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Review article

Negatively charged L5 as a naturally occurring atherogenic low-density lipoprotein

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ABSTRACT

Deranged metabolism of low-density lipoprotein (LDL) is considered the preeminent modifiable risk factor for atherosclerotic disease, and it is widely viewed as a chronic inflammatory disorder. Yet, the search for a circulating atherogenic LDL species continues, as the risk of coronary artery disease (CAD) cannot be measured by absolute LDL cholesterol concentrations in the plasma. Oxidized LDL (oxLDL) and small, dense LDL are associated with CAD, but neither has been retrieved from human plasma for mechanistic scrutiny. Electronegative LDL, a subclass of human plasma LDL, exhibits atherogenic properties in cultured vascular cells. L5, the most negatively charged subfraction of LDL, is an extreme form of electronegative LDL that we isolated through anion-exchange chromatography from the plasma of patients with increased cardiac risk (active smoking, hypercholesterolemia, type 2 diabetes mellitus, and metabolic syndrome). L5, which is scant in healthy normal patients, is as potent as artificially prepared oxLDL in inducing endothelial cell (EC) apoptosis by disrupting fibroblast growth factor 2 autoregulation that involves protein kinase B. Unlike oxLDL, however, L5 is not oxidized. Among subfractions L1–L5, which were separated by our chromatographic method, L1 is the most abundant and least negatively charged. It represents harmless normal LDL. Compared with L1, L5 has a greater content of total protein and triglycerides but a lesser amount of cholesteryl esters. Size exclusion chromatography and equilibrium density gradient ultracentrifugation indicated that L5 is neither smaller nor denser than L1. Negative charge on the particle surface has made L5 unrecognizable by the normal LDL receptor. Instead, L5 signals through and is internalized by lectin-like oxidized LDL receptor-1 (LOX-1), which has high affinity for negatively charged ligands. LOX-1 is also inducible by L5 but not L1. Through LOX-1, L5 disturbs homeostasis between the prosurvival and proapoptotic members of the Bcl-2 family, leading to mitochondrial destabilization. Additionally, it induces overexpression of various adhesion molecules and chemokines, thus promoting monocyte-EC adhesion, an early event during atherosclerosis development. Endothelial progenitor cells (EPCs) are important construction units for vascular repair and endothelial regeneration. Adding to the damage, L5 impairs EPC differentiation from

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mononuclear cells by inhibiting the induction of needed growth factor receptors. It also accelerates EPC senescence by suppressing the enzymatic activity of telomerase, which is essential for chromosome preservation. Thus, L5 is a naturally occurring, negatively charged but not oxidized LDL entity that is neither smaller nor denser than normal LDL but possesses a capacity for inducing a spectrum of atherogenic responses in vascular cells. Further investigation aimed at establishing its clinical relevance is warranted to confirm its atherogenic role. Subsequent efforts in L5 research will be directed toward the development of new diagnostic and treatment methods for CAD and other ischemic vascular diseases.

1. Introduction

There is no further doubt that low-density lipoprotein (LDL) abnormality is a primary etiology of atherosclerosis and associated complications, especially coronary artery disease (CAD). Under this notion and in view of the heterogeneity of LDL, investigators have searched extensively for the LDL species responsible for atherogenesis. Oxidized LDL (oxLDL) and small, dense LDL receive the greatest attention. Although it is supported by accumulating experimental evidence and the localization of oxidized lipids in lesions [1–3], it remains uncertain whether LDL oxidation is a cause of atherosclerosis [4]. One major reason for this doubt is that most experimental data have been derived from oxLDL that has been artificially prepared *in vitro*. Circulating small, dense LDL has been statistically related to atherosclerosis and CAD events [5,6]. Despite a possible correlation with CAD, neither form of LDL has been isolated from human plasma, to allow investigation of their effects in vascular cells. By contrast, interest is growing in another possible candidate: negatively charged LDL, a subclass of circulating LDL that is defined as either unoxidized or minimally oxidized yet potentially atherogenic.

