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PATHOPHYSIOLOGY OF ISCHEMIC DISORDERS: I- LDL CHOLESTEROL AND ISCHEMIC STROKE

Nazish Iqbal Khan and Zahir Hussain*

*Department of Physiology, University of Karachi, Karachi-75270, Pakistan.*E-Mail: zahussai@yahoo.ca*

ABSTRACT

Blood lipids, particularly total and LDL cholesterol levels, are associated with all subtypes of brain infarction. Furthermore, many of the effects of elevated or modified low density lipoproteins on endothelial cells and endothelial cell processes could be expected to contribute to the development of atherosclerosis and therefore, to the association between lipids and atherosclerotic, particularly coronary and cerebrovascular disease. However, the extent to which "endothelial dysfunction" accounts for the known relationships between serum lipid concentrations, ischemic disease and endothelial progenitor cell (EPC) pathophysiology is yet to be established. On the basis of the mentioned and other reports, we hypothesize that higher levels of plasma LDL cholesterol not only directly impair endothelial cells, but also effect EPC number and function at the same time, thus influencing the endothelial repair process and disturbing the balance between the magnitude of injury and the capacity for repair, which leads to endothelial dysfunction and promotes the progression of stroke. Because EPCs have been implicated in various events requiring endothelialization, we further hypothesized that LDL-C could influence the action of EPCs and sensory neurons and that this could be important to know how LDL-C effects repair, injury and vasculoprotection . In order to corroborate this proposal, quantification is required for EPCs and DRG neurons treated with various concentrations of LDL-C and characterizing the underlying molecular mechanisms. The relative contributions of apoptotic and necrotic death to ischemia-induced neuronal loss may provide us information for understanding the underlying mechanisms. Conclusively, the aggressive management of risk factors, like cholesterol, could have a significant and positive impact on the natural history of atherosclerotic cerebrovascular disease.

Key words: LDL cholesterol, ischemic stroke, pathophysiology

INTRODUCTION

Blood lipids, particularly total and LDL cholesterol levels, are associated with all subtypes of brain infarction, and LDL above 2.59 mmol/l is considered highly prevalent (Amarenco *et al*., 2006). Increasing evidence points to a role for circulating endothelial progenitor cells, including populations of CD34- and CD133-positive cells present in peripheral blood, in maintenance of the vasculature and neovascularization. Immature populations, including CD34-positive cells, have been shown to contribute to vascular homeostasis, not only as a pool of endothelial progenitor cells but also as a source of growth/angiogenesis factors at ischemic loci. We hypothesize that diminished numbers of circulating immature cells might impair such endothelial progenitor cells (EPCs) that are circulating precursor cells and have been implicated recently in vascular and cardiac regeneration.

There is an ongoing discussion on the immunocytological definition of EPCs, based on various surface markers, and currently different cell types are included in the term 'EPC' (Brixius *et al*., 2006). Public awareness of the connection between cholesterol, lipids, and ischemic diseases has increased dramatically in recent years, and in particular because of the updated report of the National Cholesterol Education Program (NCEP) in US (Monte and Pascal, 2002). A major focus of the NCEP report is on low-density lipoprotein cholesterol (LDL-C), which is the primary target of therapy. LDL-C has also been the focus of major clinical trials. Indeed, the outcome benefits in terms of morbidity and mortality are related to the reduction of LDL-C (Monte and Pascal, 2002). Recent clinical trials have indicated that lowering low-density lipoprotein cholesterol (LDL-C) levels below currently recommended targets results in favourable surrogate and clinical end points (Fitchett *et al*., 2006). Frequently, hypercholesterolaemia is a major risk factor for atherosclerosis. It has been shown that endothelial dysfunction ultimately represents a balance between the magnitude of injury and the capacity for repair (Shi *et al*., 1998), and a variety of evidence suggests that circulating EPCs constitute one aspect of the repair process (Shi *et al*., 1998). EPCs are cell population that have the capacity to circulate, proliferate and differentiate into mature endothelial cells, but have not yet acquired the characteristic mature endothelial markers and have not yet formed a lumen (Handgretinger *et al*., 2003). Laboratory evidence suggests that these precursors participate in postnatal neovascularization and re-endothelialization (Camargo *et al*., 2003; Urbich *et al*., 2003; Zhao *et al*., 2003). Hence, we

hypothesize that the numbers of circulating EPCs and their activity are effected in patients at risk for cerebral ischemia.

LDL effects have been demonstrated at numerous sites of the nitric oxide signaling pathway including receptor-G protein coupling, nitric oxide synthase and NO bioactivity, with evidence for enhanced superoxide formation and the consequent production of the less potent dilator peroxynitrite (Dart *et al*., 1999). The effects of lipids have been documented on many of the endothelial processes, and there is particularly strong evidence for effects on the vasodilatation mediated by endothelium derived nitric oxide and on the interaction between leukocytes and the endothelial surface. Both LDL cholesterol and triglyceride rich lipoproteins impair endothelium dependent vasodilatation (Dart *et al*., 1999). However, controversy exists about the intracellular mechanism of LDL-C activity in the apoptosis, proliferation, and expression of nitric oxide synthase and other proteins in EPCs. The adhesion of leukocytes to the endothelial surface is stimulated by low density and triglyceride rich lipoproteins. Although effects have been shown on endothelial cell growth and apoptosis and on endothelial processes related to thrombosis and fibrinolysis, these effects have been less extensively studied than endothelial dependent vasodilatation and leukocyte-endothelial cell interaction.

Furthermore, many of the effects of elevated or modified low density lipoproteins on endothelial cells and endothelial cell processes could be expected to contribute to the development of atherosclerosis and therefore, to the association between lipids and atherosclerotic, particularly coronary and cerebrovascular disease. However, the extent to which "endothelial dysfunction" accounts for the known relationships between serum lipid concentrations, ischemic disease and EPC pathophysiology is yet to be established. (Dart *et al*., 1999; Chen *et al*., 2004). Atherosclerosis and its complications represent the most common cause of death However, in the past few years we have witnessed a paradigm shift in our understanding of the underlying principles of atherosclerosis. This new view supports the concept that inflammation is the central to the atherosclerotic lesion formation, progression, and eventual rupture (Libby and Aikawa, 2002; Libby, 2002). Chronic inflammation results in endothelial dysfunction and facilitates the interactions between modified lipoproteins, monocyte-derived macrophages, T cells, endothelial cells, neuronal processes, and normal cellular elements of the arterial wall, inciting early and late atherosclerotic processes.

