are believed to sequester the proteins within caveolae and to modulate or suppress their catalytic activities, thus resulting in growth arrest.

While caveolin-1 has been implicated in both signal transduction and in cholesterol transport, it is difficult at present to produce a convincing unifying hypothesis to combine all these observations. However, recent observations have provided fundamental new insights into the way by which signalling pathways are organized in mammalian cells, the role of caveolin and

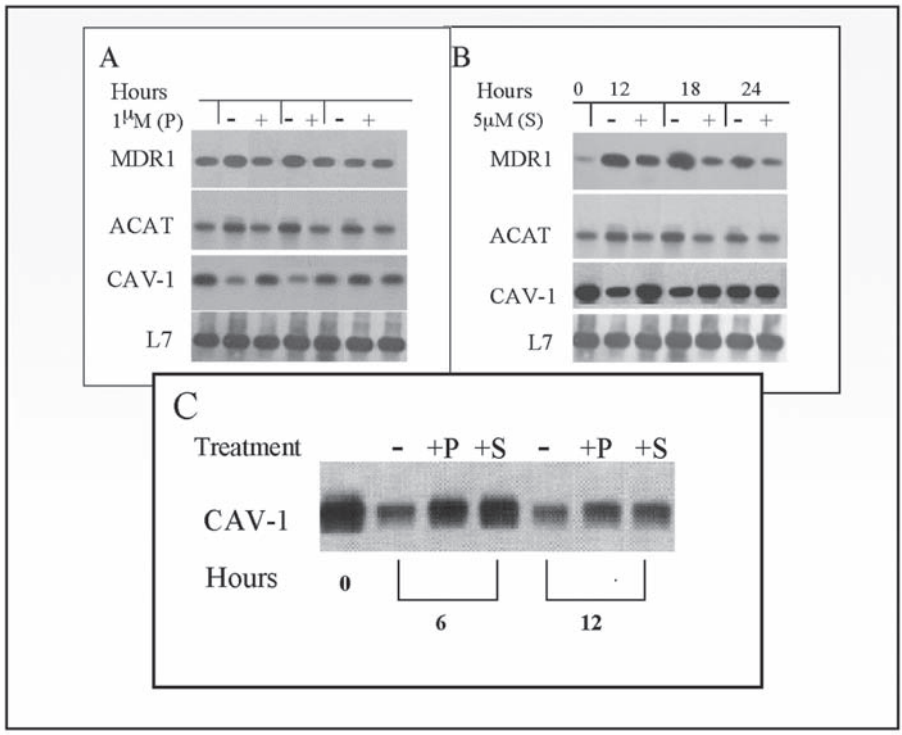

Fig. 10.3. Up-regulation of MDR1 and ACAT and down-regulation of caveolin-1 by cholesterol esterification inhibitors. Quiescent VSMC were stimulated to growth by adding 10% FCS. 1mM of progesterone (P) or 5mM of Sandoz 58035 (S) were added to quiescent VSMC (0h) simultaneously with serum. Cells were harvested at specific points following treatments. Panel A and B show representative autoradiograms of MDR1, ACAT, caveolin-1 and L7 mRNA levels after treatment of cells by 1mM P and 5mM S, respectively. Panel C shows the levels of caveolin-1 protein, as determined by Western blotting, in the cells treated with P or S for 6 and 12 hours.

cholesterol in this organization, and the dependence of specific signaling pathways on these lipid-based sorting platforms.

Depletion of cholesterol from the plasma membrane has been shown to cause a reduction in components of the MAPK cascade associated with a caveolar fraction. Remarkably, cholesterol depletion alone causes a reversible stimulation of MAPK, suggesting that reduced cholesterol in caveolae is sufficient to activate pathway leading to cell division.[44,45]

These findings raise the possibility that caveolin-1, by playing a role as a cholesterol transporter involved in maintaining caveolae cholesterol, might specifically influence raft-dependent signal transduction, at least in part, by regulating the amount of cholesterol available for raft formation in the plasma membrane.

Hypothetical Model by Which Cholesterol Esterification Induces VSMC

Although further studies will be necessary to provide a complete mechanistic understanding of exactly how cholesterol esterification regulates cell cycle progression, based on the above mentioned studies, we present a model (Fig. 10.4) to describe the hypothetical role of cholesterol esterification in the regulation of proliferation of VSMCs.