2. Electronegative LDL

Hoff, Gotto, and associates [7–9] first used the term "electronegative LDL" when they noted that LDL isolated from human atherosclerotic lesions exhibited greater mobility toward the anode end in agarose gel electrophoresis than LDL isolated from normal plasma. Using fast protein liquid chromatography (FPLC) equipped with an ion-exchange column, Avogaro and colleagues [10] first divided human plasma LDL dichotomously into electropositive LDL(+) and electronegative LDL(−) in 1988. They were also the first to report that LDL(−) particles were "stickier" to one another and more toxic to vascular cells than their LDL(+) counterparts [10]. Since then, several groups have described the chemical composition and functional traits of LDL(−), which they isolated by using a similar protocol [11–26]. Among these investigators, Sánchez-Quesada and associates [19,20] have reported on a wide range of chemical and biologic features of LDL(−). In a 2004 review, they summarized that in cultured vascular endothelial cells (ECs), LDL(−) induces the production of chemokines, such as interleukin 8 (IL-8) and monocyte chemotactic protein 1, and increases tumor necrosis factor alpha (TNF-α)-induced production of vascular cell adhesion molecule 1 (VCAM-1). Most recently, this group reported that phospholipase C-like activity may play a role in higher aggregability of LDL(−) [25], the phenomenon originally noted by Avogaro [10].

3. L5 and negatively charged LDL

Using a different protocol, we used anion-exchange chromatography to divide plasma LDL from patients with familial hypercholesterolemia heterogeneity and those with moderately increased LDL cholesterol (LDL-C) into increasingly negatively charged subfractions, L1-L5 [27,28]. Strictly speaking, in chemistry, the term "electronegativity" is defined as the tendency of an atom to attract a bonding pair of electrons. On the Pauling scale, fluorine (the most electronegative element) is assigned a value of 4.0; values range down to 0.7, in the case of cesium and francium (the least electronegative elements) [29]. L5 is only relatively more negative in surface charge than other subfractions, and the complexity of an LDL particle prevents accurate calculation of its electronegativity; we decided to define L5 as the most negatively charged LDL subfraction. This allows us to avoid inaccurately implying that L4–L1 are positively charged LDL particles. Among all subfractions, L5 is the only subfraction capable of inducing apoptosis in cultured vascular ECs (Fig. 1).

4. Chemical basis of L5 negative charge and its biologic implications

We previously reported that L5 accounts for approximately 2% of total plasma LDL in asymptomatic patients with type 2 diabetes mellitus (DM) [30]. L1, the least negatively charged subfraction, represents a majority (> 85%) of total LDL. The content of both protein and triglyceride (TG) increased progressively in the direction of L1 to L5, whereas that of cholesteryl esters decreased. The content of phospholipids and free cholesterol was largely the same among all subfractions [30]. These findings concur with those reported for LDL(−) [12]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed higher total protein content in L5 particles, which is partially explained by the inclusion of apolipoprotein (apo)-AI, apoE, and apoCIII, as well as apoB-100, which is the sole apolipoprotein of L1 [30]. Additional experiments with L1–L5 isolated from hypercholesterolemic human plasma yielded

