Developmental Endothelial Locus-1 (Del-1) Inhibits Oxidized Low-Density Lipoprotein Activity by Direct Binding, and Its Overexpression Attenuates Atherogenesis in Mice

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Background: Modified low-density lipoprotein (LDL) binding to scavenger receptors has been implicated in atherosclerosis. It is hypothesized that a third molecule may affect modified LDL binding, therefore, this study focuses on the soluble endogenous protein, developmental endothelial locus-1 (Del-1), as an inhibitor of oxidized LDL (oxLDL) interactions.

Methods and Results: Del-1 preferentially bound oxLDL over native LDL in a cell-free binding assay. Del-1 also inhibited Dil-labeled oxLDL uptake by scavenger receptors irrespective of the receptor type (LOX-1, SR-AI, CD36, or SR-BI) expressed in COS-7 cells, and independent of cell type (human coronary artery endothelial cells (HCAECs) or THP-1-derived macrophages). Furthermore, Del-1 suppressed oxLDL-induced MCP-1 and ICAM-1 expression and endothelin-1 secretion in HCAECs. Then, male Del-1 transgenic (Del-1Tg) and wild-type mice (WT) mice were established and fed a Paigen diet for 20 weeks from the age of 24 weeks. While plasma lipid concentrations did not differ between WT and Del-1Tg mice, plasma LOX-1-ligand activity was significantly lower in Del-1Tg than in WT mice. Moreover, lipid accumulation in aortic roots was significantly less in the Del-1Tg mice, evaluated with Oil red-O. Taken together, Del-1 appears to block the activity of oxLDL pharmacologically by direct binding in vitro, and attenuates atherogenesis in vivo, although its role in physiological settings are yet to be resolved.

Conclusions: Del-1 intercepted oxLDL before its receptor binding to reduce atherogenesis. (Circ J 2016; 80: 2541 – 2549)

Key Words: Atherosclerosis; Developmental endothelial locus-1 (Del-1); LOX-1; Oxidized low-density lipoprotein (LDL)
Methods

Cell-Free Binding Assay

Either LDL or oxLDL were added to 384-well plates coated with anti-ApoB antibody. After washing with phosphate buffered saline (PBS), the plate was blocked with 3% bovine serum albumin (BSA). After washing, Del-1 was added to the plate and incubated for 1 h at room temperature. Bound Del-1 was quantified by an ELISA using anti-Del-1 monoclonal antibody. (B-E) Effect of Del-1 on the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocyanine perchlorate (DiI)-LDL or DiI-oxLDL by LDL receptor (LDLR) or oxLDL receptor (LOX-1)-transfected COS-7 cells. Cells were incubated with 3 μg/ml DiI-LDL or DiI-oxLDL (red) in the absence or presence of Del-1 or control BSA for 2 h at 37°C. Nuclei were stained with DAPI (blue). Uptake of DiI-LDL or DiI-oxLDL indicates fluorescence per nucleus in each group. (F) Effects of Del-1 on oxLDL uptake by scavenger receptors (SRs). The SR-AI, CD36, or SR-BI expression vector were transfected into COS-7 cells. Cells were incubated with 3 μg/ml DiI-oxLDL in the absence or presence of Del-1 or control BSA for 2 h at 37°C. Data for SR-AI, CD36, and SR-BI are plotted together with data for LOX-1. All assays were performed in triplicate, and average values are plotted with SEM. Asterisks represent significant differences (P<0.05) relative to the control at the same concentration. Scale bar=50 μm.

oxLDL Uptake Assay in Scavenger Receptor-Transfected Cells or in HCAECs or THP-1 Cells

For expression of scavenger receptors, COS-7 cells were

Given that oxLDL is a harmful ligand acting through these receptors, it is possible that physiological mechanisms have evolved to interrupt the oxLDL-oxLDL receptor interaction. However, despite the potential significance of such factors, no endogenous molecules that block these interactions have yet been identified.