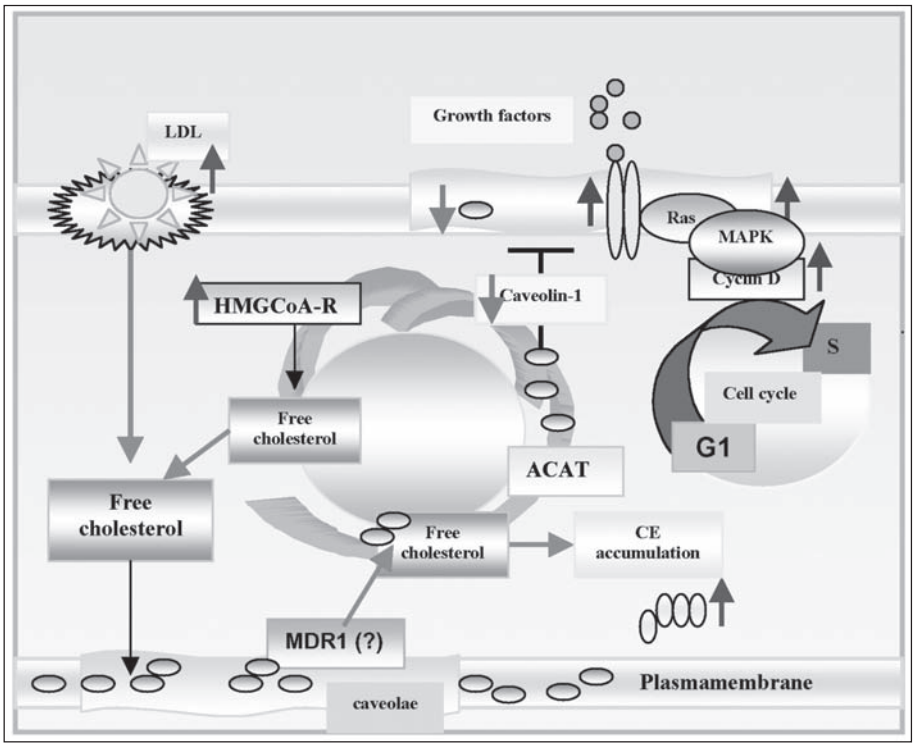

Fig. 10.4. When cells are stimulated to divide there is an increase in both endogenous cholesterol synthesis and cholesterol uptake via LDL receptor. Endogenously synthesized cholesterol in the ER and that in LDL move to the PM, first appears in caveolae, and then is rapidly distributed within the plasma membranes (PM). Excess FC initially accumulates within the plasma membrane and after exceeding a critical threshold, it is transported to the ER (P-gp-MDR1 protein?), where it is esterified by ACAT and accumulate in the cytoplasm as lipid droplets. This results in a strong decrease in the expression of caveolin-1 with consequent decrease in cholesterol efflux. The low expression of caveolin-1 coupled with lower levels of free cholesterol into caveolae, might trigger hyperactivation of signalling pathways involved in cell division, such as MAPK cascade, therefore contributing to accelerate cell cycle progression.

When cells are stimulated to divide, there is an increased endogenous cholesterol synthesis as well as an increased uptake of cholesterol via LDL receptor. Both endogenously synthesized cholesterol and LDL-cholesterol move to the caveolae, from which it is rapidly distributed along the entire surface of the plasma membrane.

Excess FC initially accumulates within the plasma membrane and, after exceeding a critical threshold, it is transported to the ER (P-gp-MDR1 protein?), esterified by ACAT, and accumulated in the cytoplasm as lipid droplets. The accumulation of cholesterol esters drastically reduces the amount of FC to be transported to the caveolae for excretion. In fact, the strong decrease of cholesterol HDL observed in experimental models as well as in human tumors, associated with the strong decrease in the expression of caveolin-1 seen in proliferating cells, including VSMC cells, support this interpretation. Moreover, the low expression of caveolin-1 in proliferating cells, coupled with lower levels of FC into caveolae, might trigger hyperactivation of signalling pathways involved in cell division, such as MAPK cascade, therefore contributing to accelerate cell cycle progression. These conclusions are consistent with the

slowing down of cell cycle progression seen in VSMC treated with inhibitors of cholesterol esterification as well as in caveolin-1 overexpressing fibroblasts.[46]

Conclusions

To date, studies utilizing in vitro VSMC models have yielded a plethora of information regarding the pathology of several vascular proliferative diseases such as atherosclerosis, postangioplastic restenosis as well as graft atherosclerosis after coronary transplantation. Researchers are progressively gaining insight into the mechanisms governing VSMC proliferation. Indeed, it appears that the biochemical factors involved in cholesterol ester trafficking might function via a similar signal transduction pathway. It seems that cholesterol esterification and correlated genes might be important in VSMC growth regulation not only by providing a rapid supply for membrane biogenesis, but also by playing a direct role in the balance of the growth regulator factors.