On the basis of the mentioned and other reports, we hypothesize that higher levels of plasma LDL cholesterol not only directly impairs endothelial cells, but also effect EPC number and function at the same time, thus influencing the endothelial repair process and disturbing the balance between the magnitude of injury and the capacity for repair, which leads to endothelial dysfunction and promotes the progression of stroke. To test this hypothesis, the characterized patient is required to be carried out on clinical basis, and to measure the number and activity of EPCs from peripheral blood of patients with ischemic stroke.

The clinical evidence for the effect of LDL-C, and mechanisms of action in cerebral ischemia and stroke might be provided by studying the endothelial progenitor cell (EPCs) and dorsal root ganglion neurons. Furthermore, the role of LDL-C and ischemia in neuronal structures including dorsal root ganglia also emphasises for further studies to be carried out. The relative contributions of apoptotic and necrotic death to ischemia-induced neuronal loss provide us information for understanding the underlying mechanisms.

Because EPCs have been implicated in various events requiring endothelialization, we hypothesized that LDL-C could influence the action of EPCs and sensory neurons and that this could be important to know how LDL-C effects repair, injury and vasculoprotection . In order to corroborate this proposal, quantification is essentially needed for EPCs and DRG neurons treated with various concentrations of LDL-C and that provides characterization of the underlying molecular mechanisms.

ENDOTHELIUM AND ITS CIRCULATING CELLS

Asahara and colleagues (Asahara *et al*., 1997) first reported the isolation and characterization of endothelial progenitor cells from peripheral blood. The exact phenotype and lineage of endothelial progenitor cells (EPCs) are still a matter of debate and different expansion protocols are used to obtain them (Bellik *et al*., 2005). Experimentally, preplating may be a way to reduce the heterogeneity of the cultivated EPCs, because this excludes rapidly adhering cells such as differentiated monocytic or possible mature endothelial cells (Shi *et al*., 1998).

The ex vivo expansion of purified CD14+ mononuclear cells yielded cells with an endothelial characteristic, which incorporated in newly formed blood vessels in vivo (Urbich *et al*., 2003). These data would suggest that myeloid cells can differentiate (or transdifferentiate) to the endothelial lineage.

Interestingly, lineage tracking showed that myeloid cells are the hematopoietic stem cell-derived intermediates, which contribute to muscle regeneration (Camargo *et al*., 2003) suggested that myeloid intermediates may be part of the repair capacity after injury. Thus, CEPCs appear to be a heterogenous group of cells originating from multiple precursors within the bone marrow and present in different stages of endothelial differentiation in peripheral blood. For this reason, the accurate characterization of EPCs is difficult because many of the cell surface markers used in phenotyping are shared by adult endothelial cells and hematopoietic stem cells.

The EPCs in culture emerge as late (>2 weeks) outgrowth colonies after plating in endothelium specified medium. Typically, the cells are defined on the basis of expression of cell surface markers such as CD34, Flk-1, and CD-133. CD133 may be the most specific marker of endothelial progenitor cells (EPCs), which are thought to be largely confined to the bone marrow milieu (Suuronen *et al*., 2006). Recently, tissue-resident stem cells have been isolated from the heart, which are capable to differentiate to the endothelial lineage (Beltrami *et al*., 2003). The cells have high proliferative potential, albeit for a finite number of cell divisions. As the cells differentiate, they acquire endothelial lineage markers, vascular endothelium-cadherin, PECAM-1 (CD31), von Willebrand factor, endothelial nitric oxide synthase (eNOS), and E-selectin, and incorporate acetylated low-density lipoprotein cholesterol.

It is known that the loss of hematopoietic stem cell marker CD133 expression coincides with EPC differentiation into cells with functional characteristics of adult endothelial cells. These data support the notion that it will be difficult to define the "true" endothelial progenitor cells. In the light of recent findings, we may have to redefine our thinking of endothelial cells as well as of perivascular mural cells (Kopp *et al*., 2006). The relative abundance of CEPC is low in basal conditions. However, the number of circulating cells increases several fold after exogenous stimulation with cytokines and hormones.

Unfractionated mononuclear cells (MNCs) are cultivated in medium enriched with endothelial specific growth factors such as vascular endothelial growth factor (VEGF). Within days of plating, colonies of adherent cells proliferate rapidly to form a monolayer with the cobblestone morphology typical of endothelium. After 2 weeks, the cells adopt endothelial-like characteristics such as expression of von Willebrand factor, uptake of acetylated low-density lipoprotein cholesterol, and the ability to assemble into vascular tube-like structures. Using this approach, we are able to expand the circulating cells in culture to yield sufficient number for autologous transplantation onto injured blood vessels and prosthetic grafts in rabbits. Furthermore, EPCs are highly amenable to genetic modification with viral vectors, rendering them useful as vehicles for delivery of therapeutic genes (Iwaguro *et al*., 2002). The pseudotyped murine stem cell retroviral vector vector transduces EPC with nearly 100% efficiency, without any noticeable effects on cell phenotype or engraftment in vivo. Furthermore, because the retroviral genome integrates into the host genome, it can lead to long-lasting transgene expression.

ENDOTHELIAL INVOLVEMENT IN ISCHEMIA

The vascular endothelium represents a dynamic border between circulating blood and the surrounding tissue. This monolayer of endothelial cells (ECs) acts as a nonadhesive surface for platelets and leukocytes and produces a variety of important regulatory factors, such as prostaglandins and NO (Cines *et al*., 1998). The endothelium is a single-cell lining covering the internal surface of blood vessels, cardiac valves, and numerous body cavities that plays an important role in thrombogenicity, anticoagulation, leukocyte and platelet adherence, and vessel wall contraction and relaxation. Endothelial dysfunction predisposes to vasoconstriction, thrombosis, and atherosclerosis. EPCs may play an important role in endothelium maintenance, being implicated in both reendothelialization and neovascularization.