Here, we focused on a molecule to seek such possibility, which is originally described as an endothelium-derived secreted protein developmental endothelial locus-1 (Del-1). Del-1 is a 54 kDa glycoprotein, expressed by endothelial cells and a subset of macrophages, which consists of 3 epidermal growth factor repeats and 2 C-terminal discoidin I-like domains. Previous work showed that Del-1 inhibits leukocyte adhesion in acute models of inflammation, prevents neutrophil-mediated inflammatory bone loss in mice, and has important functions in vascularization and phagocytosis. No reports, however, have shown the anti-oxLDL activity of Del-1. Here, we have shown anti-oxLDL and anti-atherogenic effects of Del-1 in vitro and in vivo.
transfected with LOX-1, SR-A, CD36, and SR-BI expression vector using Lipofectamine 2000 (Invitrogen, Life Technologies), and cultured for 48 h. Then, either the transfected cells, HCAECs, or THP-1 cells differentiated into macrophages, were incubated with oxLDL labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocyanide perchlorate (DiI-oxLDL) (3.0 μg/ml) and Del-1 for 2 h at 37°C in serum free Dulbecco’s Modified Eagle Medium (DMEM). After washing with PBS, the cells were fixed with 4% (w/v) phosphate-buffered formaldehyde (Wako) at room temperature for 15 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1.0 μg/ml; Sigma-Aldrich). Images were acquired on a fluorescence microscope (Axiovert 200M; Zeiss). Quantitative fluorescence cell image analysis was performed on the INCell Analyzer 1000 system (GE Healthcare).

**Analysis of Atherogenesis**

Male mice were maintained on a regular chow diet (CE-2; CLEA Japan). Starting at 24 weeks of age, the mice were fed a high-fat, high-cholesterol, bile salt diet (Paigen diet) (D12336; Research Diets) for 20 weeks. After high-fat loading, the mice were euthanized and subjected to analyses. Isolated aortic roots were fixed with 4% (w/v) phosphate-buffered formalde- hyde and washed with PBS. After flushing with 60% isopropanol for 5 min at room temperature, blood vessels were incubated for 15 min at room temperature with 0.6% Oil red O (Merck KGaA) in 60% isopropanol. Next, the aortic roots were serially rinsed with 60% isopropanol, 30% isopropanol, and PBS. Images were acquired on an AZ100M microscope (Nikon). The Oil red O positive area was measured using ImageJ software.

**Measurement of Hemodynamic Parameters and Plasma Lipid and Protein Concentrations**

Blood pressure and heart rates were measured by tail cuff plethysmography (BP-98A; Softron). Total cholesterol, triglycerides, phospholipids, and non-esterified fatty acids (NEFA) in EDTA plasma were measured by using kits (T-Chol E, TG...
E, PL C, and NEFA C, respectively; Wako). Plasma oxLDL and human Del-1 concentrations were measured using the appropriate ELISA kits (CSB-E07933m and CSB-EL007399HU, respectively; Cusabio Biotech). The plasma LAB concentrations were measured by using a sandwich ELISA as described previously. Briefly, murine plasma specimens were applied to a plate coated with extracellular domain of human LOX-1 protein and detected by using anti-apoB (HUC20) as the primary antibody, and HRP-conjugated goat anti-chicken IgG (KPL) as the secondary antibody. Peroxidase activity was detected with a TMB peroxidase EIA substrate kit, and quantified by measuring the absorbance at 450 nm on a microplate reader.

**Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from murine tissues by using a TRizol reagent (Invitrogen, Life Technologies). Complementary DNA was synthesized by using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Assays-on-Demand primers and probes (Applied Biosystems) were used to detect the transcripts of human Del-1 (edil3) (Hs00174781_m1), mouse ICAM-1 (Mm00516023_m1), mouse MCP-1 (Mm00441242_m1), and 18S. Real-time PCR was performed by using the TaqMan Gene Expression Master Mix (Applied Biosystems), and the products were quantified on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Levels of all mRNAs were normalized to the corresponding levels of 18S rRNA.

**Statistical Analysis**

A Student’s t-test was used for comparison of 2 data sets. One-way ANOVA in conjunction with Tukey’s post-hoc test was used for comparisons among multiple data sets. Statistical calculations were performed in GraphPad Prism, version 6.0 for Windows (Graph Pad Software). P values <0.05 were considered statistically significant.

**Other Methods**

Other methods including materials, cell lines and cell culture, production of recombinant Del-1, preparation of lipoproteins, analysis of endothelin-1 secretion, and generation of human Del-1 transgenic (Del-1Tg) mice are described in Supplementary File 1.