Future investigations should improve our understanding on proliferative mechanisms of VSMC and the relevance of cholesterol esterification pathway to the progression of atherogenesis.

In addition, the ability of cholesterol esterification inhibitors to reversibly block the growth of VSMC in vitro with no apparent citotoxicity indicate that these compounds may be developed as therapeutic agents against vascular proliferative diseases.

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Chapter 11

MDR, Cell Growth and Cholesterol Esterification:

Implications for Cancer Therapy

Alessandra Pani and Sandra Dessì

In the long fight against cancer chemotherapy is one of the major therapeutic weapons. Radiation therapy and surgery are also employed, but often in conjunction with chemotherapy. What makes chemotherapy very effective is that it has the ability to treat widespread (metastatic) cancer. This ability makes chemotherapy very important in a patient's fight to overcome cancer because radiation therapy and surgery are only suitable for treating cancer in localized areas. Chemotherapy is now well established for most cancers as the standard of care. However, with the exception of some types of acute leukemia and lymphomas, and unless chemotherapy is started at an early stage of the disease, the percentage of complete and life-lasting cancer remission is still below 50% for most types of cancer.[1]

Chemotherapy uses chemical agents to eradicate or control the growth of cancer. Current anti-cancers, also regarded as cytotoxic drugs, work by interfering with phases of the cell life cycle, thus impairing cancer cell's ability to grow. Normal cells grow, divide and die in a precisely controlled way, while cancer occurs when the process becomes abnormal and cells divide without control and order. Cancer cells typically proliferate more rapidly than normal cells invading surrounding tissues, and spread to secondary sites throughout the body (e.g., liver, brain, lungs, bone) where they cause hemorrhage and tissue destruction.[1,2] The neoplastic cells fail to differentiate normally and show alterations of cell surface components, such as antigens and enzymes.[2] However, since molecular targets of the current chemotherapeutics are not peculiar of cancer cells, drugs do not distinguish between them and normal cells, both types of cells being affected.[3] Selectivity of antineoplastic agents is, in fact, only based on the higher rate of proliferation of cancer compared to normal cells. Anticancer drugs exhibit a significant toxicity for tissues such as the bone marrow, gastrointestinal epithelium, hair follicles, and gonadal tissue, all characterized by high growth fractions. Toxicity of chemotherapeutics to normal cells is then cause of unpleasant and sometime severe side effects, and represent the major liming factor to the doses that can be administered. However, the great ability of most normal tissues to regenerate themselves makes the toxic effects reversible and the relationship risk/benefit of chemotherapy generally positive.

In this chapter we summarize the basic principles of anticancer chemotherapy and the problems that still hamper its full success. In particular, the major mechanism of multiple drug resistance (MDR) in cancer will be discussed in the light of the current clinical strategies to overcome it. Further, on the basis of independent findings recently obtained in our as well as in others' laboratories and that correlate cholesterol esters and the MDR efflux pump P-gp with cell proliferation, we will also discuss a novel potential approach for the control of cancer.

Cell Growth and Cholesterol Esters, edited by Alessandra Pani and Sandra Dessì.

Anti-Cancer Chemotherapy

All living cells go through a four phase cycle in order to replicate themselves.[4] The first phase called G1 is when cells prepare to replicate their chromosomes. The second stage is called S and is when DNA synthesis occurs and chromosomes are duplicated. The next phase is the G2 phase, when the RNA and protein duplicate. The final stage is the M stage, culminating in cell division. There is also a fifth phase, called G0 or resting phase, in which the cells display only basic metabolism and do not progress into cell cycle. Following appropriate stimuli, however, resting cells can reenter active growth phases by starting G1 phase.

Depending on the drug chosen, current chemotherapy will affect cell cycle progression in one of the following ways:

i. damaging the DNA of cells so they can no longer reproduce. This is done by altering the DNA structure in the nucleus;
ii. inhibiting the synthesis of new DNA strands during the S phase of cell cycle, so that no cell replication is possible. This is obtained through a direct interaction of the drug with DNA or through the inhibition of the enzymes involved in DNA synthesis (e.g., topoisomerases);
iii. affecting enzymes involved in the metabolic pathways leading to formation of nucleotide pools;
iv. stopping the mitotic processes so that the cell cannot complete division.

There are many different types of drugs that qualify as chemotherapeutic agents.[5] The drugs are classified into five major categories based on the way the drugs affect cell chemistry, and which stage of the cells life cycle they target. Drugs in the same class start processes leading to cell death by attacking the same target within the cell. The current drug classes can be summarized as follows.