In healthy subjects, a low basal level of endothelial turnover, respectively very low amounts of circulating, vessel wall-derived ECs (1 to 3/mL blood), has been described (Dignat-George and Sampol, 2000). However, acute stress injury of the vascular endothelium, which is often followed by programmed cell death (apoptosis) of ECs and leads to the loss of the antithrombotic properties of the vessel wall, rapidly enhances the number of circulating ECs. In addition, EC dysfunction is a critical event in the initiation of atherosclerotic plaque development (Ross, 1999). Thus, regeneration of the vascular endothelium is of particular importance. This endothelial reconstruction can occur by migration and proliferation of surrounding mature ECs. However, mature ECs are terminally differentiated cells with a low proliferative potential, and their capacity to substitute damaged endothelium is limited. Therefore, the endothelial repair may need the support of other cell types. Accumulating evidence in the past 5 years indicates that peripheral blood of adults contains a unique subtype of circulating, bone marrow-derived cells

with properties similar to those of embryonal angioblasts. These cells have the potential to proliferate and to differentiate into mature ECs. Therefore, they were termed endothelial progenitor (precursor) cells (EPCs). Recent studies in animals and humans suggest the ability of EPCs to ameliorate the function of ischemic organs possibly by both induction and modulation of vasculogenesis and angiogenesis in areas with reduced oxygen supply or by stimulating the reendothelialization of injured blood vessels.

Differences in EPC number and function have been observed in a number of pathologies (Vasa *et al*., 2001). An inverse correlation was recently reported between the number and migratory activity of CEPCs and risk factors for coronary artery disease (Vasa *et al*., 2001). In a group of 45 men with various degrees of cardiovascular risk, as defined by the combined Framingham risk factor score, the number of CEPCs correlated with endothelial function (Hill *et al*., 2003). Interestingly, these investigators found that the number of CEPC was a better predictor of endothelial function than the presence or absence of traditional risk factors, suggesting that the abundance of CEPC may be a useful marker of vascular function and overall cardiovascular risk. In patients with severe coronary artery disease, the colony-forming capacity and migratory activity of bone marrow-derived CD34+/CD133+ MNCs was markedly reduced and associated with reduced neovascularization after transplantation in ischemic hind limb of nude rats, despite no difference in the total number of hematopoietic progenitor cells between patients and healthy control subjects (Heeschen *et al*., 2004).

EPC (CD133+/Flk-1+) was also reported to be inversely related to the severity of congestive heart failure (Heeschen *et al*., 2004). Diabetes, a major condition associated with cardiovascular diseases, also adversely affects endothelial function and number (Loomans *et al*., 2004). EPC are markedly reduced in patients with either type I or type II diabetes. Furthermore, the EPCs from diabetic patients showed reduced capacity to induce angiogenesis in vitro. These defects in EPC function may underlie some of the vascular complications associated with diabetes, such as endothelial dysfunction, that predisposes to diffuse atherosclerosis and impaired neovascularization after ischemic events.

The physiological significance of EPC mobilization was illustrated in studies in which the thoracic aortae of adult dogs that underwent BM transplantation were implanted with Dacron grafts. After 3 months, the grafts were retrieved and found to be colonized with CD34+ endothelial cells of donor origin, suggesting that endothelialization arose exclusively from BM-mobilized EPCs. In humans, the surfaces of left ventricular assist devices were found to be colonized with CD133+/VEGFR-2+ cells. Together, these studies suggest the existence of a population of EPCs in the peripheral circulation that contributes to rapid endothelialization to prevent thrombotic complications.

The mobilization and incorporation of BM-derived EPCs has recently been shown to modulate reendothelialization at sites of endothelial cell damage. Both studies induced carotid artery endothelium injury and subsequently identified the incorporation of BM-derived endothelial cells at the site of injury. Thus, by restoring an intact endothelium, EPCs may participate in the maintenance of vascular homeostasis.

In relation to mentioned information, Schatteman and colleagues (Schatteman *et al*., 2000) showed that transplantation of CD34+-derived angioblasts from nondiabetic mice markedly accelerates blood flow restoration in ischemic hind limb of diabetic mice in association with enhanced neovascularization. Chronic renal failure, another disease well known to predispose to coronary artery disease and heart failure, is characterized by enhanced coronary atherosclerosis and impaired angiogenesis. In patients with renal failure on hemodialysis, the number and colony-forming capacity of EPCs recovered from venous blood was decreased by >40% (Choi *et al*., 2004). In addition, the EPCs from these patients showed reduced migratory activity and impaired ability to assemble into vascular tubes, suggesting that EPC deficiency may play a role in the progression of the disease. The age-related deficiency in the number of CEPCs was not related to differences in cardiovascular risk factor or cardiac function and it may be caused, at least in part, by reduced levels of angiogenic and mobilizing cytokines. This deficiency may be at the root of impaired neovascularization of ischemic tissues and attenuated re-endothelialization of injured tissues commonly observed in older patients (Rivard *et al*., 1999). Evidence for this hypothesis was provided by an elegant study by Eldeberg and associates (Edelberg *et al*., 2002) who showed that neovascularization of cardiac allografts in aged mice occurred only after transplantation of bone marrow-derived EPCs from young animals. Cardiovascular disease further compounds the effects of aging on EPC number and function. The presence of cardiovascular risk factors increases the rate of EPC senescence, even in the absence of overt disease.

In the past, the vascularization of adult tissue was considered to occur via angiogenesis, the sprouting of new vessels from existing ones. It relies on the proliferation, migration, and remodeling of fully

differentiated endothelial cells. However, with the discovery of circulating EPCs, it is currently believed that a combination of angiogenesis and a modified type of vasculogenesis contributes to neovascularization . Vasculogenesis refers to the formation of blood vessels de novo out of primitive EPCs during embryological development. With evidence suggesting EPC incorporation into the vasculature, EPC-dependent neovascularization in adults may represent a third means of blood vessel formation, paralleling developmental vasculogenesis in the embryo. EPCs may serve as the substrate for new vessel formation and simultaneously exert a paracrine effect to promote angiogenesis.

