CARDIOPROTECTIVE FUNCTIONS OF HDL

by

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Abstract

Multiple human population studies have established the concentration of high density lipoprotein (HDL) cholesterol as an independent, inverse predictor of the risk of having a cardiovascular event. Furthermore, HDLs have several well-documented functions with the potential to protect against cardiovascular disease. These include an ability to promote the efflux of cholesterol from macrophages in the artery wall, inhibit the oxidative modification of low density lipoproteins (LDLs), inhibit vascular inflammation, inhibit thrombosis, promote endothelial repair, promote angiogenesis, enhance endothelial function, improve diabetic control and inhibit hematopoietic stem cell proliferation. There are also undoubtedly other beneficial functions of HDLs that have yet to be identified. The HDL fraction in human plasma is heterogeneous, consisting of several subpopulations of particles of varying size, density and composition. The functions of the different HDL subpopulations remain largely unknown. Given that therapies that increase the concentration of HDL cholesterol have varying effects on the levels of specific HDL subpopulations, it is of great importance to understand how different HDL subpopulation distributions contribute to the potentially cardioprotective functions of this lipoprotein fraction. This review summarizes current understanding of the relationship of HDL subpopulations to their cardioprotective properties and also serves to highlight the gaps in current knowledge regarding this important aspect of HDL biology.
Introduction

The proposition that high density lipoproteins (HDLs) protect against the development of cardiovascular diseases is based on a number of robust and consistent observations: (i) Numerous human population studies have shown that the plasma concentrations of both HDL cholesterol and the major HDL apolipoprotein, apoA-I, are independent, inverse predictors of the risk of having a cardiovascular event (1-5). Moreover, a low concentration of HDL cholesterol remains predictive of increased cardiovascular risk, even when low density lipoprotein (LDL) cholesterol has been reduced to very low levels by treatment with statins (6); (ii) HDLs have several well-documented functions with the potential to protect against cardiovascular disease (7, 8); (iii) Interventions that increase the concentration of HDLs inhibit the development and progression of atherosclerosis in several animal models (9-12) and (iv) In “proof-of-concept” studies in humans, intravenous infusions of reconstituted HDLs (rHDLs) consisting of apoA-I complexed with phospholipids promote regression of coronary atheroma as assessed by intravascular ultrasound (13, 14).

However, interventions that increase the concentration of HDL cholesterol in humans have not yet been shown to translate into a reduction in clinical cardiovascular events. Indeed, recent human clinical trials investigating the effects of raising the level of HDL cholesterol by treatment with cholesteryl ester transfer protein (CETP) inhibitors or with niacin failed to demonstrate any clinical cardiovascular benefit (15-17) (http://www.thrivestudy.org) and in one case, the treatment caused harm (15). A reasonable assumption that has been made from these studies is that the cholesterol content of HDLs is not the factor that protects. Thus, if HDLs do have direct cardioprotective properties, it follows from the human population studies that, while the concentration of HDL cholesterol is generally an excellent marker of the HDL functions that do protect, it does not invariably reflect their cardioprotective
functions. This is consistent with the finding in a recent Mendelian randomisation study that some genetic mechanisms that raise the concentration of HDL cholesterol appear not to lower the risk of myocardial infarction (18). The authors of this analysis concluded that, while this result challenges the concept that raising the concentration of HDL cholesterol will translate into a reduction in risk of myocardial infarction, it did not address the possibility that an enhancement of HDL function would be protective.

For example, in some clinical circumstances a high concentration of HDL cholesterol is not accompanied by a decrease in cardiovascular risk (19). This may reflect the fact that an intervention that raises the level of HDL cholesterol is not necessarily accompanied by an increase in the functionality of the HDL fraction. This leads to an obvious question: given the robust evidence from animal and human population studies that HDLs are anti-atherogenic, why have recent clinical trials using agents that increase the level of HDL cholesterol failed to demonstrate a reduction in clinical cardiovascular events?

There are several potential explanations for this. It is possible that the inverse relationship between the concentration of HDL cholesterol and cardiovascular risk observed in human population studies is an epiphenomenon rather than a direct cardioprotective effect. However, this is not supported by many animal studies showing that atherosclerosis is inhibited by interventions that increase the level of HDL cholesterol (9-12). It is also possible, but unlikely, that the ability of HDLs to inhibit the development of atherosclerosis in animals, and to promote regression of atherosclerosis in humans, does not necessarily translate into an ability of HDLs to reduce clinical cardiovascular events in humans. There is also evidence that some agents that increase HDL cholesterol levels have adverse off-target effects that may negate the benefit conferred by the HDL elevation. This may well have been the case with the
CETP inhibitor, torcetrapib (15). It is also possible that a niacin-induced insulin resistance (20) may partially negate the effects of this agent on HDLs.