**Results**

**Del-1 Binds to oxLDL**

As LOX-1 binds to phosphatidylserine (PS) as well as to oxLDL, we hypothesized that some PS binding proteins might bind to oxLDL and interfere with its action. In this study, we focused on a PS binding protein, Del-1. First, we investigated whether Del-1 binds directly to oxLDL in a cell-free system. Del-1 bound to oxLDL-coated plates (Kd = 5.7 nmol/L) much more than to native LDL-coated plates (Figure 1A). It is possible that the binding of Del-1 to LDL might be due to its binding to oxLDL, which was originally present in the LDL fraction or produced during the assay.

**Del-1 Inhibits DiI-oxLDL Uptake by LOX-1-Transfected Cells**

We next investigated whether Del-1 affects oxLDL-receptor binding, using COS-7 cells transfected with the oxLDL receptor, LOX-1. While Del-1 did not affect the uptake of LDL by LDL receptor (LDLR)-transfected cells (Figures 1B,D), Del-1 inhibited oxLDL uptake by LOX-1 in a dose-dependent manner, while the control BSA did not (Figures 1C,E). These
results indicate that Del-1 selectively blocked oxLDL.

Besides LOX-1, several oxLDL receptors are known, including SR-A, CD36, and SR-BI. Therefore, we investigated whether Del-1 blocks oxLDL uptake by these scavenger receptors as well. Del-1 suppressed oxLDL uptake by all these oxLDL receptor-expressing cells, while the control BSA did not (Figure 1F). Thus, Del-1 blocked oxLDL uptake, regardless of the type of oxLDL receptors at least examined in the present study.

Del-1 Inhibits Dil-oxLDL Uptake by Endothelial Cells and Macrophages

To determine whether Del-1 blocks oxLDL receptors natively expressed by cells, we examined human coronary artery endothelial cells (HCAECs) and THP-1 cells that differentiated into macrophages. Treatment with Del-1 suppressed oxLDL uptake by HCAECs (Figures 2A,C) or THP-1-derived macrophages (Figures 2B,D) in a dose-dependent manner, while treatment with the control BSA did not.

Del-1 Inhibits oxLDL-Induced Cellular Responses in Endothelial Cells

Next, we investigated whether Del-1 inhibits the pro-atherogenic action of oxLDL, such as induction of the expression of MCP-1, ICAM-1, and endothelin-1 in endothelial cells. An oxLDL-induced increase in MCP-1 and ICAM-1 expression in HCAECs was significantly suppressed by Del-1, but not by the control BSA (Figure 2E). oxLDL-induced endothelin-1 secretion in HCAECs was also significantly suppressed by Del-1, but not by the control BSA (Figure 2F). Thus, Del-1 not only inhibited the binding of oxLDL to the receptors, but also the cellular reactions to oxLDL.

Two Arginine Residues in Del-1 Are Crucial for the oxLDL Inhibitory Function

To characterize the structural requirement for oxLDL binding and inhibition of oxLDL uptake by Del-1, we constructed various Del-1 cDNAs containing point mutations. In D98E, the aspartic acid at residue 98 of the RGD motif was replaced by glutamic acid (Figure 3A). In R195A and R375A, either arginine at residue 195 or 375 in the phospholipid-binding domain was replaced by alanine; we also constructed the double-point mutant, R195A/R375A (Figure 3A).

In the cell-free system, the binding of R195A or R375A to oxLDL was significantly reduced compared with native Del-1 (Figure 3B), and the binding of R195A/R375A was reduced further. By contrast, D98E bound oxLDL almost equally to that of native Del-1, despite its mutation.

Next, we examined the effects of mutations on the inhibition of Dil-oxLDL uptake by LOX-1-transfected cells (Figure 3C). The inhibitory effect of Del-1 was significantly reduced in R195A or R375A relative to native Del-1, and the effect was further reduced in the R195A/R375A double mutant. By contrast, the D98E mutant did not affect the inhibitory effect of Del-1. These observations suggest that the phospholipid-binding domain plays a crucial role in the anti-oxLDL activity of Del-1, whereas the integrin-binding function does not seem to be essential.

