Published in final edited form as:

J Cardiovasc Transl Res. 2010 October ; 3(5): 448–453. doi:10.1007/s12265-010-9205-7.

Phenotypic High-Throughput Screening in Atherosclerosis Research: Focus on Macrophages

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Abstract

Atherosclerosis is a complex disease characterized by arterial lesions consisting of macrophage foam cells, smooth muscle cells, lymphocytes and other cell types. As atherosclerotic lesions mature, they can rupture and thereby trigger thrombosis that can result in tissue infarction. Macrophage foam cells develop in the subendothelial space when cells take up cholesterol from modified forms of low-density lipoprotein (LDL) and other apolipoprotein B-containing lipoproteins. Current therapies to limit atherosclerosis focus on altering the plasma lipid composition, most commonly by reducing circulating LDL levels. No current therapy is specifically designed to alter the cellular composition of atherosclerotic lesions. To address this deficit, phenotypic high-throughput drug screens have been developed to identify compounds that reduce the uptake of oxidized LDL by macrophages or to identify compounds that increase the efflux of cholesterol from macrophages. Additional phenotypic screens can be envisaged that address cellular processes in active atherosclerotic lesions including macrophage apoptosis and efferocytosis.

Keywords

Atherosclerosis; Chemical Biology; High-Throughput Screen; Phenotypic Screen

Introduction: Atherosclerotic Vascular Disease

Atherosclerotic vascular disease is responsible for a tremendous amount of suffering and death, and despite advances in medical therapeutics and surgical interventions, remains an enormous societal problem. A widely held model is that atherosclerotic lesions form in large arteries at the site of endothelial activation or dysfunction [1]. These endothelial alterations are hypothesized to occur as a consequence of high circulating LDL and other apolipoprotein B-containing lipoprotein levels, toxins in cigarette smoke, hypertension, hyperglycemia, viral infection, and disturbances in laminar flow [2–5]. In the setting of high circulating LDL levels, some of the LDL particles are deposited in the subendothelial space of vessels. In response to lipoprotein-retention-induced endothelial dysfunction and inflammation, monocytes are recruited from the bloodstream, and these cells bind to the endothelium and then interdigitate into the subendothelial space where they differentiate into macrophages [1,6]. Once differentiated, the macrophages may take up cholesterol from LDL particles, especially when the LDL is modified by oxidation (oxLDL) or other processes (Fig. 1). Macrophages that are filled with cholesterol inclusions are called “foam cells.” As

atherosclerotic lesions mature, foam cells become surrounded by smooth muscle cells, lymphocytes and other cell types.

In most cases, atherosclerotic lesions do not rupture and do not trigger thrombosis. However, in a small percentage of lesions, perhaps 2% or 3%, the plaque can rupture and trigger thrombosis resulting in tissue necrosis [7,8]. The causes of plaque instability and rupture are not well understood, but macrophage apoptosis and necrosis may play a significant role in this process [9]. Macrophage foam cells may undergo apoptosis as a consequence of persistent oxidative stress that leads to dysfunction of the endoplasmic reticulum and triggers the unfolded protein response [10]. Persistent ER stress of macrophages, in concert with additional stimuli, such as oxLDL binding to CD36, leads to macrophage apoptosis. In addition, efferocytosis may be defective in advanced atherosclerotic lesions. Efferocytosis is the process by which macrophages, possibly of the M2 subset that promotes resolution of inflammation, engulf and dispose of apoptotic cells, including macrophage foam cells [10]. In the setting of defective efferocytosis, apoptotic macrophages can become secondarily necrotic and large areas of extracellular debris can accumulate in lesions, called the “necrotic core.” Therefore, defective resolution of inflammation may be a critical factor in the progression and instability of atherosclerotic lesions.

Critical biochemical research decades ago led to the identification of drugs that inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase, a hepatic enzyme that is rate-limiting in the mevalonate pathway for the synthesis of cholesterol [11,12]. Inhibition of HMG CoA reductase activity leads to a reduction in circulating levels of low-density lipoprotein (LDL) particles [11,12]. The HMG CoA reductase inhibitors, commonly known as “statins,” remain the mainstay of medical therapy for the treatment for atherosclerotic vascular disease. In addition, drugs have been developed that increase the production of the potentially-beneficial high-density lipoprotein (HDL) particles by inhibiting the activity of cholesteryl ester transferase protein, but the clinical utility of these agents remains unproven [13,14]. Furthermore, agents have been developed that are ligands for the nuclear hormone receptor peroxisomal proliferator-activated receptor α , called fibrates, that reduce the hepatic production and release of free fatty acids that are typically bound to albumin in the bloodstream and that also increase the hepatic production of HDL particles [15]. Therefore, agents in clinical use today are designed to modify the plasma lipoprotein and fatty acid content.