Evidence for the potential of EPCs to participate in new blood vessel formation emerged from studies that demonstrated the formation of capillary-like structures from isolated HSCs or ex vivo expanded EPCs. The contribution of BM-derived cells to neovascularization, after ischemic injury in vivo, was shown in experiments using labeled populations of stem cells to reconstitute lethally irradiated mice.Three weeks after photocoagulation of the retinal vasculature to induce retinopathy, the damaged retinal vessels were replaced with a new, developing GFP+ capillary network. BM-derived EPCs also contribute to neovascularization during the newborn period, are present in corpus luteal and endometrial neovasculature after inductive ovulation, and are incorporated into vessels during wound healing (Young *et al*., 2002).
However, pathological tumor angiogenesis also depends on the recruitment of hematopoietic and circulating endothelial precursor cells. Thus, blockade of EPC migration may serve as a new target for antiangiogenesis therapy (Rafii *et al*., 2002). If EPC-mediated neovascularization could be medically influenced and optimized to restore sufficient blood flow to ischemic areas, therapeutic revascularization would be possible. But before the therapeutic potential of cell-based therapies is realized, the mechanisms underlying EPC differentiation, mobilization, and recruitment must be identified.

VEGF and nitric oxide production have been reported to decrease with age (Scheubel *et al*., 2003) and it is known that these two factors play synergistic roles in the mobilization, migration, proliferation, and survival of endothelial cells.The alterations in EPC number and properties seen in aging and cardiovascular disease may be caused by a combination of factors. The chronic exposure to risk factors and presence of underlying cardiovascular disease accentuates endothelial injury, which may require continuous replacement of damaged endothelial cells. This may lead to exhaustion of the pool of progenitor cells available in the bone marrow, which may be exacerbated by accelerated senescence and apoptosis of the remaining cells.

In addition, the reduced availability of mobilizing, homing, and differentiation/survival signals may limit the ability of EPC to repair injured tissues. Paradoxically, the functional impairment of EPC by cardiovascular disease and aging may limit their therapeutic usefulness in the patients who need it most. Multiple lines of evidence suggest that EPC are recruited to sites of injury where they participate in the repair of damaged tissues (Asahara *et al*., 1997). For example, the bone marrow-derived mononuclear cells (BM-MNCs), containing also the EPC population, home to ischemic myocardium (Massa *et al*., 2005) **,** brain, (Zhang *et al*., 2002) and hind limb (Asahara *et al*., 1997). They participate in neovascularization. Marked increase in mobilization and homing of CD34+ cells was seen in patients with myocardial infarction. The role of endothelial progenitor cells in endothelial cell homeostasis and their putative role in atherogenesis have been recently investigated (Werner and Nickenig , 2006). Therapeutic neovascularisation by endothelial progenitor cells (EPCs) mediated vascular regeneration is becoming a novel option for the treatment of ischaemic diseases (Zhang *et al*., 2006). It has been noted that improvement of neovascularization is a therapeutic option to rescue tissue from critical ischemia (Isner *et al*., 1999).

The finding that bone marrow-derived cells can home to sites of ischemia and express endothelial marker proteins has challenged the use of isolated hematopoietic stem cells or EPCs for therapeutic vasculogenesis. Correspondingly, initial pilot trials indicate that bone marrow-derived or circulating blood-derived progenitor cells are useful for therapeutically improving blood supply of ischemic tissue (Tateishi-Yuyama *et al*., 2002; Assmus *et al*., 2002).

The transplantation of ex vivo expanded endothelial progenitor cells significantly improved coronary flow reserve and left ventricular function in patients with acute myocardial infarction (Assmus *et al*., 2002).
Regeneration of endothelium is a fundamental process in vascular repair. Mature endothelial cells have limited ability to regenerate damaged endothelium because these cells are terminally differentiated. Accessory mechanisms such as EPCs may play a significant role in vascular repair and healing, and strategies aimed at rapid endothelial recovery should reduce cardiovascular events associated with endothelial cell loss, including thrombosis, restenosis, and hypertension. EPCs were reported to repopulate

implanted vascular grafts and damaged blood vessels as part of endogenous repair mechanism. Bone marrow-derived circulating endothelial progenitor cells have been successfully used to enhance angiogenesis after tissue ischemia (Werner and Nickenig , 2006). Besides models of peripheral ischemia, the angiogenic potential of EPCs was also investigated in animal models of tumor angiogenesis. Thereby, the inhibition of VEGF-responsive bone marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis (Lyden *et al*., 2001).

The overall functional improvement appear similar, when isolated human CD34+, CD133+, EPC, MAPC, or murine Sca-1+ cells were used (Grant *et al*., 2002; Urbich *et al*., 2003). Likewise, early spindle-like cells and late outgrowing EPCs showed comparable in vivo vasculogenic capacity (Hur *et al*., 2004). These results suggest that the functional activity of the cells to augment neovascularization is rather independent of the type of (endothelial) progenitor cell used. However, the CD34- fractions of freshly isolated bone marrow- or blood-derived mononuclear cells showed a reduced incorporation and functional activity. These data indicate that the number of cells capable to augment neovascularization is low in this crude fraction of freshly isolated uncultivated CD34- cells. Remarkably, terminally differentiated mature endothelial cells (HMVECs, GEAECs, and SVECs) did not improve neovascularization (Hur *et al*., 2004). suggesting that a not-yet-defined functional characteristic (eg, chemokine or integrin receptors mediating homing) is essential for EPC-mediated augmentation of blood flow after ischemia. The Ex vivo cultivated EPCs from CD14+ mononuclear cells or CD14- mononuclear cell starting population improved neovascularization to a similar extent, whereas the same number of freshly isolated mononuclear cells taken from the starting culture did not (Urbich *et al*., 2003). Interestingly, these experimental data are supported by first clinical trials showing that freshly isolated mononuclear cells are not well suited to improve neovascularization in patients with peripheral vascular diseases. However, monocytic cells may play a crucial role in collateral growth (arteriogenesis). Thus, the attraction of monocytic cells by monocyte chemoattractant protein-1 (MCP-1) enhanced arteriogenesis (van Royen *et al*., 2003).