Del-1 Overexpression Inhibits Atherosclerosis

These in vitro observations prompted us to test whether the anti-oxLDL activity on Del-1 can effectively suppress atherosclerosis in vivo. To test this hypothesis, we generated Del-1Tg mice. The human Del-1 protein is 95% homologous to mouse Del-1, and is expected to work similarly as the mouse one. We designed the Del-1 transgene to be driven by a cytomegalovirus (CMV) promoter (Figure 4A), and obtained 2 independent lines of transgenic mice-expressing human Del-1 (Del-1Tg mice). CMV, CMV (cytomegalovirus) promoter; poly(A), polyadenylation signal. (B) Expression of human Del-1 in Del-1Tg mice, as determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA isolated from Del-1Tg mouse tissues were subjected to qRT-PCR. Amounts of human Del-1 mRNA were normalized against the corresponding levels of 18S rRNA. Data are presented as means±SEM. (C) Expression of endogenous mouse Del-1 in Del-1Tg mice. Total RNA isolated from wild-type (WT) or Del-1Tg mouse tissues were subjected to qRT-PCR. The amounts of mouse Del-1 mRNA were normalized against the corresponding levels of 18S rRNA. Data are presented as means±SEM. (D) Baseline plasma concentrations of human Del-1 in Del-1Tg mice, as determined by an ELISA. Scatter plots show individual measurements and means±SEM. n=4, each group. *P<0.05 vs. WT.

To examine the in vivo effects of Del-1 on atherosclerosis, we fed male Del-1Tg and WT mice a Paigen diet for 20 weeks from when the mice were 24 weeks old. After Paigen diet
feeding, plasma concentrations of transgenic human Del-1 in both Del-1Tg #1 and #2 lines were still significantly high compared with WT mice (Figure 5A). oxLDL concentration, determined by conventional antibody-based assay, did not differ between Del-1Tg mice and WT mice (Figure 5B). Other lipid concentrations (total cholesterol, HDL cholesterol, phospholipids, and NEFA) were also comparable between WT mice and Del-1Tg mice, although plasma triglyceride was

![Figure 5.](image)

**Figure 5.** Developmental endothelial locus-1 (Del-1) overexpression decreased plasma LOX-1 ligand containing apoB (LAB) activity and inhibited atherosclerosis in mice. (A–C) A comparison of plasma concentration between wild-type (WT) or human Del-1Tg mice after feeding them a 20-week Paigen diet from the age of 24 weeks: (A) transgenic human Del-1, (B) oxidized low-density lipoprotein (oxLDL), and (C) LAB (n=6, each group). Scatter plots show individual measurements and means±SEM. *P<0.05 vs. WT. (D) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of the expression of MCP-1 and ICAM-1 in the aorta of mice fed a Paigen diet, as described in (A–C). Results are normalized against the corresponding levels of 18S rRNA (means±SEM). *P<0.05 vs. WT. (E) Representative images of Oil red O staining for lipid deposition and quantitative analysis of atherosclerotic lesion size in the aortic root of mice fed a Paigen diet, as described in (A–C). The Oil red O-positive areas were quantified and expressed as percentages of the aortic root area. Scatter plots show individual measurements and means±SEM. *P<0.05 vs. WT. Scale bar=1 mm.

| Table. Hemodynamic and Plasma Lipid Indices of WT, Del-1Tg#1, and Del-1Tg#2 Mice After a 20-Week Paigen Diet (44 Weeks Old) |
|-----------------|-----------------|-----------------|
|                 | WT              | Del-1Tg#1       | Del-1Tg#2       |
| Body weight, g  | 30.45±1.45      | 31.83±0.40      | 33.13±1.64      |
| SBP, mmHg       | 101.0±1.8       | 101.9±2.0       | 102.0±4.4       |
| MBP, mmHg       | 71.7±2.0        | 71.1±2.0        | 73.8±2.9        |
| DBP, mmHg       | 57.2±2.5        | 55.9±2.5        | 59.5±3.1        |
| Heart rate, beats/min | 627.3±20.6 | 638.3±26.2 | 647±31.9 |
| Total cholesterol, mg/dl | 191.6±11.1 | 187.2±17.6 | 179.7±10.2 |
| Phospholipid, mg/dl | 172.4±12.2 | 154.3±9.3 | 160.8±10.7 |
| Triglyceride, mg/dl | 29.9±3.7 | 17.8±2.8* | 21.4±4.4 |
| NEFA, mEq/L      | 1.3±0.1         | 1.5±0.2         | 1.2±0.1         |
| HDL, mg/dl       | 49.8±3.9        | 54.2±4.3        | 50.4±6.8        |

WT, wild-type; Del-1Tg, developmental endothelial locus-1 transgenic mice; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; NEFA, non-esterified free fatty acid; HDL, high-density lipoprotein. Values are expressed as the means±SEM. *P<0.05 vs. WT.
slightly lower in Del-1Tg#1 than in WT mice (Table). In concordance with Del-1 production, plasma LAB activity, which reflects receptor-binding activity of a modified LDL solution, was significantly lower in Del-1Tg mice than WT mice under these conditions (Figure 5C). Expression of adhesion molecules, MCP-1 and ICAM-1, was decreased in the aorta (Figure 5D), and the Oil red O-positive atheromatous area at the aortic roots was dramatically smaller in Del-1Tg than in WT mice (Figure 5E). These results demonstrated that Del-1 decreased plasma LAB activity and inhibited atherogenesis in vivo.