It has, furthermore, been suggested that increasing HDL cholesterol levels may be ineffective in statin-treated patients in whom the concentration of LDL cholesterol is very low (21), although there is little evidence to support this. It is also possible that in some studies the ability of HDL cholesterol raising agents to reduce cardiovascular events was tested in people in whom HDL function was compromised. This may well have been the case in the population of acute coronary syndrome patients studied in the Dal-OUTCOME trial, where, even in the placebo group, there was no evidence of a relationship between HDL cholesterol levels and cardiovascular events (17). This observation raised the possibility, as has been reported in ex vivo studies (22), that the cardioprotective properties of the HDLs are compromised after an acute coronary syndrome. If this is true, it follows that increasing endogenous HDL levels in these individuals may not lead to a reduction in cardiovascular events.

These observations raise several questions and highlight major gaps in current knowledge about the relationship between HDLs and cardiovascular disease. For example, it is not known which HDL functions are clinically important; nor is it known whether some functions are more cardioprotective than others. Moreover, we know little about the functionality of individual HDL subpopulations. Nor do we know whether therapeutic interventions that increase the concentration of HDL cholesterol are necessarily accompanied by an enhancement of HDL function.

This review is concerned with what is currently known about the potentially cardioprotective
functions of HDLs and will highlight the gaps in current knowledge regarding this important aspect of HDL biology. It should be noted that in the interest of keeping the size of the review manageable, it has not been possible to cite all of the references that would otherwise be worthy of inclusion.

**Overview of HDL functions that have the potential to reduce cardiovascular risk**

Several well-documented functions of HDLs and apoA-I have the potential to protect against cardiovascular disease. The most extensively studied of these relates to the ability of HDLs to promote efflux of cholesterol from macrophages in the artery wall (23). HDLs also inhibit vascular inflammation (8, 24) and have anti-oxidant (8) and anti-thrombotic (25) properties. They enhance endothelial function (26), promote endothelial repair (27, 28), increase angiogenesis (29), suppress the production and mobilization of monocytes and neutrophils from bone marrow (30) and have recently been reported to have anti-diabetic properties (31) (31, 32). Which of these functions of HDLs are clinically important is not known. Nor is it known which HDL component(s) or which HDL subpopulations are responsible for these potentially cardioprotective properties. This lack of insight is largely due to the fact that HDLs consist of multiple subpopulations of particles that are continually being interconverted from one to another by a range of plasma factors (33), as well as by the fact that it is currently not feasible to isolate specific HDL subpopulations in amounts that are sufficient to investigate their functionality in a systematic manner.

**Heterogeneity of the HDL fraction in human plasma**

The HDLs in human plasma are heterogeneous in terms of particle density, size, shape, surface charge and composition (34). When isolated on the basis of density, human HDLs can be resolved into two major subfractions: HDL$_2$ which comprises large, less dense particles
and HDL₃, which consists of smaller and more dense particles. Separation of the total HDL fraction on the basis of particle size by non-denaturing polyacrylamide gradient gel electrophoresis has identified at least five distinct HDL subpopulations (35).

HDLs also vary in surface charge and can be resolved on this basis by agarose gel electrophoresis into several subpopulations. According to this approach, α-migrating particles equate with the spherical HDL particles that predominate in human plasma, while pre-β migrating particles comprise either a single molecule of lipid-poor apoA-I, a single molecule of apoA-I complexed with a small number of phospholipid molecules, or discoidal particles that contain two or three molecules of apoA-I complexed with multiple phospholipid molecules plus a small amount of unesterified cholesterol (36).

Discrete HDL subpopulations have also been identified on the basis of their apolipoprotein composition: those that contain apoA-I but are deficient in apoA-II, the second most abundant HDL apolipoprotein, (A-I HDLs), and those that contain both apoA-I and apoA-II (A-I/A-II HDLs) (37). A minor subpopulation of apoE-containing, γ-migrating HDL particles has also been reported (38).

It has been suggested that A-I HDLs may be superior to A-I/A-II HDLs in their ability to protect against atherosclerosis (39), although evidence for this is associative and requires confirmation in large prospective population studies. It has also been suggested that particles in the HDL₂ subfraction may be more cardioprotective than those in the HDL₃ subfraction (40), while other studies have concluded that a very high concentration of large, cholesterol-rich HDL₂ particles, when not accompanied by a correspondingly high level of apoA-I-containing HDL, may be associated with increased rather than decreased cardiovascular risk.
Thus, the evidence linking cardioprotection to specific HDL subpopulations in humans is both limited and conflicting.