The basal incorporation rate of progenitor cells without tissue injury is extremely low (Crosby *et al*., 2000). In ischemic tissue, the incorporation rate of genetically labeled bone marrow-derived cells, which coexpress endothelial marker proteins, differs from 0% to 90% incorporation. (Massa *et al*., 2005) High amounts (>50%) were predominantly detected in models of tumor angiogenesis (Lyden *et al*., 2001). Some studies only detected bone marrow-derived cells adjacent to vessels, which do not express endothelial marker proteins (Ziegelhoeffer *et al*., 2004). A reasonable explanation might be that the model of ischemia (eg, intensity of injury or ischemia) significantly influences the incorporation rate.

Indeed, therapeutic application of cells by intravenous infusion of ex vivo purified bone marrow mononuclear cells or expanded endothelial progenitor cells led to a higher incorporation rate approx 7% to 20% incorporation rate) as compared with the endogenously mobilized bone marrow-engrafted cells (2%) (Urbich *et al*., 2003). The number of incorporated cells with an endothelial phenotype into ischemic tissues is generally quite low. How can such a small number of cells increase neovascularization? A possible explanation might be that the efficiency of neovascularization may not solely be attributable to the incorporation of EPCs in newly formed vessels, but may also be influenced by the release of proangiogenic factors in a paracrine manner.

Moreover, adherent monocytic cells, which were cultivated under similar conditions, but do not express endothelial marker proteins, also release VEGF, HGF, and G-CSF. The release of growth factors in turn may influence the classical process of angiogenesis, namely the proliferation and migration as well as survival of mature endothelial cells (Folkman, 1995). However, EPCs additionally incorporated into the newly formed vessel structures and showed endothelial marker protein expression in vivo. In contrast, infusion of macrophages, which are known to release growth factors, (Berse *et al*., 1992) but were not incorporated into vessel-like structures, induced only a slight increase in neovascularization after ischemia, indicating-but not proving-that the capacity of EPCs to physically contribute to vessel-like structures may contribute to their potent capacity to improve neovascularization (Urbich *et al*., 2003). Further studies will have to be designed to elucidate the contribution of physical incorporation, paracrine effects and possible effects on vessel remodeling and facilitating vessel branching to EPC-mediated improvement of neovascularization. The EPCs are nonleukocytes derived from the bone marrow that are believed to have proliferative potential and may be important in vascular regeneration (Boos *et al*., 2006).

The regeneration of injured endothelium has been attributed to the migration and proliferation of neighboring endothelial cells. More recent studies, however, indicate that additional repair mechanisms may exist to replace denuded or injured arteries. Thus, implanted Dacron grafts were shown to be rapidly

covered by bone marrow-derived cells deriving from CD34+ hematopoietic stem cells in a dog model (Shi *et al*., 1998). In humans, the surface of ventricular assist devices was covered by even more immature CD133-positive hematopoietic stem cells, which concomitantly express the VEGF-receptor 2. Additionally, Walter and coworkers demonstrated that circulating endothelial precursor cells can home to denuded parts of the artery after balloon injury (Walter *et al*., 2002). Bone marrow transplantation experiments revealed that bone marrow-derived cells can contribute to reendothelialization of grafts and denuded arteries (Walter *et al*., 2002). However, in a model of transplant arteriosclerosis, bone marrow-derived cells appear to contribute only to a minor extent to endothelial regeneration by circulating cells. These data again indicate that there might be at least two distinct populations of circulating cells that principally are capable to contribute to reendothelialization, namely mobilized cells from bone marrow and non-bone marrow-derived cells. The latter ones may arise from circulating progenitor cells released by non-bone marrow sources (eg, tissue resident stem cells) or represent vessel wall-derived endothelial cells (Walter *et al*., 2002).

Indeed, enhanced incorporation of ß-galactosidase-positive, bone marrow-derived cells were associated with an accelerated reendothelialization and reduction of restenosis (Walter *et al*., 2002). Similar results were reported by Griese *et al*, who demonstrated that infused peripheral blood monocyte-derived EPC home to bioprosthetic grafts and to balloon-injured carotid arteries, the latter being associated with a significant reduction in neointima deposition (Walter *et al*., 2002). Likewise, infusion of bone marrow-derived CD34-/CD14+ mononuclear cells, which are not representing the population of the "classical hemangioblast," contributed to endothelial regeneration. The regenerated endothelium was functionally active as shown by the release of NO, which is supposed to be one of the major vasculoprotective mechanisms. Consistently, neointima development was significantly reduced after cell infusion. Whereas the regeneration of the endothelium by EPCs protects lesion formation, bone marrow-derived stem/progenitor cells may also contribute to plaque angiogenesis, thereby potentially facilitating plaque instability (Griese *et al*., 2003). However, in a recent study, no influence of bone marrow cell infusion on plaque composition was detected in nonischemic mice (Hu *et al*., 2003).

An increase in plaque size was only detected in the presence of ischemia, suggesting that ischemia-induced release of growth factors predominantly accounts for this effect (Silvestre *et al*., 2003). Overall, these studies implicate that regardless of the origin of circulating endothelial progenitor cells, this pool of circulating endothelial cells may exert an important function as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer by replacing denuded parts of the artery. One can speculate that these cells may also regenerate the low grade endothelial damage by ongoing induction of endothelial cell apoptosis induced by risk factors for coronary artery disease (see review: Rossig *et al*., 2001). The maintenance of the endothelial monolayer may prevent thrombotic complications and atherosclerotic lesion development. Although this concept has not yet been proven, several hints from recently presented data are supportive. Thus, transplantation of ApoE-/- mice with wild-type bone marrow reduced atherosclerotic lesion formation (Rauscher *et al*., 2003).