Alkylating agents. These were among the first anti-cancer drugs and still are the most commonly used agents in chemotherapy today. They act directly on DNA, causing cross-linking of DNA strands, abnormal base pairing, or DNA strand breaks, thus preventing the cell from dividing. These drugs can work at any time of the cell cycle, but are most effective during DNA synthesis. They are used to treat Hodgkin's disease, lymphomas, chronic leukemias, and certain carcinomas of the lung, breast, prostrate, and ovary. Examples of drugs in this category are Cyclophosphamide, Mechlorethamine, and Cisplatin.

Nitrosoureas are similar to alkylating agents, and work by inhibiting the changes necessary for DNA repair. A very important feature of this class of drugs is that they can cross the blood-brain barrier which makes them very useful for treating brain tumors. They can also be used to treat lymphomas and melanomas. Examples of drugs in this category are Carmustine and Lomustine.

Antimetabolites block cell growth by interfering with DNA synthesis. These drugs work by mimicking a physiologic metabolite involved in DNA synthesis. These drugs affect the S phase of the cell cycle. Antimetabolites are used to treat tumors of the gastrointestinal tract, breast, and ovary. Examples of these drugs are 6-Mercaptopurine and 5-Fluorouracil.

Antitumor antibiotics work by binding to DNA to prevent both RNA transcription and DNA replication. This category of drugs is used to treat a wide variety of cancers including testicular cancer and leukemia. Some examples of this category are Doxorubicin and Mitomycin-C.

Vinca alkaloids work by preventing cell division. During metaphase, mitotic spindles hold the two sets of DNA the cell needs to divide. The spindles are formed using a protein called tubulin. Plant alkaloids bind to tubulin, which prevents the formation of mitotic spindles. These drugs are derived from plants and are used to treat Wilm's tumor, and cancers of the lung, breast, and testes. Some examples of this category are Vincristine and Vinblastine.

Steroid Hormones. These drugs modify the growth of hormone dependant cancers. They induce a change in the three dimensional shape of the receptor on a cell preventing the cell's binding to the needed estrogen response element on the DNA. These drugs are most commonly used in treating breast cancer. Some examples of this category are Tamoxifen and Flutamide.

Monotherapy based on any of the above drugs have proved not effective against cancer, while administration of cocktails composed of different drugs has led, in general, to positive results and, in a few cases, to the total and permanent remission of cancer.[1,5] The use of combinations of drugs belonging to different classes can be regarded as one of the major advances made in anticancer chemotherapy during the last seventy years. Polychemotherapy has, in fact, several important advantages. It usually results in a decreased incidence of drug resistance (see below). There is often a greater than additive or synergistic effect of the combined drugs. Furthermore, by using combination of drugs with different targets, it is possible to achieve the same extent of inhibition with lower doses of each drugs, and thus the overall toxicity, or at least that to any one organ system, results decreased.

Variables of the Cancer Response to Chemotherapy

Beside the overall toxicity of a given drug, or of a drug combination, that limits the dose administration, cancer response to chemotherapy is subjected to several other variables, that are:[1,6]

Growth fraction. That is the proportion of dividing cells in a tumor or normal tissue, e.g., the fraction of cells that are actively dividing with respect to the entire population of viable cells. The higher it is, the better the cancer response to chemotherapy would be.

Mass doubling time. That is the time it takes for the tumor to double in size. Tumors with shorter mass doubling times are more amenable to treatment with drugs. As tumors get larger, the mass doubling time increases and the growth fraction decreases.

Cell cycle phase. The response to certain phase-specific drugs depends on the percentage of cells in a susceptible phase during the time of exposure to pharmacologically effective drug concentrations.

Tumor heterogeneity. Tumors are made by heterogeneous populations of cells; i.e., some of cells are proliferating, some are dormant (G0 phase of the cell cycle), some are dying. Cells in the resting stage are not responsive to action of anticancer drugs, which target only actively growing cells, and thus represent a reservoir of cancer since they can reenter the active growth cycle any time.

Drug delivery to cancer cells. Tumors are supplied by blood vessels that can have many abnormalities resulting in reduced delivery of drug.

Drug resistance. As many as 40-45% of patients' cancers may have or may develop resistance to anticancer drugs. To date, several biochemical mechanisms have been identified. The increased intracellular inactivation of cytotoxic drugs[7,8] or changes in intracellular drug metabolism[9,10] are two examples. The glutathione S-transferase enzyme system is naturally present in the body as a detoxification mechanism. When cancer cells are exposed to anticancer drugs, the levels of these enzymes increase. The enzymes combine with the drugs, forming a complex conjugate that is less toxic and ineffective.