There is compelling evidence from human studies that interventions that raise the level of HDL cholesterol have varying effects on specific HDL subpopulations. For example, statins (41, 42), niacin (43) and CETP inhibitors (44) tend to increase the concentration of large, apoA-I containing HDLs. Fibrates, on the other hand, tend to increase levels of smaller HDL particles that contain both apoA-I and apoA-II (45). The clinical implications of these differences are not known. There is also evidence from animal studies that different approaches to raising the concentration of HDL cholesterol may vary markedly in terms of their ability to inhibit atherosclerosis. For example, there is robust and consistent evidence that over-expression of apoA-I in mice (10, 46) and rabbits (11), as well as intravenous infusions of native HDLs or rHDLs into these animals (9, 47), inhibits the development of atherosclerosis. There is also consistent evidence that increasing endogenous HDL cholesterol levels by inhibiting CETP (48, 49) or by over-expressing LCAT in cholesterol-fed rabbits (50) is anti-atherogenic. However, there are situations in some genetically engineered mouse models where an increase in HDL cholesterol level is not accompanied by an inhibition of atherosclerosis. These include mice that over-express LCAT (51) or lack endothelial lipase (52).

So, until we know which HDL components and which HDL subpopulations relate to specific, potentially cardioprotective HDL functions, and until we have much more information about the effects of HDL-raising therapies on HDL composition, HDL subpopulation distribution and HDL function, it will be difficult to predict how any specific HDL-targeted therapy will impact on human cardiovascular risk. The following sections summarize what is currently
known about the potentially cardioprotective functions of HDLs, how they relate to specific HDL lipid and apolipoprotein constituents and, wherever possible, the relationship of specific HDL functions to specific HDL subpopulations.

**HDLs and cholesterol efflux**

The efflux of cholesterol from a variety of cell types, including macrophages, to HDLs in the extracellular space is mediated by four distinct processes (53). These are (i) the efflux of cholesterol to apoA-I from cells expressing the ATP binding cassette transporter A1 (ABCA1) (54, 55), (ii) the efflux of cholesterol to HDLs from cells expressing the ATP binding cassette transporter G1 (ABCG1)(56, 57), (iii) the bi-directional exchange of cholesterol between HDLs and cell membranes expressing scavenger receptor-B1 (SR-B1) (58, 59) and (iv) passive, aqueous diffusion of cholesterol from cell membranes to HDLs (58).

ABCA1 translocates phospholipids and cholesterol from the inner to the outer leaflets of cell membranes, from where they are exported to lipid-free or lipid-poor apolipoproteins in the extracellular space (60). This progressively lipidates the apolipoproteins, leading to the generation of discoidal HDL particles that can also act as acceptors of the cholesterol that effluxes from ABCA1-containing cells (Fig. 1). Discoidal HDLs are also excellent substrates for lecithin:cholesterol acyltransferase (LCAT), the plasma enzyme that esterifies the newly acquired cholesterol. The resulting cholesteryl esters are hydrophobic and are sequestered into the centre of the particles in a process that converts discoidal HDLs into the spherical HDL particles that predominate in human plasma. Both discoidal and spherical HDL particles act as acceptors of the cholesterol that is exported from cells by ABCG1 (Fig. 1).
The third cholesterol efflux mechanism is mediated by the binding of both discoidal and spherical HDLs to SR-B1. As the rate of SR-B1-mediated cholesterol efflux from cells is positively correlated with the concentration of HDL cholesterol (59), it follows that the contribution of this process to total efflux may increase when HDL levels are increased either by pharmacological inhibition of CETP, by infusions of rHDLs, or by other means. Evidence in support of this notion comes from a recent report showing that SR-B1 accounts, at least in part, for the rapid mobilization of cellular cholesterol into the plasma compartment in mice infused with discoidal rHDLs (61).

Passive aqueous diffusion of cholesterol from the cell surface to HDL particles in the extracellular space reflects the movement of cholesterol down the concentration gradient that is formed when LCAT depletes the HDL surface of cholesterol by generating cholesteryl esters that move into the centre of the particle. This LCAT-generated cholesterol concentration gradient enables HDL particles to acquire additional cholesterol from cell membranes, as well as from other lipoproteins.