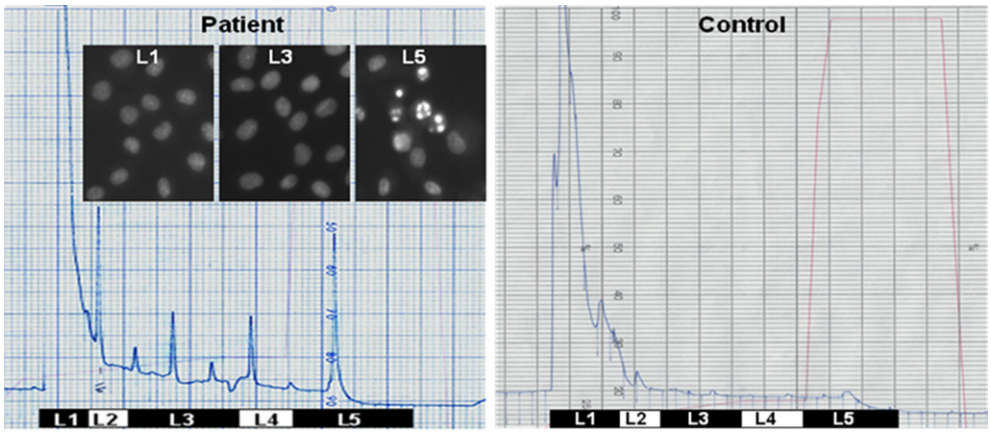

Fig. 1 – Isolation of L5 and other LDL subfractions by FPLC. L5 is present in patients with elevated LDL-C (>160 mg/dL) but not in nondiabetic, normolipidemic healthy subjects. L5 is the only subfraction that induces marked EC apoptosis, as demonstrated by nuclear condensation and fragmentation. C = cholesterol; EC = endothelial cell; FPLC = fast protein liquid chromatography; LDL = low-density lipoprotein.

similar results; Fig. 2 shows a representative SDS-PAGE gel. Fig. 3 illustrates similarities and differences between L5 and L1. Because L5 contains twice the amount of TG than L1, it can be regarded as a TG-rich LDL by comparison. Compatible with our findings, Bancells and colleagues [26] demonstrated recently by proteomics analysis that the amounts of apoE, apoAI, apoC-III, apoAII, apoD, apoF, and apoJ are higher in LDL(–) than in LDL(+). In our latest experiments with liquid chromatography/mass spectrometry (LC/MS^E), we quantitated in detail all apolipoproteins and other low molecular weight proteins associated with L5. Selective association of these molecules with low isoelectric points in L5, but not L1, in part explains the relatively high negative charge on the surface of L5 (unpublished data), which may contribute to the altered affinity of L5 for the normal LDL receptor (LDLR). The chemically oriented shift of L5's affinity from LDLR to other receptors constitutes the basis of its unique pathologic functionality.

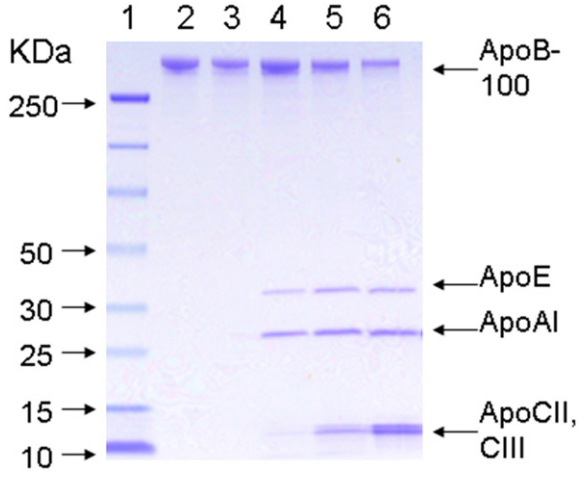

Fig. 2 – Representative 4-20% SDS-PAGE gel that shows the apolipoprotein distribution in L1–L5 from hypercholesterolemic human plasma. Protein profiles are shown for L1-L5 (from left to right: Lanes 2-6); apoB-100 is the sole protein in L1, whereas L5 also contains apoE, apoAI, and apoCII/CIII. apo = apolipoprotein; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