Alternatively, changes in the activity of target enzymes[11,12] can occur, as for topoisomerase I and II, thymidylate synthase, and dihydrofolate reductase. Topoisomerases are nuclear enzyme responsible for many aspects of DNA functioning, so they are targets of many anticancer drugs. In order to be effective, the drug must form a three-way complex with the enzyme and DNA. Anything that interferes with the formation of this complex will result in resistance.

Reductions in the levels of topoisomerase can result in loss of drug sensitivity by depriving the drug of a target, while mutations in the gene for the enzyme can result in a drug resistant variant. Thymidylate synthase and dihydrofolate reductase, instead, are two cytoplasmic enzymes essential for the formation of the deoxynucleotide pools necessary for DNA synthesis. Since rapidly dividing cancer cells have a great demand for DNA synthesis, anticancer drugs were developed that target the synthetic pathway at two points: 5-flurouracil blocks the action of thymidylate synthase, while methotrexate blocks dihydrofolate reductase.[1] The cancer cell often overcomes these inhibitions by simply increasing the production of the two enzymes.

Another relevant mean of resistance involves the inhibition of those processes culminating in the programmed cell death called apoptosis. Most, if not all, anticancer drugs ultimately kill cancer cells by apoptosis.[2,5] The decision whether a cell continues to survive or undergoes apoptosis is dependent on a complex interplay of genes and proteins that exert a regulatory role in cellular events. Normally, DNA damage caused by anticancer drugs will stimulate apoptosis, particularly under the direction of p53 protein. Resistance can develop with a loss of genes required for apoptosis, or with an overexpression of genes that block apoptosis.

Among all mechanisms of drug resistance, great importance assumes the emergence (or amplification) of cell functions leading to the drug elimination from the cell interior. These mechanisms lead to the reduction of the intracellular drug concentrations below those necessary to exert inhibition on the specific target.[13-17] Further, in this type of drug resistance the cell ability to efflux one drug implies a similar ability on a series of functionally and structurally unrelated drugs (multidrug resistance, MDR).[13,18] To date, MDR phenomenon in cancer represents the most undesirable mechanism of resistance and one of the major limitations to the success of chemotherapy since leaves the clinician with no alternative therapeutic options and the patient with an unfavorable prognosis.

MDR and Cancer

Intrinsic or acquired MDR mediated by P-gp and encoded by the MDR1 gene is one of the best understood mechanisms of multiple resistance to anticancer drugs which confers upon cells the ability to resist lethal doses of certain cytotoxic drugs by pumping the drugs out of the cells.[19,20] This resistance is obtained through stepwise selection and it reflects the amplification (and/or mutation) of the MDR1 gene that encodes the transmembrane glycoprotein P-gp pumping the drugs out of the cell.

Gastrointestinal, hepatobiliary and renal cancers which are largely unresponsive to chemotherapy, show high levels of intrinsic MDR, whereas leukemias, lymphomas, ovarian and breast cancers often respond to treatment initially, but acquire resistance during the course of the chemotherapy cycles. There has bee found a negative correlation between P-gp expression and chemosensitivity or survival in leukemia,[21,22] lymphomas,[23] osteogenic sarcoma,[24] small-cell lung cancer,[25] breast cancer[26,27] and pediatric solid tumors.[28,29]

P-gp has a broad specificity for cytotoxic substrates (e.g., doxoribicin, taxol, etoposide, vincristine, vinblastine), and several noncytotoxic drugs may competitively inhibit efflux of the cytotoxic drugs by P-gp thereby downmodulating MDR (see Chapter 8).[30] Some of these compounds added simultaneously with anticancer drugs proved, in fact, useful in treating drug resistant tumors in animals, thus suggesting the possibility to overcome the problem of MDR also in humans. Several classes of MDR modulators have been identified among drugs that were originally developed for other therapeutic indications, including calcium channel blockers, calmodulin antagonists, steroid hormonal agents and immunosuppressive agents,[31] all being currently evaluated in the clinic.

Table 11.1. MDR modulators in clinical trials

Verapamil and dexverapamil
Cyclosporin A and PSC-833
Several dihydropyridines
Phenothiazines
Quinolines
Tamoxifen, trifluoperazine, nifedipine, and progesterone