The contributions of the various efflux pathways in cholesterol-normal and -enriched cells were evaluated by Adorni et al. (62). They reported that ABCA1 had little effect on total efflux when cell cholesterol levels were normal. However, efflux was decreased by 50% in macrophages from ABCA1-deficient mice, while studies of macrophages from ABCG1-deficient mice indicated that approximately 20% of the efflux from cholesterol-loaded monocyte derived macrophages was attributable to ABCG1. In this study, SR-B1 was shown to make only a minor contribution to the total cholesterol efflux. In contrast, aqueous diffusion was found to be a major contributor to efflux, particularly in cells with normal cholesterol levels.
Despite significant efforts in recent years, the relative contributions of these four processes to the net efflux of cholesterol from macrophages in the artery wall remain poorly defined. The effects of variations in HDL composition and subpopulation distribution on the regulation of cholesterol efflux are also not well understood.

The importance of understanding such factors is highlighted by the recent observation that HDLs isolated from different human subjects vary in their ability to promote cholesterol efflux from macrophages \textit{ex vivo} (63). Furthermore, the cholesterol efflux promoted by HDLs isolated from different individuals correlates inversely with measures of atherosclerosis, independent of the plasma concentration of HDL cholesterol (63). So, the question arises: how does HDL composition and HDL subpopulation distribution impact on the ability of HDL to promote the efflux of cholesterol from cells?

\textit{Roles of specific HDL constituents and specific HDL subpopulations as mediators of cellular cholesterol efflux}

Lipid-free apoA-I, lipid-poor apoA-I and discoidal HDL particles, all of which migrate to a pre-\(\beta\) position when subjected to agarose gel electrophoresis, are the preferred acceptors of the cholesterol that effluxes from cells that express ABCA1 (60, 64). There is evidence that, in addition to apoA-I, other HDL apolipoproteins that contain multiple amphipathic \(\alpha\)-helical domains (apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III and apoE) can also accept the cholesterol and phospholipids released from cells that express ABCA1 (65). The relative contributions of these acceptors to ABCA1-mediated cholesterol efflux \textit{in vivo} is completely unknown. The fact that these apolipoproteins all contain multiple amphipathic \(\alpha\)-helical motifs suggests that there is likely to be a similarity in the mechanism by which they mediate
efflux. It is thus tempting to speculate that their capacity to remove cholesterol from cells may simply be a reflection of their relative abundance in plasma.

Larger, spherical HDL particles, whether containing apoA-I without apoA-II or containing both apoA-I and apoA-II, are the preferred acceptors of the cholesterol released from cells by ABCG1 (56) and by SR-B1 (53). It should be noted, however, that the physiological importance of the ABCG1 and SR-B1 pathways is unknown, as is the role of large, spherical HDL particles as acceptors of cell cholesterol in vivo. It is also noteworthy that the efflux of cholesterol mediated by both ABCG1 and SR-B1 is regulated by the phospholipid content and size of the acceptor particles (66) (67).

The final point worthy of consideration in this section is that the results of in vitro measures of cholesterol efflux need to be interpreted with care. Many of these studies involve quantifying the export of radiolabelled cholesterol from cells. This provides a reliable estimate of efflux only when the donor cells are cholesterol loaded (68). Under circumstances where donor cells are not cholesterol loaded, an accurate estimation of cholesterol efflux depends on the quantification of cholesterol mass in the medium and the donor cells (68).

**Anti-oxidant activity of HDLs**

HDLs possess anti-oxidant properties that have the capacity to inhibit the pro-atherogenic oxidative modification of LDL (69). The mechanism by which HDL inhibit oxidation is uncertain. The most extensively studied aspect of this process involves activity of paraoxonase-1 (PON-1), which is transported in the plasma as a component of the HDL fraction (70). However, as outlined below, HDL apolipoproteins also have intrinsic anti-oxidant effects that are independent of PON-1.
Roles of specific HDL constituents and specific HDL subpopulations as mediators of the anti-oxidant properties of HDL

ApoA-I (71), apoA-II (71), apoA-IV (72, 73) and apoE (74) all have anti-oxidant properties in vitro. Which of these apolipoproteins, if any, are also anti-oxidant in vivo is not known. While the PON-1 that is transported as a component of HDL particles is widely considered to be a major contributing factor to the anti-oxidant potential of HDLs, there is also evidence that HDLs have intrinsic anti-oxidant properties that are PON-1-independent (71, 75). According to these reports, the lipid hydroperoxides that are associated with HDLs are reduced to less deleterious lipid hydroxides via a process that is accompanied by the concomitant oxidation of apoA-I methionine residues (71, 75). These events, which are initiated by the transfer of lipid hydroperoxides from LDLs to HDLs by CETP (76), represent a PON-1-independent anti-oxidant property of HDLs. There is evidence that the antioxidant activity of HDL₃ is superior to that of HDL₂ (77). It has also been reported that phospholipids modulate the anti-oxidant properties of HDLs (75).