5. L5 vs. oxLDL and small, dense LDL

Although L5 and copper-oxidized LDL are equally potent in suppressing the transcription of fibroblast growth factor 2 (FGF2) and inducing apoptosis in vascular ECs [28,31], they differ widely in their chemical and physical characteristics. The production of thiobarbituric acid-reactive substances (TBARS) is often used as a measure of oxidative lipid modification. After copper and oxygen exposure for 24 hours, the TBARS value of oxLDL often reaches the high concentration of 18–22 nmol/mg protein. In contrast, the TBARS value of L5 is mostly less than 1 nmol/mg protein, which is no different than that of L1–L4 [28]; this is in accordance with observations made in LDL(–) [11,20,22]. Various artificial oxidation methods increase the negative charge of normal LDL and turn it into electronegative LDL [17,32]. In preliminary experiments, copper oxidation of L1 yielded ox-L1, which exhibited increased electrophoretic mobility on agarose gel, as well as proapoptotic effects, as seen with non oxidized L5 (data not shown). However, neither oxLDL nor ox-L1 has the same chemical composition as naturally occurring L5. Therefore, L5 is a nonoxidized, naturally occurring atherogenic LDL.

L5 is a subfraction of plasma LDL, which is defined by density. The high TG content and the association with apoCIII, apoE, and apoAI suggest L5's close resemblance to remnant-like particle cholesterol [33,34]. Size exclusion chromatography and equilibrium density gradient ultracentrifugation showed that L5 is no different from L1–L4 in either particle size or density [35]. This suggests that L5 is neither smaller nor denser than other particles in the same lipoprotein class defined by density. Small, dense LDL is defined by particle size on gradient gel electrophoresis or nuclear magnetic resonance (NMR) [36]. Small, dense LDL is considered atherogenic chiefly because it has a greater propensity for oxidation, and it may be concluded that it is an etiologic agent in atherosclerosis. The association of

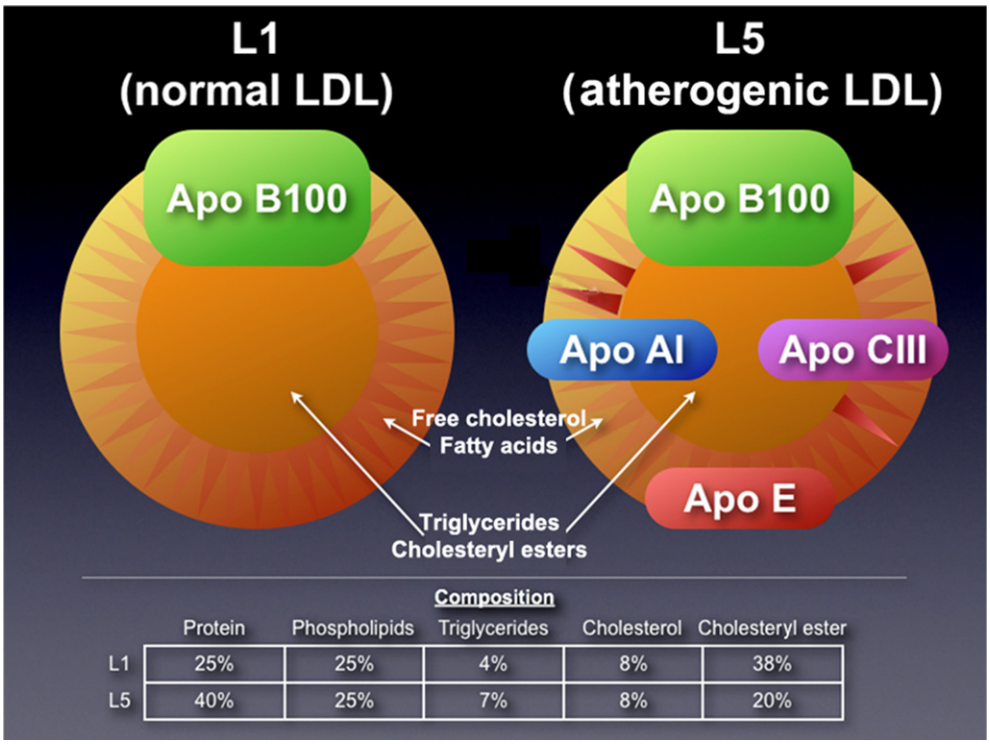

Fig. 3 – Schematic comparison between L1 and L5 isolated from hypercholesterolemic human plasma. L5 and L1 differ in their lipid and protein compositions. LDL = low-density lipoprotein; apo = apolipoprotein.