**Discussion**

**Endogenous oxLDL-Blocking Proteins**

In this study, we found that Del-1 inhibited oxLDL uptake by scavenger receptors and suppressed oxLDL-dependent cellular responses (Figure 6). Furthermore, in vivo, Del-1Tg mice exhibited attenuated atherogenesis.

Generally speaking, vascular function is physiologically maintained by the balances between actions and counteractions, such as vasoconstrictors and vasorelaxants, and prothrombotic and anti-thrombotic substances. Similarly, in the context of atherosclerosis, many substances act as pro-atherogenic or anti-atherogenic. Among pro-atherogenic substances, studies of oxLDL, on which we focused in this study, have evolved along with the understanding of the biology of its receptors; namely, SR-A and CD36 for foam-cell formation and LOX-1 for endothelial dysfunction.

Given the importance of oxLDL in the initiation and progression of atherosclerosis, there must exist mechanisms that counter-balance the activity of oxLDL. To date, however, no such molecules have been reported. Most studies have focused on antioxidants, which inhibit oxLDL generation and activity indirectly, or on HDL, which functionally antagonizes the activity of oxLDL.

Although a report showed C-reactive protein (CRP) binds to oxLDL and inhibits its uptake by macrophages, it is also reported that CRP promotes oxLDL activity by forming an oxLDL-CRP complex that is recognized by CRP receptors on macrophages. In this study, we clearly demonstrated for the first time the presence of an endogenous molecule, Del-1, that can bind to oxLDL and inhibit binding activity to oxLDL receptors.

**Mechanisms of Action**

Besides their inhibitory activity against oxLDL, Del-1 has several other functions. First, via its RGD motif, Del-1 competes with ICAM-1 for binding to integrin LFA-1. Hence, Del-1 works as an anti-adhesion molecule for leukocytes in order to inhibit inflammation. Second, via their phospholipid-binding domains, Del-1 recognizes PS exposed on the surface of apoptotic cells, and simultaneously binds to integrin via the RGD sequence. Then, Del-1 promotes clearance of apoptotic cells by phagocytosis.

In this study, mutations in residues in the phospholipid-binding domain effectively inhibited the anti-oxLDL activity of Del-1, whereas a mutation in the RGD sequence did not affect the activity. Therefore, the anti-oxLDL activity is ascribed to the phospholipid-binding domain of Del-1. Previous studies showed that oxidized phospholipids in oxLDL might be important in the pathogenesis of atherosclerosis. Del-1 may recognize oxidized phospholipids in oxLDL via its phospholipid-binding domain.

Thus, Del-1 uses its 2 domains separately depending on the function; that is, inhibition of leukocyte adhesion, promotion of phagocytosis, or inhibition of receptor binding by oxLDL. It is unclear what makes these differences in the domain activity of Del-1. Possible explanations would be the expression level and activation status of target integrins, and the presence or absence of ligand phospholipids.

In this study, in good agreement with the anti-oxLDL function of Del-1, we demonstrated that overexpression of Del-1 greatly suppressed the Paigen diet-induced aortic lipid deposition in mice. As described above, Del-1 has 2 additional functions reported to date. Therefore, the effect of Del-1 overexpression on atherosclerosis should be a result of complex actions of these different kinds of functions. However, considering the importance of oxLDL in the pathogenesis of atherogenesis and the direct activity of Del-1 against oxLDL, it is likely that the anti-oxLDL function of Del-1 plays a major role in the suppression of atherogenesis.

Here, we found that Del-1 bound to oxLDL via its phospholipid binding domain. With the same domain, Del-1 binds to microparticles via the PS exposed on their surface. Finn et al reported that while the circulating level of Del-1 in coronary artery disease (CAD) patients is higher than that of healthy subjects, there was less Del-1 bound to microparticles from CAD patients.