Anti-inflammatory activity of HDLs

Inflammation plays a pivotal role in both the genesis and the instability of atherosclerotic plaques. HDLs have the capacity to inhibit this inflammation by multiple mechanisms. They inhibit the binding of monocytes to cultured endothelial cells in vitro (69). They also reduce the cytokine-induced expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin in cultured endothelial cells in a concentration-dependent manner (24). The ability of HDLs to inhibit inflammation in endothelial cells in vitro varies according to the phospholipid composition of the particles (78), with their constituent apolipoproteins exhibiting much lower specificity (79).
HDLs also inhibit inflammation in vivo. For example, intravenous infusions of apoA-I-containing rHDLs reduce endothelial adhesion molecule expression in cholesterol-fed, apo E-knockout mice in which vascular inflammation has been induced by insertion of a periarterial carotid cuff (80). Studies of apoE-knockout mice transgenic for human apoA-I have established that increasing the concentration of HDLs reduces ex vivo leukocyte adhesion to endothelial cells (81). In a study conducted in rabbits in which aortic atherosclerosis was induced by endothelial denudation of the abdominal aorta followed by 17 weeks of intervention with a high cholesterol diet, intravenous infusions of relatively small amounts of HDLs given only during the last week of the study significantly reduced inflammation and decreased the macrophage/smooth muscle cell ratio in the aortic wall (12).

HDLs also have vascular anti-inflammatory properties in vivo in the absence of atherosclerosis, with intravenous infusions of rHDLs inhibiting the development of a local inflammatory infiltrate following the subcutaneous administration of interleukin-1 in a porcine model (82). In studies of experimental stroke in rats, pre-treatment with rHDLs has also been shown to substantially reduce the brain necrotic area (83).

In other studies conducted in normcholesterolemic rabbits in which arterial inflammation was induced by placing a non-occlusive silastic collar around the common carotid artery, intravenous infusions of small amounts of rHDLs containing apoA-I markedly inhibited the infiltration of neutrophils into the intima/media of the artery wall, the collar-induced reactive oxygen species formation, and the collar-induced endothelial expression of adhesion molecules and chemokines (84). It was subsequently shown that this inhibition of peri-arterial collar-induced acute vascular inflammation in rabbits could be achieved with a single
injection of remarkably small amounts (2mg/kg) of apoA-I that were not sufficient to increase circulating levels of HDL cholesterol (85).

The latter observation suggests that other events, such as increased expression of key genes that prevent inflammation, are likely to be important for mediating the anti-inflammatory effects of HDL and apoA-I. This notion is further supported by the observation that the ability of rHDLs to inhibit VCAM-1 expression in tumor necrosis factor-α (TNF-α)-activated endothelial cells persists, even when the rHDLs are removed from the culture medium several hours prior to the cytokine activation (86).

These findings led to the hypothesis that exposure of endothelial cells to rHDLs alters the expression of proteins that have the potential to protect against a subsequent inflammatory insult. One such protein that is induced both in vitro (87) and in vivo (88) by rHDLs is the anti-oxidant protein, 3β-hydroxysteroid-Δ24 reductase (also known as 24-dehydrocholesterol reductase, or DHCR24). Silencing DHCR24 expression in endothelial cells not only increases NF-κB activation and VCAM-1 protein levels in both non-activated and TNF-α-activated endothelial cells, but it is also associated with a loss of the anti-inflammatory effects of rHDLs (87). The rHDL-induced expression of DHCR24 in cultured endothelial cells is dependent on SR-B1, but not ABCA1 or ABCG1 (87). This effect appears not to be related to the ability of SR-B1, ABCA1 and ABCG1 to maintain intracellular cholesterol homeostasis, but is associated with the activation of intracellular signal transduction pathways that are involved in inflammation (89-91).

Expression of DHCR24 can also be induced in rabbits with acute vascular inflammation by administering a single intravenous infusion of lipid-free apoA-I 24 hours prior to inserting a
non-occlusive peri-arterial silastic collar (88). The results from that study also established that apoA-I-containing rHDLs inhibit vascular inflammation \textit{in vivo} and adhesion molecule expression in TNF-\(\alpha\)-activated endothelial cells \textit{in vitro} by increasing DHCR24 expression and that this mediated downstream activation of the PI3K/Akt signal transduction pathway with subsequent induction of the cardioprotective enzyme, heme oxygenase-1 (HO-1) (Fig. 2) (88).