Phenotypic High-Throughput Screening

The traditional high-throughput screening method for the identification of agents that are effective at treating human disease is the use of a biochemical assay with a purified target protein [16,17]. In this method, the ability of a compound to alter the enzymatic activity or binding properties of a target protein is assayed. This approach has been applied successfully in many cases, and the biochemical activity of the compounds selected as “positive hits” is immediately known after completion of the screen. However, the relationship between the biochemical activity of an identified compound and its potential clinical utility remains uncertain in advance of animal or human testing.

An alternative approach to pure protein high-throughput screens is to use complex phenotypes in cells, tissues or whole organisms as the key assay for drug discovery. In phenotypic high-throughput screens, a biological process is evaluated so that a library of compounds can be screened to identify specific compounds that modify the biological process [16–18]. It is very helpful if the biological assay is quantitative and reproducible. In phenotypic high-throughput screens, cells, tissues or small organisms are placed in

microtiter plates and specific compounds from chemical libraries are dispensed into unique wells by a robotic liquid-handling device. Superior biological assays for this type of screen involve the use of fluorescent or luminescent reagents that allow for the use of automated microtiter plate readers with advanced data processing. Active compounds are identified because of their ability to modify the results of the biological assay (see my previous review for a more detailed discussion of the development and statistical analysis of phenotypic high-throughput screens in cardiovascular research [19]).

The Use of Phenotypic Screens to Interrogate Macrophage LDL Uptake and Foam Cell Formation

A critical step in atherosclerotic lesion formation is the generation of sub-endothelial macrophage-derived foam cells (Fig. 1) [1]. My group recently completed a phenotypic high-throughput screen to evaluate the uptake of oxidized low-density lipoprotein (oxLDL) particles by J774 monocyte/macrophage cells [19,20]. While the uptake of oxLDL by macrophages is one mechanism by which foam cells form in lesions, it is likely that other mechanisms, such as phagocytosis of aggregated LDL and pinocytosis of native LDL may also be significant in vivo [21,22]. In our screen, J774 cells were cultured in 96-well microtiter plates and 480 compounds from the ICCB Known Bioactives Library were added. For each compound in this library, at least one specific biochemical activity is known. "Known bioactives" libraries are useful in that they can supply both phenotypic and biochemical information in a single high-throughput screen [19]. Human oxLDL particles were labeled with the fluorescent lipophilic dye 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), and they were added to every well in the screen. After 2 h, the total fluorescence was measured to determine auto-fluorescence and quenching. The J774 cells were then washed and fluorescence was measured again to quantify the specific uptake of DiI-oxLDL. In previous work, the JNK pathway inhibitor (SP600125) was shown to block oxLDL uptake by macrophages [23]. Therefore, SP600125 was used as a positive control in this high-throughput screen of DiI-oxLDL uptake.

Twenty-two compounds from the ICCB Known Bioactives Library were identified in the phenotypic screen that had a significant impact on oxLDL uptake by the cultured J774 cells [20]. Among the positive hits, several had been previously implicated in oxLDL uptake, including the JNK pathway inhibitor SP600125, the Src tyrosine kinase inhibitors PP1 and PP2, ikarugamycin and bafilomycin. Several drugs were previously identified that had not been previously shown to inhibit oxLDL uptake, including three inhibitors of the NF- κ B transcription factor pathway, two protein kinase C inhibitors, and one phospholipase C inhibitor. Furthermore, the μ opioid receptor agonist loperamide increased oxLDL uptake at low doses. Taken together, these results support the model that the uptake of oxLDL by macrophages is dependent on the sequential or parallel activation of several signal transduction cascades that promote actin-mediated internalization. Modification of the initial screening methods permitted the determination of dose-response curves in primary peritoneal macrophages for most of the identified agents [20]. To provide more biological evidence for the efficacy of identified drugs, the long-term administration of these compounds to hypercholesterolemic mice or other mammalian models of atherosclerosis is an important objective. In vivo testing of identified compounds is also needed to exclude compounds with systemic toxicity.

Recently, a second high-throughput phenotypic screen was performed to identify inhibitors of the uptake of modified forms of LDL by macrophages [24]. In this screen, Sf9 insect cells were infected with a baculovirus encoding human class B scavenger receptor CD36. Sf9 cells were plated in 96-well microtiter plates, infected with the baculovirus encoding CD36, and 72 h later, 3,200 compounds from a library of the Institute of Medicinal Biotechnology,

Chinese Academy of Medical Sciences, were added. After 1 h of incubation with the compounds, DiI-labeled acetylated LDL (DiI-acLDL) was added to each well. After 5 h, auto-fluorescence and quenching was evaluated. The Sf9 cells were washed multiple times and fluorescence was measured again to quantify the specific uptake of DiI-acLDL. OxLDL was used as a positive control in the assay as a way to inhibit DiI-acLDL uptake. Two compounds were found to significantly inhibit DiI-acLDL uptake by Sf9[hCD36] cells, 3-cinnamoyl indole and 13-pentyl berberine [24]. These two compounds were also found to increase oxLDL uptake in RAW 264.7 macrophage-like cells. However, the ability of these agents to inhibit foam cell formation in vivo has not been demonstrated.