LDL particle size with cardiovascular diseases has been tested for magnitude and independence in many studies, e.g., clinical intervention trials, and cross-sectional and prospective epidemiologic studies. Nearly all show a significant univariate association of small, dense LDL with increased CAD risk, but LDL size is seldom a significant and independent predictor of CAD risk after multivariate adjustment for confounding variables, in particular, plasma TG and high-density lipoprotein cholesterol (HDL-C) concentration. Hence, it may be that the increased risk associated with smaller LDL size in univariate analyses arises from broader pathophysiologic causes, of which small, dense LDL is a part, rather than a reflection, of intrinsic increased atherogenic potential. Thus, a clear causal relationship between small, dense LDL and increased cardiovascular risk has not been proven [37]. This further accentuates the importance of L5, which is not a small, dense LDL, in atherogenesis.

6. Receptors for and active components of L5

Our data suggest that at least two receptors, platelet activating factor (PAF) receptor (PAFR) and lectin-like oxidized LDL receptor-1 (LOX-1), are involved in transducing L5-elicited signaling of ECs and endothelial progenitor cells (EPCs) [31,38,39]. Protein kinase B (Akt) exerts multiple prosurvival and vasomotor effects by activating downstream targets, such as endothelial nitric oxide synthase (eNOS), after its own activation by phosphatidylinositol 3-kinase (PI3K) [40,41]. L5 inhibits EC proliferation and induces EC apoptosis, disrupting FGF2 autoregulation via an FGF2-PI3K-Akt loop [31]. These effects are significantly attenuated by pharmacological blockage of PAFR or by inhibiting G protein incorporation into PAFR, a G protein-coupled receptor [28,31,42].

LOX-1, though originally cloned against copper-oxLDL, has a strong affinity for negatively charged particles through an electrostatic interaction [43,44]. We reported that, unlike L1, L5 is not recognized by LDLR on the plasma membrane of ECs and EPCs, but it is internalized by LOX-1 in a competitive manner against artificially prepared oxLDL [31,38]. Preliminary experiments suggest that the low pI values of non–apoB-100 protein molecules contribute to the switch in affinity from normal LDLR to LOX-1 (unpublished data), which is also inducible by L5 but not L1 [31,38].

The active components of L5 are not yet fully identified, but our current data suggest that they reside on lipids that accumulate in L5 particles. The total lipid extract derived from L5 is as efficient as L5 in inducing intracellular calcium transient in polymorphonuclear neutrophils (PMNs) [28]. Pretreatment of L5 with PAF acetylhydrolase to degrade PAF and PAF-like lipids via hydrolysis of *sn*-2 residues removes L5's capacity to downregulate FGF2 and induce EC apoptosis. Also, the lipid extract of degraded L5 fails to elicit calcium transient in PMNs, which is readily restored by adding exogenous PAF [28]. Findings suggest that protein configuration is important for receptor recognition but that lipid components, especially certain phospholipids, are responsible for signaling.

7. Evidence of L5's atherogenicity *in vitro*

Apoptosis of the vascular endothelium contributes to increased transendothelial permeability [45,46]; released microparticles

enhance tissue factor expression and thus provoke coagulation [47,48]. Evidence that L5 induces EC apoptosis in a concentration- and time-dependent manner indicates L5's involvement in both early and late stages of atherothrombosis [28,30,31,35,39]. In addition to EC apoptosis, L5 induces monocyte-EC adhesion, another early event in atherosclerosis development [27,28]. Cell-to-cell adhesion is promoted by EC secretion of adhesion molecules, including VCAM-1, IL-8, and CXC chemokines [49], which is in agreement with what has been described for LDL(−) [20,50,51].