MDR Modulators in Clinical Trials

As stated in the previous paragraph, intrinsic or acquired MDR mediated by P-gp can be reversed by the action of a group of compounds known as MDR modulators or chemosensitizers. In general, these substances are suggested to compete with antitumor drugs for the same binding sites, in this way inhibiting the P-gp mediated drug efflux. The calcium channel blocker verapamil was the first agent that was shown to modify MDR in vivo and in vitro,[32] but unfortunately the MDR modulating activity required concentrations that are associated with severe cardiac toxicity in patients.[33] The immunosuppressive agent cyclosporin A (CsA) has also been shown to be a highly potent inhibitor of P-gp both in cell lines[34] and in animal models.[35] Although CsA inhibits P-gp at clinically tolerable concentrations in these experiments, the immunosuppression restricts its utility in clinical oncology. In the recent years, a new generation of modulators with substantially improved potency for inhibition of P-gp in vitro has developed. These agents are highly specific and effective at low nM concentrations. The toxic potential is regarded as generally low in these agents. They are usually a product of specific drug discovery programs and include the nonimmunosuppressive cyclosporin derivative PSC 833,[36] the cyclopeptolide 280-446,[37] and the cyclopropyldibenzosuberane LY 335979.[38]

This paragraph will focus on efforts in the clinic to overcome MDR by using P-gp modulators (Table 11.1). Key elements used in the clinical experience with P-gp modulators include:

i. initial treatment with the anti-cancer drugs alone;
ii. co-administration of modulator and anti-cancer drugs;
iii. pharmacokinetic studies of both compounds. The tolerability of combined treatment with chemotherapeutic agents and MDR modulators firstly gave moderately encouraging results in both phase I and II trials by using first generation modulators such as calcium channel blockers (verapamil) and cyclosporins. These trials have shown that the early modulators frequently exercise unacceptable toxicity at plasma concentrations equal to the dose level required to circumvent MDR in vitro.[39] Furthermore, these trials have demonstrated certain pharmacokinetic interactions with anticancer drugs.

Other agents which have been used in clinical trials to modulate MDR include tamoxifen, quinidine, trifluoperazine, quinine, nifedipine, and progesterone.[40-43] These trials vary considerably in their design and the conclusions which may be derived with regard to MDR modulation. Interpretation of these trials is complicated by many factors. Adequate concentrations of many potential modulating agents may not be achievable because of toxicity. Second, the pharmacokinetic consequences of the drug interactions which are produced often have not been well characterized (drug levels of both modulator and cytotoxins). Third, the modulating drug may not be bioavailable in vivo due to binding to factors such as serum proteins. Finally, proper controls may not be included (clinical resistance to prior therapy, or a randomized control group without the modulator). In general, the doses of the modulators were limited by their toxic effects below those required to achieve effective inhibition of P-gp in vivo.

To date, only limited randomized phase III studies of P-gp modulators have been published. No beneficial effect was observed from the addition of oral verapamil to the combination chemotherapy employing vincristine, doxorubicin (adriamycin) and dexamethasone (VAD) for the treatment of drug-resistant myeloma patients.[44] As above stated, the clinical applications of both verapamil and CsA have been limited by severe heart, kidney, and immunosuppression at doses that could effectively inactivate the active P-gp pump. Currently, a variety of second-generation MDR modulators are being investigated, some of which may prove to have a more favorable toxicity profile compared with these first-generation products. One of the most promising second generation agents for modulation of MDR1 is the cyclosporins analogue PSC 833 or valspodar.[45-47] In contrast to CsA, PSC 833 is a nonimmunosuppressive, nonnephrotoxic cyclosporine derivative, which is approximately two to tenfold more potent than CsA.[48] Preclinical and phase I clinical studies have shown that PSC 833 reverses MDR at dose levels that are well tolerated in animals and humans.[49] The principle toxicity associated with administration of PSC 833 is a moderate and reversible ataxia. Subsequently, a phase II trials was conducted and some data are now available. As reported by Case at the annual meeting of The American Society of Hematology (1998), a total of 41 patients have been treated, and 36 patients are valuable for safety. The most common PSC 833 (valspodar)-associated toxicity were mild to moderate unsteady gate (36%), with no reports of severe symptoms. Objective responses were observed in 4 of 41 (10%) patients. Furthermore, a recent update has confirmed that another 15% of the patients have responded for a total of 25%. This study has further demonstrated that the combination of valspodar and reduced-dose VAD is safe and can induce responses in VAD-refractory patients. However, continued follow-up is necessary to establish the efficacy of this regimen. Valspodar is a prime example of an agent that has emerged from the culmination of intensive research into the molecular mechanisms of MDR and novel drug development. Based on these encouraging results, this agent is currently in pivotal phase III trials in acute myeloid leukemia, multiple myeloma, and ovarian cancers, and is the leading candidate as the first successful drug developed specifically to modulate MDR. The results of these trials should be available in the coming year and are awaited with considerable interest.