In addition, factors that reduce cardiovascular risk such as statins 56 (Walter *et al*., 2002) or exercise elevate EPC levels, which contribute to enhanced endothelial repair. The balance of atheroprotective and proatherosclerotic factors, thus, may influence EPC levels and subsequently reendothelialization capacity. Much emphasis has been given on the role of cardiovascular risk factors on endothelial cell apoptosis and EPCs with its pathophysiological consequences for atherogenesis and a regenerative therapy approach that highlight the role of EPCs as a marker for cardiovascular mortality and morbidity (Werner *et al*., 2006). Because EPCs contribute to reendothelialization and neovascularization, increasing the number of these cells may be an attractive therapeutic tool. The mobilization of stem cells in the bone marrow is determined by the local microenvironment, the so-called "stem cell niche," which consists of fibroblasts, osteoblasts, and endothelial cells (see review: Papayannopoulou *et al*., 2004). Basically, mobilizing cytokines hamper the interactions between stem cells and stromal cells, which finally allow stem cells to leave the bone marrow via transendothelial migration. Thereby, activation of proteinases such as elastase, cathepsin G, and matrix metalloproteinases (MMPs) cleave adhesive bonds on stromal cells, which interact with integrins on hematopoietic stem cells. MMP-9 was additionally shown to cleave the membrane-bound Kit ligand (mKitL) and induces the release of soluble Kit ligand (KitL; also known as stem cell factor, SCF) (Heissig *et al*., 2002).

Physiologically, ischemia is believed to be the predominant signal to induce mobilization of EPCs from the bone marrow. Ischemia thereby is believed to upregulate VEGF or SDF-1, which in turn are released to

the circulation and induce mobilization of progenitor cells from the bone marrow via a MMP-9-dependent mechanism (Heissig *et al*., 2002).

Although the preclinical and clinical studies reviewed here generally lend support to the therapeutic potential of autologous EPCs in the treatment of tissue ischemia and repair of injured blood vessels, the clinical application of EPCs is limited by several factors. First, the scarcity of CEPCs makes it difficulty to expand sufficient number of cells for therapeutic application without incurring the risk of cell senescence and change in phenotype (Walter *et al*., 2002).

Furthermore, EPCs from patients with cardiovascular diseases display varying degrees of functional impairment (Heeschen *et al*., 2004; Loomans *et al*., 2004). Aging and diabetes markedly reduce the availability and impair the function of EPCs (Schatteman *et al*., 2000; Edelberg *et al*., 2002; Scheubel *et al*., 2003) Because older and diabetic patients are the most vulnerable populations for cardiovascular diseases, this severely restricts the ability to treat with autologous EPCs the patients who theoretically need them most. Mobilization could potentially accelerate progression of atherosclerotic plaque by recruiting inflammatory and vascular smooth muscle cell progenitor cells into the plaque, contributing to neointima hyperplasia and transplant arteriopathy. Increased rate of in-stent restenosis led recently to the cancellation of the MAGIC clinical trial using G-CSF for endogenous mobilization of progenitor cells in patients with myocardial infarction (Kang *et al*., 2004).

In the early embryogenesis, the vascular system develops from vasculogenesis in which angioblasts differentiate into endothelial cells to form a primitive capillary network, whereas angiogenesis, the sprouting of capillaries from preexisting blood vessels, is involved in the late stage of embryogenesis and in the adult. Angioblast-like circulating endothelial progenitor cells are present in the peripheral blood and have been isolated from adult animals. Injection of circulating endothelial progenitor cells into animals with hindlimb or myocardial ischemia results in incorporation of circulating endothelial progenitor cells into neovasculature at the site of ischemia, suggesting that circulating endothelial progenitor cells contribute to formation of new blood vessels in the adult tissue.

The endothelial cells of cerebral capillaries differ functionally and morphologically from those of noncerebral capillaries. During embryonic development, the cerebral vascular system originates from the perineural plexus when vascular sprouts invade the proliferating neuroectoderm, indicating that the cerebral vascular system is primarily developed by angiogenesis and not by vasculogenesis. The cerebral endothelial cells are linked by complex tight junctions that form the blood brain barrier (BBB). In the adult brain, proliferation of the cerebral endothelial cells ceases, and the turnover rate of endothelial cells is approximately 3 years (Robertson *et al*., 1985).

Studies from human and experimental stroke indicate that neovascularization is present in the adult brain after ischemia (Zhang *et al*., 200). However, development of new blood vessels in ischemic adult brain is incompletely understood, and it remains unknown whether newly formed vessels are induced by proliferation of preexisting vascular endothelial cells or by recruitment of circulating endothelial progenitor cells to the brain after stroke.

These data indicate neovascularization occurs in the adult ischemic brain (Zhang *et al*., 200) This is consistent with previous reports that functional imaging of stroke patients shows increased cerebral blood flow and metabolism in tissue surrounding focal brain infarcts (Cramer and Chopp , 2000) whereas increases in proliferated endothelial cells in the ischemic vessels may reflect angiogenesis. Formation of new vessels may arise from the proliferation and migration of endothelial cells from the adjacent tissue and from circulating endothelial progenitor cells.

Taken together, these data suggest that circulating endothelial progenitor cells contribute to the microvascular structure of the choroid plexus. Recent studies suggest that active vascular recruitment and endothelial cells provide cues mediating neurogenesis in the brain (Palmer *et al*., 2000). Neurogenesis takes place in the subventricular zone and the dentate gyrus throughout of the rodent life. Observation of presence of circulating endothelial progenitor cells in the choroid plexus suggest that these cells may support neurogenesis in the subventricular zone and the dentate gyrus by secreting growth factors associated with neurogenesis, such as vascular endothelial growth factor or brain-derived neurotrophic factor (Palmer *et al*., 2000).

Cells derived from bone marrow can migrate into the brains of adult mice and differentiate into astrocytes, microglia, and neurons, indicating that bone marrow-derived progenitor cells are reservoirs of normal tissues. Exogenous angioblasts might be used to augment compromised vascularization in ischemic brain. Incorporation of circulating endothelial progenitor cells into sites of neovascularization suggests that

recruitment of circulating endothelial progenitor cells induced by focal cerebral ischemia may be regulated by cytokines, soluble receptors, and adhesive molecules released from the ischemic lesion.