\textbf{Roles of specific HDL constituents and specific HDL subpopulations as mediators of the anti-inflammatory properties of HDL}

It has been reported that the HDL\(_3\) subfraction, which predominantly consists of A-I/A-II HDL, inhibits TNF-\(\alpha\)-induced inflammation in endothelial cells more effectively than the HDL\(_2\) subfraction, which is mostly A-I HDL (79). The HDL constituents that are responsible for this difference have yet to be identified. Existing evidence suggests that this observation is unlikely to be a consequence of variations in HDL apolipoprotein content as HDL preparations that contain apoA-I or apoA-II as the sole protein appear to be equally effective at inhibiting the TNF-\(\alpha\)-induced increase in adhesion molecule expression in cultured endothelial cells (79).

Phosphatidylcholine vesicles that do not contain apolipoproteins also inhibit adhesion molecule expression \textit{in vitro} in TNF-\(\alpha\)-activated endothelial cells (78). Of interest, lipid-free apoA-I is unable to inhibit endothelial cell inflammation \textit{in vitro} (92). \textit{In vivo}, by contrast, intravenous infusions of lipid-free apoA-I (84) inhibit the acute inflammation induced by inserting non-occlusive carotid collars into rabbits as effectively as the corresponding rHDL preparations. The explanation for the difference between these \textit{in vitro} and \textit{in vivo} findings is not known, but may reflect the fact that intravenously injected lipid-free apoA-I rapidly
acquires phospholipids and is incorporated into the endogenous HDL fraction as discoidal HDL particles that resemble the rHDLs that effectively inhibit endothelial inflammation \textit{in vitro}.

**Inhibition of thrombosis by HDLs**

HDLs inhibit thrombosis by attenuating expression of tissue factor and selectins, by down-regulating thrombin generation via the protein C pathway, and by directly and indirectly blunting platelet activation (25). Inhibition of platelet activation is also dependent on SR-B1 (93, 94). SR-B1 and eNOS knockout mice, which have increased susceptibility to deep vein thrombosis, have also been used to establish that activation of the SR-B1-eNOS signalling pathway by apoA-I is important for inhibiting thrombosis (95). HDLs also have the potential to protect against arterial and venous thrombosis by activating prostacyclin synthesis (25) and can reduce the risk of recurrent venous thrombosis (96).

**Roles of specific HDL constituents and specific HDL subpopulations as mediators of the anti-thrombotic properties of HDL**

The HDL constituents and subpopulations that inhibit thrombosis have not been investigated extensively. There is, however, one report of venous thrombosis being associated with low apoA-I levels and a reduction in HDL size (97).

**HDLs and endothelial repair**

HDLs enhance endothelial repair by two distinct mechanisms. They stimulate endothelial cell migration from healthy to damaged endothelium via the SR-B1-mediated activation of Rac GTPase in a process involving activation of Src kinases, PI3K, and p44/42 mitogen-activated protein kinases (27). In addition, HDLs can promote endothelial repair \textit{in vivo} by enhancing
the engraftment of endothelial progenitor cells into areas of damaged endothelium (28). These topics are covered in detail in the companion review article from this series by Riwanto et al.

Roles of specific HDL components and specific HDL subpopulations in promoting endothelial repair

There is little information regarding which HDL components and which HDL subpopulations are responsible for enhancing endothelial repair, other than an adenoviral-mediated increase in apoA-I expression increases circulating endothelial progenitor cell numbers and facilitates their incorporation into the endothelium of apoE-deficient mice (98). There is also evidence that the involvement of SR-B1, Src kinases, PI3K and p44/42 mitogen-activated protein kinases in endothelial repair is dependent on apoA-I (27).

HDL and endothelial function

HDLs are known to play an important and beneficial role in endothelial function. A single intravenous infusion of rHDLs into human subjects with hypercholesterolemia (99), or with low levels of HDLs secondary to partial deficiency of ABCA1 (26), normalizes endothelial function by a mechanism that involves the restoration of endothelial nitric oxide production. *Ex vivo* studies of endothelial cells incubated with HDLs isolated from healthy subjects have also indicated that they can induce eNOS expression, stimulate endothelial cell nitric oxide production and reduce endothelial oxidant stress (100). Of interest, these potentially beneficial effects of HDLs are not observed in preparations of HDLs isolated from patients with type 2 diabetes (101), or from patients with coronary artery disease (22). The ability of HDLs to induce expression of eNOS in endothelial cells is partly dependent on SR-BI (100). Binding of apoA-I to SR-B1 in endothelial cells initiates a signalling cascade that involves PDZK1-
dependent activation of the Src family kinases, PI3K and AKt, which leads to phosphorylation of eNOS and a subsequent increase in enzyme activity (102).