Clinical Significance of P-gp Expression in Cancer

Overall, clinical studies have shown that the most positive results for modulation of MDR could be obtained among hematolymphoid malignancies: acute myeloid leukemia (AML),[50,51] lymphomas,[52,53] and multiple myeloma.[54-58] Other types of cancers, in fact, are moderately responsive to MDR-related drugs and do not usually express MDR1 at diagnosis, although some relapsed or refractory tumors (30-50%) express the gene. These include breast, ovarian, and small cell lung cancers, sarcomas, and some pediatric cancers.[59-63] A third group of cancers are those which constitutively express P-gp in almost all cases, especially colorectal and renal cancers. These are known to be refractory to MDR-related cytotoxins, and the experience thus far with MDR1 modulation has been largely negative.[40-42] These negative results may be due to the use of sub-optimal modulators in these early clinical trials, and/or because multiple, redundant mechanisms of resistance may be present in these tumors. Reversal of resistance to colorectal and renal carcinomas may thus require modulation of more than one type of MDR mechanism.

As outlined, despite the potential role of P-gp inhibitors in resolving MDR, clinical trials attempting to reverse or modulate MDR have generally failed. To date, there is no completely satisfying explanation for limitation to the success of this strategy. It is, however, apparent that in many cases there may be a coordinated involvement of many systems. The main reasons evoked for the failure of MDR modulators in the clinic are reported in Table 11.2.

Table 11.2. Reasons for the failure of MDR modulators in the clinic

Modulator is too toxic
Modulator may increase the toxicity of the co-administered anticancer drug
Modulator may not reach tumor
Tumors may have multiple mechanisms of resistance
Modulator may block the physiological function of P-gp
Inappropriate patients may have been used in clinical trials
Pharmacokinetic interactions between cytotoxic drugs and MDR modulators

Among others, pharmacokinetic interactions of combination therapy consisting of MDR modulators and MDR-related anticancer agents, appear as one of the major mechanisms responsible for the failure of the attempts to modulate MDR in the neoplastic patient. The interactions, in fact, lead to increased toxicity such as myelosuppression. One could question whether adequate concentrations of an MDR modulator have been achieved in clinical trials which do not manifest such interactions or alterations in toxicity. Understanding these pharmacokinetic effects is essential in the design and interpretation of clinical trials of MDR modulation.

Other Mechanisms by Which P-gp Inhibitors May Block Cancer Progression

The classical approach to circumvent P-gp-mediated MDR by using co-administration of modulators and chemiotherapics could not represent the only way to block cancer progression. During our in vitro studies on MDR tumor-cells (see Chapter 8),[64] we found that these cells showed increased susceptibility to the antiproliferative effects of the P-gp inhibitors tested. In particular, progesterone was the most potent being able to determine cell death at concentrations up to ten times lower than those giving the same effects on parental, drug-sensitive, cells. Even though at a minor extent, similar results were obtained with other two P-gp modulators, verapamil and CsA. Although the mechanism of the selective cytotoxicity of these P-gp inhibitors for MDR cells it is not clear at present, these results open to the exploration of an alternative approach that implies the use of P-gp modulators separately to anticancer drugs. The potentiality of this novel approach is supported by several independent evidences appeared in recent literature. First, P-gp modulators separately inhibit cell growth and induce apoptosis in leukemia, epidermoid carcinoma, and breast carcinoma cell lines.[65-68] It has been also demonstrated the ability of the MDR modulators PSC 833, 280-446 and LY335979 to provoke cytokinesis failure and G2/M cell cycle arrest in leukemia cells with elevated expression levels of P-gp.[65] Although other mechanisms separate from the efflux activity cannot be excluded, the Authors suggested that the interruption of the ability of P-gp to act as an efflux pump of endogenous substrate(s) conferring survival signals may play a role. Chaudary and Roninson have proposed that P-gp in hematopoietic stem cells may participate in the export of a growth regulatory molecule.[69] Accordingly, interruption of a possible autocrine pathway supporting cell growth by endogenously produced and exogenously secreted cytokines may thus inhibit cell proliferation.[70] Another explanation for the antiproliferative effect of P-gp inhibitors has evolved from recent experiments that have disclosed increased intracellular content of ceramide in MDR cancer cells exposed to the P-gp inhibitors CsA, PSC 833 and tamoxifen.[67,68] Ceramide is a potentially toxic sphingolipid which is involved in signaling events that result in G0/G1 cell cycle arrest and apoptosis.[71] The capacity to glucosylate ceramide is enhanced in MDR cancer cells resulting in elevated levels of glucosylceramide and corresponding decline in the level of ceramide.[72] Therefore, there are several candidates for a growth regulatory molecule

maintained by P-gp, but at present no study has provided proof of such a mechanism or identified the effector molecule.