LDL CHOLESTEROL AND CEREBRAL ISCHEMIA

Although it had traditionally been assumed that replacement of damaged endothelium resulted only from outgrowth of preexisting vasculature, it is now identified endothelial progenitor cells (EPCs) that appear to contribute to vascular homeostasis and repair. Clinical trials to assess the therapeutic potential of bone marrow-derived mononuclear cells, a rich source of immature cells including EPCs have been initiated and have, thus far, provided promising results. Furthermore, immature cells, including CD34-positive (CD34+) cells, have been shown to contribute to maintenance of the vasculature, not only as a pool of EPCs but also as the source of growth/angiogenesis factors. Bone marrow-derived immature cells have also been shown to participate in neovascularization of ischemic brain after experimental stroke (Beck *et al*., 2003).

In rodents, the improvement in outcome after myocardial infarction or stroke by medication is dependent on endothelial NO synthase but independent of LDL cholesterol because the therapy have little effect on LDL cholesterol in mice or rats (Landmesser *et al*., 2004). It has, however, never been unequivocally demonstrated in humans that prolonged treatment exerts effects independently of LDL cholesterol because their application always results in reduced LDL cholesterol levels. Of note, in patients with ischemic conditions, several recent studies have shown that lower serum cholesterol levels are independently associated with an impaired prognosis (Rauchhaus *et al*., 2003) which raises concerns about whether cholesterol lowering is beneficial in these patients.

Interestingly, there is evidence from both experimental and clinical studies that reduced endothelial NO availability may play an important role in the pathophysiology of heart failure and stroke. In fact, reduced endothelial NO availability results in impaired neovascularization and EPC function (Landmesser *et al*., 2004).

Inhibitors of HMG-CoA reductase (statins) lower the level of circulating LDL-C by blocking the activity of HMG-CoA reductase. Their efficiency to prevent cardiovascular events was demonstrated in several clinical trials for primary and secondary prevention. Possible repair of ischemic tissues through enhancement of mobilization of endothelial progenitor cells are also described (Nalbone *et al*., 2003) although more investigation are needed to clearly identify the role and safety. These are therefore a valuable tool not only for the clinician but also for the biologist, allowing to investigate the regulation of gene expression that is controlled by the intracellular activity of membrane-anchored signaling proteins.

Inspite of so much awareness, an unacceptably high proportion of the individuals with dyslipidemia are neither on lipid lowering therapy nor manage aggressively enough to achieve recommended target cholesterol levels (Joseph *et al*., 1999). This is an important issue since mounting evidence indicates that aggressive lipid-lowering, particularly with therapy, will be of benefit to the majority of patients with ischemic stroke (Stroke Council, 2004). Lowering the LDL-C and using a multivitamin containing high-dose folic acid, pyridoxine, and cobalamin given to lower total homocysteine levels would reduce the incidence of recurrent cerebral infarction in patients with a nondisabling cerebral infarction (Toole *et al*., 2004). In this study, the aim will be to describe lipid profiles among all individuals with an ischemic stroke and to identify associated factors.

We need to analyse lipid levels in individuals who have experienced an ischemic stroke and to find that how many of the patients out of high vascular risk persons are within target levels of LDL-Cl (LDL cholesterol concentration 100 mg/dL or less). Numerous studies of individuals with cardiac disease have implied that appropriate treatment of cholesterol levels has been suboptimal (Eaton *et al*., 1994; Stafford *et al*., 1997; Frolkis *et al*., 1998). It is suggested that the same may be true for patients with ischemic stroke at high vascular risk. Perhaps even more worrisome is that almost half of the high vascular risk subjects are not prescribed a therapy even though they have LDL-C concentrations above the standard range.

One possible reason for the underutilization of lipid treatment in ischemic stroke survivors may be decades of prior research which have yielded inconsistent results regarding the role of cholesterol in ischemic stroke (Shahar *et al*., 2003) and this may have generally fostered a reluctance on the part of clinicians to take the treatment of lipid levels in patients with ischemic stroke very seriously. For preventing coronary events among ischemic stroke survivors aggressive lipid lowering treatment can be recommended for those with indicator conditions such as diabetes, and other CAD risk equivalents.The approach may shed further light on the role of lipid-lowering in patients with ischemic stroke without known CAD.

The implementation of vascular protection strategies as a whole among patients with stroke has been variable and suboptimal. It is not uncommon to find that among health care professionals attending to patients with stroke, the focus is primarily on secondary stroke prevention. It is true that shortly after an ischemic stroke the highest vascular risk is for another stroke (Hankey, 2003). However, patients with stroke are at a greater long-term risk of death due to recurrent cardiac events than recurrent stroke (Sacco *et al*., 1994). Aggressive management of risk factors, like cholesterol, could have a significant and positive impact on the natural history of atherosclerotic cardiovascular disease.

As such, the issue of underdiagnosis and undertreatment of high vascular risk individuals may be compounded. It would appear that a number of directed quality improvement interventions are needed to improve the quality of cholesterol management in patients with ischemic disorders. Alongside the implementation of prompt diet and lifestyle modification, the screening and initiation of treatment before hospital discharge may be a simple and effective intervention with the potential to substantially reduce cardiovascular morbidity and mortality in this vulnerable population. Indeed, the systematic predischarge treatment initiation strategy has been shown to be feasible (Fonarow *et al*., 2001; Ovbiagele *et al*., 2004) predict longer-term community care (Aronow *et al*., 2003) and result in improved vascular outcomes (Fonarow *et al*., 2001; Ovbiagele *et al*., 2004).

However, if recent stroke studies (UHS Consortium Ischemic Stroke Clinical Benchmarking Project, 2002; Paul Coverdell National Acute Stroke Registry, 2003) showing that fewer than 50% of hospitalized patients with stroke receive a lipid panel are relevant, it would appear unlikely that there has been substantial improvement. Another important consideration is that we do not know if and how many patients might have been offered and declined, started and discontinued, or received inadequate doses of statins (Frolkis *et al*., 2002) in the outpatient setting. Furthermore, we lack information on potential complications of statin therapy, such as myalgias, that may have limited statin use. We excluded investigation of use of lipid lowering agents other than statins from this study, because most of the landmark lipid lowering trials used statins, recent guidelines focus on statins (Stroke Council, 2004) and statins remain the most effective and best-tolerated drugs for lowering LDL-C. Finally, we may have underestimated the proportion of high-risk individuals because some individuals identified as low risk by Framingham score might had been on statins, and the effect of statins on total cholesterol concentration may have led to their misclassification. In order to reduce the burden of vascular disease, the diagnosis of dyslipidemia and treatment with therapeutic targets must be improved.