The Use of Phenotypic Screens to Interrogate the Macrophage Cholesterol Efflux Pathway

Phenotypic high-throughput screening techniques were also utilized in a project to identify inhibitors of the transfer of cholesterol between HDL particles and cells [19,25]. This transfer process is mediated by the scavenger receptor class B, type I (SR-B1) (Fig. 1). Cholesterol can be transferred from plasma HDL particles to cells and from cells to HDL particles via SR-B1. While promoting efflux of cholesterol from cells to HDL particles is an important objective of atherosclerosis research, this particular screen focused on the cellular uptake of cholesterol from HDL [25]. HDL particles were labeled with DiI so that they could be used as a detection reagent in a phenotypic screen since the cellular uptake and accumulation of DiI from DiI-labeled HDL is apparently a reliable surrogate for SR-B1-mediated uptake of the cholesteryl esters in HDL particles [25]. LDL receptor-deficient Chinese hamster ovary cells that expressed high levels of murine SR-B1 were cultured in microtiter plates and over 16,000 compounds were added to individual wells [25]. DiI-HDL was then added to each well. After an additional two hours, the fluorescence of each well was measured to determine auto-fluorescence and quenching. Next, cells were washed and fluorescence was measured again to quantify DiI-HDL uptake by cells. The cellular uptake of cholesterol from HDL was inhibited by five compounds and these compounds also inhibited the efflux of cholesterol from cells to HDL particles [25]. It is unclear whether the five compounds identified in this high-throughput screen will promote the efflux of cholesterol from macrophage foam cells in atherosclerotic lesions, so additional in vivo studies are warranted.

In an additional phenotypic high-throughput screen, compounds that promoted the expression of ATP-binding cassette transporter A1 (ABCA1) were discovered [19,26]. ABCA1 is a plasma membrane protein that stimulates HDL particle generation by promoting the cellular efflux of cholesterol and phospholipids, and that has a significant anti-atherosclerotic effect (Fig. 1) [27,28]. HepG2 cells were stably transfected with a human ABCA1 promoter-luciferase reporter construct. Transfected HepG2 cells were cultured in microtiter plates and 2,600 compounds were added [26]. Fourteen compounds were discovered that increased the activity of the ABCA1 promoter-luciferase reporter by at least 1.5-fold, and the bioactivity of some of these compounds was confirmed in a second assay. Identified compounds included two anthracycline antibiotics and two isoflavone compounds. These four drugs were studied further and they induced ABCA1 expression in cultured cells [26]. The ability of these four compounds to promote cholesterol efflux from macrophage foam cells within atherosclerotic lesions remains to be demonstrated.

The Potential Use of Phenotypic High-Throughput Screens to Investigate Macrophage Apoptosis and Efferocytosis

The vast majority of atherosclerotic lesions do not become unstable and rupture, and do not cause thrombosis resulting in myocardial infarction or stroke. Indeed, perhaps only 2% or 3% of atherosclerotic lesions ever cause detectable injury to a patient [10]. Therefore, it is reasonable to consider the biological events that occur in atherosclerotic lesions that become unstable and cause clinical symptoms for investigation in phenotypic high-throughput screens. Recent work showed that macrophage apoptosis and the failure to efficiently clear dead macrophages and other dead cells (efferocytosis) contributed to the development of unstable atherosclerotic lesions (Fig. 1) [10]. The cause of macrophage apoptosis in aging atherosclerotic lesions is probably multifactorial, but the occurrence of endoplasmic reticulum (ER) stress may be an important contributory factor. Work from one group showed that ER stress, combined with the activation of scavenger receptors, such as CD36 (plus TLR2), potently induced macrophage apoptosis [10,29]. Therefore, a hypothetical phenotypic high-throughput screen would be to treat cultured macrophages with an ER stress-inducing agent (e.g. thapsigargin), and oxLDL to activate CD36. Compounds could be screened for their ability to inhibit macrophage apoptosis in the presence of thapsigargin and oxLDL.