Roles of specific HDL components and specific HDL subpopulations in enhancing endothelial function

The ability of HDL to enhance endothelial function in humans in vivo has been observed with apoA-I containing rHDL but has not been investigated with other HDL preparations. The ability of HDL to enhance synthesis of nitric oxide in vitro has been reported in incubations of endothelial cells with HDLs isolated from human subjects (100) but, as far as we are aware, there are no reports of this effect being specifically due to apoA-I-containing HDL.

HDL and angiogenesis

Intravenous injections of rHDLs have been shown to significantly augment blood flow recovery and to increase capillary density in a murine ischemic hind-limb model of angiogenesis (29). In this model, treatment with rHDLs increased the number of bone marrow-derived cells that were incorporated into newly formed capillaries in ischemic muscle and promoted the differentiation of peripheral mononuclear cells into endothelial progenitor cells in a dose-dependent manner. The effect of rHDLs on endothelial progenitor cell differentiation is abrogated by co-administration of LY294002, an inhibitor of PI3K (29, 103). It is noteworthy that rHDLs are unable to promote angiogenesis in endothelial NO-deficient mice (29).

Low concentrations of HDLs isolated from healthy donors, but not from donors with cardiovascular disease, have also been shown to enhance EPC-mediated tubulogenesis by
processes that involve PI3K/Akt and eNOS, while higher, more physiologically relevant HDL concentrations from both types of subjects, do not (104). The mechanistic basis for this difference is not clear at present. There is also evidence from in vitro studies that the angiogenesis mediated by HDL involves Ras/MAP kinase activation (105).

**Roles of specific HDL constituents and specific HDL subpopulations in promoting angiogenesis**

The contributions of specific HDL lipid and apolipoprotein constituents to angiogenesis have not been investigated systematically. The reports published to date have focussed on the ability of apoA-I-containing rHDLs to stimulate angiogenesis (29). Studies of the HDL$_2$ and HDL$_3$ subfractions have indicated that they both mediate tubulogenesis to an extent comparable to that of the total HDL fraction (104), which suggests that there is unlikely to be specificity in terms of the apolipoprotein content of HDL. The effects of HDL lipids on this function have not yet been investigated.

**Effects of HDL on hematopoietic stem cell proliferation**

This is one of the more recently identified novel HDL functions. Increased blood levels of monocytes and neutrophils are known to predict the development of atherosclerosis (106), with evidence indicating that they play a causative role in lesion development (30). Studies conducted in mice have shown that suppression of the cholesterol efflux promoted by ABCA1 and ABCG1 in bone marrow cells leads to hematopoietic stem cell proliferation and mobilization (30). This increases monocyte and neutrophil production in bone marrow, as well as the number of circulating leukocytes, which infiltrate into multiple organs. This deleterious effect can be reduced by transgenic overexpression of apoA-I in LDL receptor-deficient mice transplanted with bone marrow from ABCA1/ABCG1-deficient mice, with the
end result of reduced leukocytosis, reduced myeloid cell infiltration into organs and reduced diet-induced atherosclerosis (30).

**Effects of specific HDL constituents and specific HDL subpopulations on hematopoietic stem cell proliferation**

In addition to the original report of the beneficial effects of transgenic overexpression of apoA-I on leucocytosis in mice transplanted with ABCA1/ABCG1 deficient bone marrow, a later report from the same group established that apoE-deficient mice also have leucocytosis that is significantly decreased by a single intravenous infusion of apoA-I-containing rHDLs (107). It is also noteworthy that global deficiency of apoE, but not apoA-I, is sufficient to induce leucocytosis in mice (107). Other HDL subpopulations that may be involved in these events have yet to be identified. These observations are relevant to the development of atherosclerosis in humans, with a strong association of leukocytosis and excessive morbidity and mortality in cardiovascular disease being reported (106).

**Anti-diabetic effects of HDL**

HDLs have recently been recognized as having a potentially important role in glucose metabolism. Increasing HDL levels with infusions of apoA-I-containing rHDLs increases plasma insulin levels and reduces plasma glucose levels in people with type 2 diabetes (31). Increasing HDL cholesterol levels by inhibiting activity of CETP has also been shown to improve glycemic control in people with type 2 diabetes (108). The outcomes of these studies suggest that HDL cholesterol raising therapies currently being assessed in humans in large scale cardiovascular clinical outcome trials may also be useful for maintaining glycemic control in people with type 2 diabetes. It should be noted, however, that not all HDL-raising therapies are anti-diabetic, with clear evidence that treatment with niacin is pro- rather than...
anti-diabetic (109).