Although an immense knowledge has accumulated concerning regulation of cholesterol homeostasis in the body, this does not include the brain, where details are just emerging.The importance of cholesterol in the nervous system was recognized as early as 1834, when Couerbe's observations lead him to regard cholesterol as *un element principal* of the nervous system (Couerbe and Du Cerveau, 1834). Despite concerted efforts in the interim, it is only during the past few decades that the brain has begun to surrender the secrets of the behavior of one of its most abundant lipids. Approximately 25% of the total amount of the cholesterol present in humans is localized to this organ, most of it present in myelin. Almost all brain cholesterol is a product of local synthesis, with the blood-brain barrier efficiently protecting it from exchange with lipoprotein cholesterol in the circulation. Thus, there is a highly efficient apolipoprotein-dependent recycling of cholesterol in the brain, with minimal losses to the circulation. Under steady-state conditions, most of the de novo synthesis of cholesterol in the brain appears to be balanced by excretion of the cytochrome P-450-generated oxysterol 24S-hydroxycholesterol. This oxysterol is capable of escaping the recycling mechanism and traversing the blood-brain barrier. Cholesterol levels and cholesterol turnover are affected in neurodegenerating disorders, and the capacity for cholesterol transport and recycling in the brain seems to be of importance for the development of such diseases. No firm conclusions can, however, be drawn from the studies presented thus far.

During the past decade, there has been a significant increase of knowledge about cholesterol homeostasis in the brain, and powerful new experimental tools have been introduced in this field of research. Overall, the current data provide tantalizing indications that modulation of intracerebral cholesterol levels may be a possible strategy for protection. At present, it is impossible to draw a firm conclusion about the value of using statins to prevent (or delay) the onset of the disorders. In addition to HMG CoA reductase, there are many other possible targets for modulation of cholesterol homeostasis in the brain, and new therapeutic strategies are likely to be tested in the near future. The brain has only begun to reveal the secrets of its cholesterol homeostasis, and many more are likely to be uncovered.

During Wallerian degeneration of rat sciatic nerve, the expression of apolipoprotein E increases and apolipoprotein E-containing endoneurial lipoproteins accumulate in the distal nerve segment. In established primary cultures dissociated from dorsal root ganglia, Schwann cells and sensory neurons internalized rhodamine-labeled lipoproteins isolated from crushed rat sciatic nerve as well as low density lipoprotein (LDL) from human serum. The uptake of endoneurial lipoproteins could be inhibited by an excess of LDL or at low temperature (4 degrees C) (Rothe and Muller, 1991).

After transection of nerve fibers in dorsal root ganglia explant cultures, the uptake of lipoproteins was markedly stimulated in Schwann cells that were in close proximity to degenerating neurites. LDL receptor immunoreactivity was expressed by cell bodies and processes of cultured Schwann cells, sensory neurons, and fibroblasts from dorsal root ganglia (Rothe and Muller, 1991). Incubation of Schwann cells and neurons with the LDL receptor antibody strongly inhibited the uptake of endoneurial lipoproteins. This provide direct evidence for the important role of the LDL receptor-mediated pathway to internalize endoneurial lipoproteins into Schwann cells and peripheral neurons required for reuse of cholesterol and other lipids in myelin and plasma membrane biogenesis during nerve repair.

Neural cell degeneration underlies central and peripheral nervous system disorders. Exposure of DRG cells to Oxidized-LDL for 24 h led to elevation of LDH in the culture medium; short term exposure (4 h) induced apoptosis, evidenced by DNA fragmentation and a positive TUNEL-reaction (Papassotiropoulos *et al*., 1996). These results suggest that Oxidized-LDL is a neurotoxin; it initiates apoptotic cell injury which progresses to necrosis and cell death.

Preliminary studies suggest that caveolae are likely to be involved in the potential transport of LDL from the blood to the brain. It has been demonstrated that oxidized LDL is present in brain parenchyma of patients with ischemic infarction and suggests a potential mechanism by which oxidized LDL may activate innate immunity and thereby indirectly influence neuronal survival (Shie *et al*., 2004).

CONCLUSIONS

The extent to which "endothelial dysfunction" accounts for the known relationships between serum lipid concentrations, ischemic disease and EPC pathophysiology is yet to be established. Hence, the characterization of the patients on clinical basis is required. The role of LDL-C and ischemia in neuronal structures including dorsal root ganglia also emphasises for further studies to be carried out. The relative contributions of apoptotic and necrotic death to ischemia-induced neuronal loss may provide interesting information for understanding the underlying mechanisms. Further studies may provide us information to know partly how LDL-C effects repair, injury and vasculoprotection.

The EPCs contribute to reendothelialization and neovascularization, increasing the number of these cells may be an attractive therapeutic tool. EPCs are a valuable tool not only for the clinician but also for the biologist, allowing to investigate the regulation of gene expression that is controlled by the intracellular activity of membrane-anchored signaling proteins. Furthermore, inspite of so much awareness, an unacceptably high proportion of the individuals with dyslipidemia are neither on lipid lowering therapy nor manage aggressively enough to achieve recommended target cholesterol levels**.** This is an important issue since mounting evidence indicates that aggressive lipid-lowering, particularly with therapy, will be of benefit to the majority of patients with ischemic stroke**.** Lowering the LDL-C could reduce the incidence of recurrent cerebral infarction in patients with a nondisabling cerebral infarction**.**. One possible reason for the underutilization of lipid treatment in ischemic stroke survivors may be decades of prior research which have yielded inconsistent results regarding the role of cholesterol in ischemic stroke and this may have generally fostered a reluctance on the part of clinicians to take the treatment of lipid levels in patients with ischemic stroke very seriously. For preventing coronary events among ischemic stroke survivors aggressive lipid lowering treatment can be recommended for those with indicator conditions. This approach may shed further light on the role of lipid-lowering in patients with ischemic stroke without known coronary disorder. Aggressive management of risk factors, like cholesterol, could have a significant and positive impact on the natural history of atherosclerotic cerebrovascular disease.

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