In this context, the data obtained by us in the last recent years indicate that the separate antiproliferative effect shown by P-gp inhibitors could be correlated with the postulated role of P-gp in the regulation of intracellular cholesterol trafficking; e.g., cholesterol being the endogenous substrate of P-gp. In a series of in vitro experiments in which cholesterol synthesis, esterification and growth rate potential were evaluated in several different types of normal and tumor cell lines characterized by different rates of cell growth, we found a positive correlation between cholesterol esterification, expression of ACAT gene, and growth rate potential, indicating the possible involvement of cholesterol esterification pathway in the regulation of cell growth and division.[73] We assumed that an increased capacity in producing and storing cholesteryl esters may facilitate new membrane biogenesis required for cell proliferation, and thus faster re-entry of cells into cell cycle.

As detailed in other chapters of this book, a direct correlation between MDR1 gene expression and cholesterol esterification has been found in a number of cultured human cells, suggesting a role for MDR in cholesterol esterification. In addition, growing evidences suggest that P-gp increases esterification by facilitating cholesterol movement from the plasma membrane to the ER.[74,75] Accordingly, in a wide range of cultured cell lines, several inhibitors of P-gp activity such as progesterone, dehydroepiandrosterone (DHEA) and verapamil, besides blocking the movement of cholesterol from the plasma membrane to ER, were also found to inhibit cholesterol esterification by ACAT. Since these compounds are all known to physically interact with P-gp inhibiting its activity, the most likely explanation for the correlation observed is that changes in cholesterol esterification are a consequence of the effect of these inhibitors on P-gp activity.

Based on these results, we deemed it of importance to test whether variations in MDRI gene expression were also present during the growth of different normal and tumor cells. In the studies performed, high levels of cholesterol esterification and of expression of ACAT gene were positively related to a markedly increased expression of MDR1 gene, suggesting that the latter might contribute towards regulation of the rate of cell growth and division by modulating intracellular cholesterol ester levels.[76] In addition, in a more recent study, using as a model system leukemia cells freshly isolated from patients in which ACAT and P-gp activities were inhibited through pharmacological treatments (i.e., Sandoz 58-035 and progesterone, respectively), we found an average reduction in proliferation rate by 60-70% in leukemia cells treated with inhibitors. In this case, the effect was not caused by cell toxicity, as cell morphology and viability were unaffected at the concentrations of inhibitors used (unpublished results). The inhibition of cell proliferation was associated with a reduction of ACAT and MDR1 gene expression and conversely, with an upregulation of caveolin-1 gene expression and of p-53 protein. These results imply that cholesterol esters not only constitute a form of lipid storage but likely have a role in dynamic processes of cell division inside the cells. We concluded that ACAT inhibitors as well as P-gp modulators administered alone, by reducing cellular cholesterol esters, may warrant consideration as antineoplastic agents, also in those tumors that are moderately responsive to MDR-related drugs and do not usually express MDR1 at diagnosis.

Conclusions

In clinical oncology the MDR phenomenon is a complex issue with profound impact on treatment outcome in the patients. P-gp is one of many resistance mechanisms at work, but represents undoubtedly the most common and best studied target for MDR circumvention at present. Although clinical studies evaluating less toxic modulators of P-gp will possibly give more encouraging results then previous trials, therapeutic success of the modulators in MDR cancer is not so close to come. Beside of the identification of other resistance factors possibly

involved, diverse aspects yet obscure of the phenomenon might influence and even change the actual therapeutic strategy. Of importance it appears the identification of the endogenous substrate of MDR1 P-gp and its precise role in the cells. Further, of interest is also to establish whether or not P-gp functionality in MDR is the same as in nonMDR cells, and thus whether MDR simply arises as a consequence of MDR1 gene amplification or if it is the results of stepwise drug selection of cells overexpressing a P-gp bearing sequence mutations affecting its substrate specificity.

As for now, besides being a potential target for drug sensitization, P-gp appears to be a target for the control of cholesterol esterification in certain cancer cells. The P-gp inhibitors may thus provide a mode for the regulation of intracellular cholesterol, which is an attractive feature suggesting a potential for therapeutic exploitation. Further studies are needed to better understand the mechanisms by which modulation of P-gp, and consequently that of cholesterol esterification, could regulate the growth of tumor cells. Most important, however, is the question whether the antiproliferative effect exerted in vitro by P-gp inhibitors may translate into clinical practice. The first step to answer this question has already been taken in our laboratories as we have initiated experiments with P-gp inhibitors monotherapy in mice transplanted with MDR and non-MDR L1210 leukemia cells for response evaluation in vivo.

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