The authors ascribed this phenomenon to the decreased affinity of microparticles from CAD patients to Del-1. However, considering the results of the present study, it is possible that the interaction between Del-1 and oxLDL-like molecules in CAD patients might interfere with Del-1 binding to microparticles. In other words, for Del-1, oxLDL and microparticles might be competing in CAD patients.

While we analyzed the effects of Del-1 on oxLDL activity in the present study, the effects of oxLDL on the function of Del-1 might also be of importance. Namely, Del-1 and oxLDL might interfere each other’s activity.

**Significance of the Evaluation of Oxidized and Modified LDL Activity**

After the proposal of the oxLDL hypothesis in the 1980s, various measurement systems using different types of anti-oxLDL antibody have been developed and used to analyze the association between circulating oxLDL concentration and atherosclerotic diseases. However, the relationships between circulating oxLDL and progression of atherosclerotic diseases remain unclear.
One of the reasons underlying this controversy is the fact that the various oxLDL measurement systems detect different molecules in micelles of heterogeneous molecules of oxLDL. Another reason might be, as we demonstrated in this study, the existence of molecules that inhibit oxLDL activity without affecting oxLDL concentrations that are determined by anti-oxLDL antibodies. Such conditions have not been taken into consideration in previous anti-oxLDL antibody-based measurement systems.

To address these problems, we developed a receptor-based system to measure oxLDL activity, designated LAB. As shown in this study, oxLDL concentrations measured by an anti-oxLDL antibody-based assay were not different between Del-1-Tg and WT mice given a Paigen diet, whereas LAB activity was significantly reduced in Del-1-Tg compared with WT mice. This observation clearly indicated that LAB, which is a measure of biological activity of (oxidatively) modified LDL, is more strongly associated with the progression of atherosclerosis than oxLDL.

In humans, we performed a cohort study and cross-sectional studies showing that LAB is an independent risk factor for cardiovascular disease, which is strongly associated with the progression of atherosclerosis. Although at the time of those reports, the presence of anti-oxLDL factors demonstrated here had not yet been considered. The strong predictive relationship between LAB and cardiovascular risk, however, suggests that a similar mechanism might also be working in humans.

Regarding the reason why overexpression of Del-1 did not alter the plasma concentration of oxLDL in vivo, we think that there are two possible explanations. First, the in vivo model we used was relatively mild in terms of loading fat/cholesterol, while clearance of oxLDL has a large capacity in vivo. In such a case, the change in the plasma oxLDL concentration would be minimal, even if it might increase. Second, there could be unknown clearance mechanism(s), which might recognize oxLDL epitope(s) unaffected by Del-1. Actually, the oxLDL receptors, SR-A, SR-BI, and LOX-1, are reportedly not significantly involved in the clearance. Although Luangrath et al showed that CD36 partly mediates oxLDL clearance in vivo, when large amounts of oxLDL are applied, the residual clearance mechanism(s) are still unknown.

In contrast, plasma LAB was decreased when Del-1 was overexpressed. This could occur even if oxLDL concentrations were the same, because LAB measures LOX-1-binding activity, which is blocked by Del-1.

Limitations of the Study
It is important to know to what extent Del-1 "physiologically" works as an anti-atherogenic molecule in a manner which we suggested in the present study. However, in this study, we focused on the "pharmacological" effects of Del-1 by applying Del-1 exogenously in vitro and overexpressing Del-1 in vivo. This means that we only analyzed the effects of Del-1 that was exogenously applied, even though Del-1 is an endogenously expressed protein. This would open, however, a new avenue of pathophysiology in that oxLDL intercepting molecules like Del-1 might suppress atherogenesis, even if Del-1 itself could have only marginal effects on atherogenesis "physiologically."

In addition, it is suggested that steatohepatitis might develop with an etiology shared with atherosclerosis. Therefore, the condition used in the present study could also be applied to analyze the possible protective effects of Del-1 on steatohepatitis. Future research is warranted for this important area.

Conclusions
A soluble protein, Del-1, binds to oxLDL to block its proatherogenic activity, regardless of the type of receptors. This could attenuate the biological activity of circulating oxLDL and progression of atherosclerosis.

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Conflicts of Interest
The authors declare that they have no conflicts of interest.

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### Supplementary Files

#### Supplementary File 1

**Methods**

Please find supplementary file(s): [http://dx.doi.org/10.1253/circjc.CJ-16-0808](http://dx.doi.org/10.1253/circjc.CJ-16-0808)