**Effects of specific HDL constituents and specific HDL subpopulations in diabetes**

To date apoA-I and apoA-II in the lipid-free form and as constituents of rHDLs have both been shown to increase insulin synthesis and secretion in the clonal MIN6 mouse β-cell line, as well as in isolated islets from Sprague-Dawley rats via processes that are dependent on ABCA1, ABCG1 and SR-B1 (110). HDL and apoA-I also protect β-cells from apoptosis *in vitro* (111), while treatment with the apoA-I mimetic peptide, L-4F, has been reported to decrease insulin resistance in obese mice (112). The C-terminal domain of apoA-I has also been shown to increase glucose uptake *in vitro* into cultured L6 skeletal muscle myotubes (113). These findings are consistent with reports of mice deficient in apoA-I and apoA-II having compromised glycemic control (62, 114) and mice overexpressing apoA-I having improved insulin sensitivity (115).

**Inhibition of apoptosis by HDLs**

Endothelial cell apoptosis may play a role in the development atherosclerosis. HDLs protect both macrophages (116) and endothelial cells (117) from apoptosis, in part by promoting the ABCG1-mediated efflux of 7-ketocholesterol and related oxysterols from the cells. It has also been found that small, dense, lipid-poor HDL₃ particles protect endothelial cells from apoptosis induced by oxidized LDLs, with evidence that apoA-I plays a key role such protection (118). The anti-apoptotic properties of HDLs appear to be lost in patients with coronary artery disease, with evidence that HDLs isolated from such patients may even be pro-apoptotic (119). In other studies, HDLs have been shown to inhibit apoptosis in pancreatic beta cells in a process that has the capacity to decrease the progression of type 2 diabetes mellitus (111, 120).
Effects of specific HDL constituents and specific HDL subpopulations on apoptosis

There is evidence that apoA-I (117) and the lysophospholipid, sphingosine-1-phosphate (S1P) (121-125) have anti-apoptotic effects comparable to those of intact HDLs. The observation that S1P enhances endothelial cell survival with effects comparable to those of native HDL, suggests that signalling by lysophospholipid components of HDL may be important for the inhibition of apoptosis (122).

Unanswered questions and future challenges

There has been a recent move towards the view that an accurate assessment of HDL function is likely to provide a more informative assessment of cardiovascular risk than simply measuring HDL cholesterol levels. This has led to considerable progress being made in recent years in understanding the known cardioprotective functions of HDLs, as well as the identification of new cardioprotective functions of HDLs.

Despite these advances, many important, as yet unanswered questions remain. For example, we do not understand (i) which, if any, of the known functions of HDLs are clinically important, (ii) which HDL constituents and which HDL subpopulations are responsible for which functions, (iii) whether specific HDL subpopulations and functions reduce cardiovascular risk better than others or (iv) how HDL-targeted interventions impact on HDL subpopulation levels and function. The answers to these questions are likely to guide future HDL-raising strategies with the potential to reduce cardiovascular risk.

Conclusions

The results from human population and preclinical animal studies demonstrating that HDLs protect against atherosclerotic cardiovascular disease is compelling. Despite this, there is still
no evidence in humans that increasing HDL cholesterol levels translates into a reduction in clinical cardiovascular events. Given that interventions that increase the concentration of HDL cholesterol may not necessarily be accompanied by an enhancement of HDL function, it is clear that we need to understand much more about how HDL function relates to HDL subpopulation distribution and cardiovascular risk in order to develop rational, HDL-based therapeutic strategies for preventing atherosclerosis.

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Figure Legends

**Figure 1. Efflux of cholesterol and phospholipids from cells to HDLs.**

Phospholipids and cholesterol are exported from cell membranes via ABCA1 to lipid-free or lipid-poor apolipoproteins, generating discoidal HDLs that accept cholesterol from ABCA1, ABCG1 and SR-B1. The cholesterol in discoidal HDLs is esterified by LCAT, which generates spherical HDLs that accept cholesterol from ABCG1 and SR-B1.

**Figure 2. Inhibition of inflammation by HDL**

Reconstituted HDLs inhibit inflammation in endothelial cells by increasing DHCR24 expression. This leads to activation of the PI3K/Akt signal transduction pathway, and induction of the cardioprotective enzyme, heme oxygenase-1 (HO-1) (adapted from Wu et al., ref. 84).


Altered activation of endothelial anti- and proapoptotic pathways by high-density lipoprotein from patients with coronary artery disease: role of high-density lipoprotein-proteome remodeling. *Circulation* **127**: 891-904.


Figure 1
Figure 2