FGF2 functions by activating downstream kinases and effectors, including Akt, Bcl-2, Bad, Bax, Bcl-xL, and eNOS [52]. These effectors are major regulators of mitochondrial function and structural integrity [53]. We recently demonstrated that L5 suppresses the expression of the mitochondria-stabilizing and prosurvival effectors Bcl-2, Bcl-xL, and eNOS, as well as eNOS phosphorylation, and upregulates the pro-apoptotic effectors Bax, Bad, and TNF-α in vascular ECs through LOX-1 [39]. The L5-initiated signaling that leads to endothelial dysfunction and atherogenesis is summarized in the schematic illustration seen in Fig. 4.

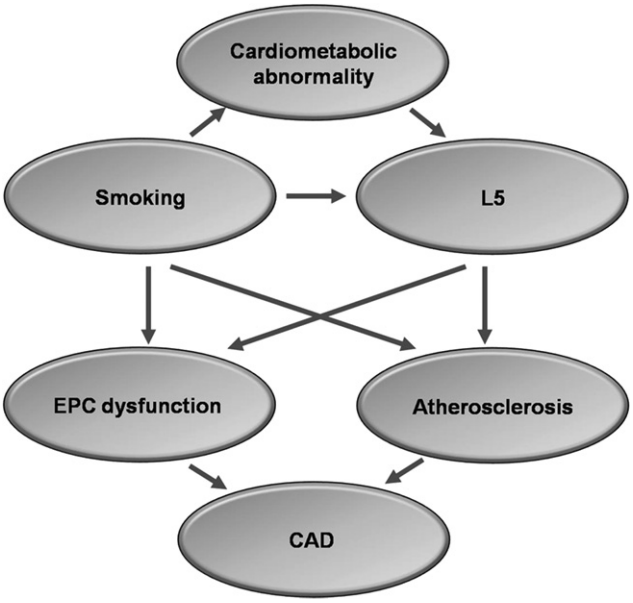

Fig. 5 – Risk factors for L5 formation and the consequent effects on cardiovascular health. Cardiometabolic abnormalities include hypercholesterolemia, type 2 diabetes mellitus, and metabolic syndrome. CAD = coronary artery disease; EPC = endothelial progenitor cell.

8. L5 and EPC physiology

Evidence suggests that bone marrow-derived EPCs play a key role in endothelial regeneration, as well as in vasculogenesis