The potential inability of live M2 macrophages in aged atherosclerotic lesions to efficiently clear dead macrophages and other dead cells suggests other potential phenotypic screens [10,30]. Recent work demonstrated that macrophage LRP1 and ABCA7 are required for effective efferocytosis, but that ABCA7 is usually located in the cytosol of macrophages [31,32]. Therefore, agents that promote the translocation of ABCA7 to the plasma membrane of macrophages may promote efficient efferocytosis. Furthermore, agents that increase the expression of LRP1 or ABCA7 may promote efferocytosis. Another important molecule in this process is the proto-oncogene tyrosine-protein kinase MER (MERTK) that is a receptor on efferocytes for apoptotic cells [10,33]. In another hypothetical high-throughput screen, drugs could be screened for their ability to promote the expression of LRP1, ABCA7 or MERTK by use of J774 cells transfected with appropriate promoter-reporter constructs [31]. Alternatively, fluorescently labeled vesicles containing phosphatidylserine could be added to macrophages to directly evaluate the efferocytosis of apoptotic cells [34]. Phosphatidylserine is a key ligand on the outer plasma membrane of apoptotic cells that triggers phagocyte engulfment [34]. Drugs could then be screened for their ability to enhance macrophage uptake of the phosphatidylserine-containing, fluorescently labeled vesicles.

Summary

Despite the widespread utilization of lipid-lowering therapeutic agents, such as statins and fibrates, atherosclerosis remains a common and highly destructive disease process. To address this great clinical need, it may be useful to alter the cellular composition and characteristics of atherosclerotic lesions. Phenotypic high-throughput screens provide a method to identify agents useful in modifying atherosclerotic lesions. Phenotypic screens use cells, tissues or organisms in complex bioassays to evaluate drug efficacy, and in this way are dramatically different from standard pure-protein screens that evaluate a drug's ability to modify a specific enzymatic activity. Phenotypic screens are dependent on the reproducibility and robustness of the bioassay, and assays that rely on fluorescent or luminescent read-outs are predicted to be superior to assays that depend on more qualitative endpoints.

The use of phenotypic screens has now been successfully applied to atherosclerosis research in several cases. Firstly, the examination of cholesterol influx from oxLDL particles into J774 macrophages has been used to screen a library of compounds [20]. Secondly, the ability of compounds to block the uptake of acLDL by CD36 in Sf9 cells has been evaluated in a high-throughput screen [24]. Thirdly, the examination of cholesterol transfer between cultured CHO cells expressing SR-B1 and HDL particles has been successfully used in a phenotypic screen [25]. Fourthly, the ability of compounds to stimulate the expression of the ABCA1 transporter, which mediates the cellular efflux of cholesterol and phospholipids, in cultured HepG2 cells has been used in another high-throughput screen [26]. Specific pharmacologic agents have been identified that may promote cholesterol efflux from macrophages to HDL particles, and that inhibit macrophage cholesterol influx from oxLDL.

Once positive hits have been identified in high-throughput automated screens, in vivo studies to examine efficacy in animal models of atherosclerosis can be performed. These animal studies have not been reported to date for the phenotypic screens mentioned above. However, some of the drugs identified in the oxLDL uptake screen were previously shown to be effective at reducing the development atherosclerosis in hyperlipidemic mouse models [20]. When treating patients, it is likely that therapy will not be initiated until atherosclerosis is well established, so the ability of compounds to prevent the establishment of lesions in hyperlipidemic mice may not reflect their clinical utility in reducing atherosclerotic lesion burden or preventing plaque rupture. However, it is possible that established lesions may exhibit plasticity and be responsive to interventions that reduce oxLDL uptake or promote cholesterol efflux by macrophages. Although the ultimate clinical utility of phenotypic screens to identify compounds for the treatment of atherosclerotic vascular disease remains unproven, it is possible that novel compounds will be developed for patient care by use of this methodology. In addition to their potential ability to identify compounds of therapeutic value, phenotypic high-throughput screens may also provide biological information that is complementary to proteomic and genomic studies, especially when “Known bioactives” compound libraries are employed.

Acknowledgments

The author thanks the Chemical Genetics Screening Core at Washington University School of Medicine for completion of the phenotypic high-throughput screen of oxLDL uptake by J774 cells.

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Fig. 1.

A simplified model of macrophage foam cell formation and death in the arterial wall. Monocytes in circulating blood adhere to endothelial cells expressing appropriate adhesion molecules, which usually occur in response to injury or infection. Monocytes interdigitate between endothelial cells into the subendothelial space where they differentiate into macrophages. Also, in the subendothelial space, low-density lipoprotein (LDL) particles from the blood are resident, and they can be oxidized by enzymes such as 12/15-lipoxygenase or myeloperoxidase. Oxidized forms of LDL are taken up by macrophage scavenger receptors, such as CD36 and SR-A. Cholesterol from oxLDL particles is retained in macrophages in inclusion bodies leading to foam cell formation. Cholesterol efflux from macrophages can occur via SR-B1 and ABCA1 to HDL. Foam cells can undergo apoptosis, in part because of endoplasmic reticulum stress, and apoptotic cells are cleared by M2 macrophages through a process called efferocytosis [10]. In advanced lesions, efferocytosis is impaired, leading to secondary necrosis of the foam cells and formation of the necrotic core