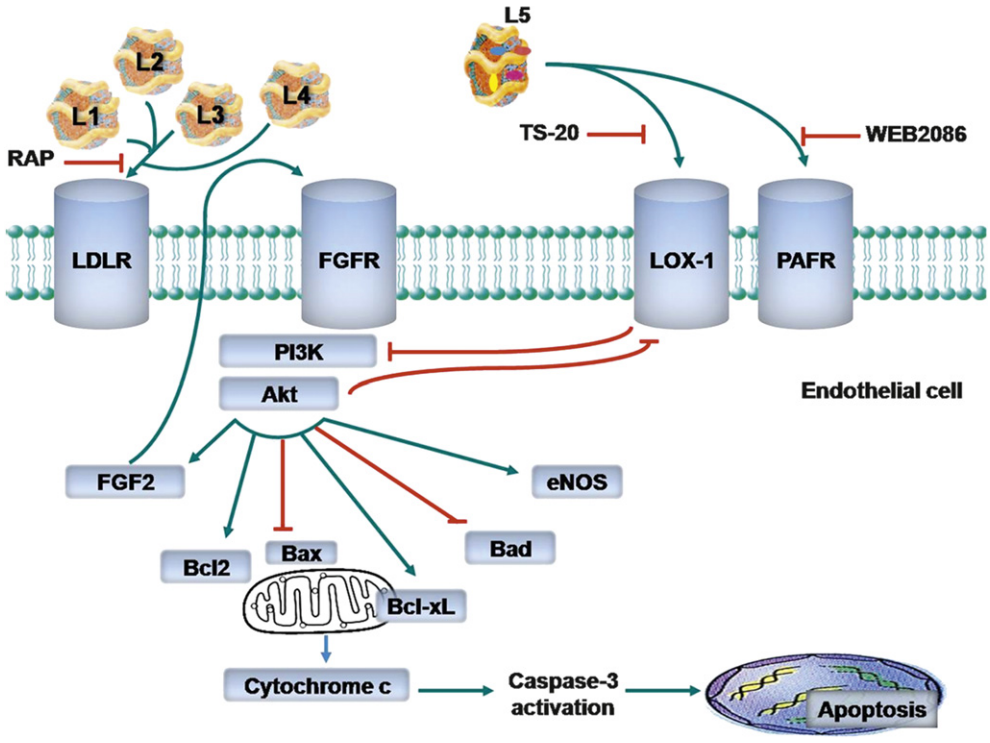

Fig. 4 – Schematic summary of L5 signaling through LOX-1 in vascular ECs. L5 signals and is internalized through LOX-1, whereas L1–L4 are endocytosed via LDLR. Green arrows indicate stimulation; blue arrows indicate release from mitochondria; red lines with end bars indicate inhibition. RAP = the LDLR inhibitor receptor-associated protein; TS-20 = LOX-1 neutralizing antibody; EC = endothelial cell; LDL = low-density lipoprotein; LDLR = LDL receptor; LOX-1 = lectin-like oxidized LDL receptor-1; FGFR = fibroblast growth factor receptor; PAFR = platelet-activating factor receptor; WEB2086 = thieno-triazolodiazepine, an antagonist of PAF; PI3K = phosphatidylinositol 3-kinase; Akt = protein kinase B; FGF2 = fibroblast growth factor 2; Bcl-2 = B-cell lymphoma 2; Bax = Bcl-2-associated X protein; Bcl-xL = B-cell lymphoma extra large; Bad = Bcl-2-associated death promoter; eNOS = endothelial nitric oxide synthase.

[54–56]. EPCs are reduced in number and/or functional activity in the presence of traditional and emerging major risk factors, whether separately or as clusters [57]. Reported correlated risk factors include aging [58], subclinical and clinical atherothrombotic disease [59,60], types 1 and 2 DM [61,62], hypercholesterolemia [63], smoking [57], and metabolic syndrome [64]. One common trait in people with these risk factors is the percentage increase of L5 in their plasma LDL [27,28,30,35,38,65,66]. At subapoptotic concentrations, L5 inhibited vascular endothelial growth factor (VEGF)-induced differentiation of human circulating monocytes into EPCs; this is achieved by suppression of Akt-mediated induction of VEGF kinase insert domain-containing receptor and other endothelial cell markers [38]. The impairment of mitogenic activity in early EPCs by abnormal LDL can accelerate EPC senescence [67]. Cellular senescence is critically influenced by telomerase, which elongates telomeres and thereby counteracts the telomere length reduction induced by each cell division [68,69]. In our setting, L5 accelerates EPC senescence by inhibiting telomerase activity [38], thus severely compromising the regenerative capacity of progenitor cells.

9. Conclusions and perspectives

Based on our findings and a review of the literature, we conclude that L5 is a non oxidized, naturally occurring atherogenic LDL that is not smaller or denser than other LDL subfractions. In preliminary experiments, repeated injection of human L5 into apoE knockout mice induced atherosclerotic changes in the aorta, and addition of L5 into organ chambers attenuated the endothelium-dependent relaxation of aortas that were removed from rats (data not shown). These *in vivo* and *ex vivo* observations support a role for L5 in endothelial dysfunction and atherosclerosis formation. Our recent data also suggest that (a) L5 is increased in patients undergoing acute coronary events and that (b) intracoronary thrombi contain tissue that exhibits strong LOX-1 expression. Further study is needed to confirm the role of L5 in atherothrombotic development in animals and in humans. Fig. 5 summarizes the links between risk factors that favor L5 formation and the consequences of L5 accumulation. Our ongoing studies aim to develop both diagnostic and therapeutic methods for the early detection and effective treatment of L5-mediated vascular disease.

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