In Vivo and In Vitro Studies Support That a New Splicing Isoform of OLR1 Gene Is Protective Against Acute Myocardial Infarction

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Abstract—Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), encoded by the OLR1 gene, is a scavenger receptor that plays a fundamental role in the pathogenesis of atherosclerosis. LOX-1 activation is associated with apoptosis of endothelial cells, smooth muscle cells (SMCs), and macrophages. This process is an important underlying mechanism that contributes to plaque instability and subsequent development of acute coronary syndromes. Independent association genetic studies have implicated OLR1 gene variants in myocardial infarction (MI) susceptibility. Because single nucleotide polymorphisms (SNPs) linked to MI are located in intronic sequences of the gene, it remains unclear as to how they determine their biological effects. Using quantitative real-time PCR and minigene approach, we show that intronic SNPs, linked to MI, regulate the expression of a new functional splicing isoform of the OLR1 gene, LOXIN, which lacks exon 5. Macrophages from subjects carrying the “non-risk” disease haplotype at OLR1 gene have an increased expression of LOXIN at mRNA and protein level, which results in a significant reduction of apoptosis in response to oxLDL. Expression of LOXIN in different cell types results in loss of surface staining, indicating that truncation of the C-terminal portion of the protein has a profound effect on its cellular trafficking. Furthermore, the proapoptotic effect of LOX-1 receptor in cell culture is specifically rescued by the coexpression of LOXIN in a dose-dependent manner. The demonstration that increasing levels of LOXIN protect cells from LOX-1 induced apoptosis sets a groundwork for developing therapeutic approaches for prevention of plaque instability. (Circ Res. 2005;97:152-158.)

Key Words: OLR1 ▪ myocardial infarction ▪ LOX-1 ▪ oxLDL ▪ apoptosis

Recent studies have shown that atherogenesis is no longer an inevitable consequence of aging but instead is a complex disease with multi-factorial etiology.1,2 Traditional risk factors associated with atherogenesis include hypercholesterolemia, smoking, male gender, hypertension, diabetes, and age. However, newly defined nontraditional risk factors are emerging as being equally important.2 Among these are hypercholesterolemia, smoking, male gender, hypertension, diabetes, and unstable angina, and the more severe lesions contain a significantly higher percentage of oxLDL–positive macrophages.6 According to this scenario, it has been shown that oxLDL is cytotoxic to cultured cells and induces apoptosis and necrosis of vascular endothelial cells, smooth muscle cells (SMCs), and macrophages.7-10 These processes have been proposed to lead to plaque vulnerability and potential rupture, which is ultimately responsible for acute atherothrombotic vascular occlusion and tissue infarction.

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Ingested by macrophages, resulting in foam cells formation.5 Increased levels of oxLDL relate to plaque instability in human coronary atherosclerotic lesions. Thus, oxLDL levels show a significant positive correlation with the severity of acute coronary syndromes such as myocardial infarction (MI) and unstable angina, and the more severe lesions contain a significantly higher percentage of oxLDL–positive macrophages.6 According to this scenario, it has been shown that oxLDL is cytotoxic to cultured cells and induces apoptosis and necrosis of vascular endothelial cells, smooth muscle cells (SMCs), and macrophages.7-10 These processes have been proposed to lead to plaque vulnerability and potential rupture, which is ultimately responsible for acute atherothrombotic vascular occlusion and tissue infarction.

Most of these effects are mediated by the interaction of oxLDL with its major receptor, named LOX-1, a type-II
membrane protein belonging to the C-type lectin family. LOX-1 consists of 4 domains: a short N-terminal cytoplasmic, a transmembrane, a connecting neck, and a lectin-like domain at the C terminus which binds oxLDL. Importantly, the integrity of the lectin-like domain is critically required for its binding activity and is highly conserved among species. LOX-1 is expressed in endothelial cells, macrophages, SMCs, and platelets. Furthermore, LOX-1 is present in atheroma-derived cells and is overexpressed in humans and animal atherosclerotic lesions in vivo. An association of polymorphisms in the human OLR1 gene and MI susceptibility has been recently reported. In particular, we have identified 7 different single nucleotide polymorphisms (SNPs), 6 of them located within introns 4, 5, and 3’ UTR (untranslated region), comprised in a linkage disequilibrium (LD) block strongly associated with the elevated risk to develop MI. Because the SNPs related to an increased risk of MI did not affect the coding sequence of the gene, we decided to explore whether the SNPs could give rise to a functional product by examining the existence of messenger RNA (mRNA) isoforms as a consequence of alternative splicing. Using a minigene approach, we show that SNPs located in the LD block regulate the level of the new fully functional transcript by modulating the retention of exon 5 of the OLR1 gene. The identification and characterization of the new splice variant of the OLR1 gene suggest that this variant may have a functional role on plaque instability and therefore in the pathogenesis of myocardial infarction.

Materials and Methods

RT-PCR Experiments
Total RNA was purified from monocyte-derived macrophages and COS-7 cells using RNeasy Mini Purification Kit (Qiagen). Poly A+ RNA were purified using the Oligotex mRNA Mini Kit (Qiagen). Reverse transcription was performed with a High-Capacity cDNA Archive Kit (Applied Biosystems). RT products were amplified using forward OLR1-FW primer (5’-TGTGTTAAGTCTGTGACGCTT-3’), reverse OLR1-RW primer (5’-TGTTGAAGTTCGTGACTGCTT-3’), and then subcloned using a TA cloning kit (Invitrogen).

DNA Constructs
To amplify the genomic sequence surrounding the alternatively spliced exon 5 of OLR1 gene, we used the primer pair OLR1-XholIF (5’-ACA GTC CTC GAG GTG AGT GTT CAT GGA TAT TTG-3’) and OLR1-EcoRIIR (5’-TGT GTG GAT ATC CTC GAG CAG GTA GGA AAA ACA AAA-3’). We used human genomic DNA homoyzous with respect to “risk” or “non-risk” haplotype at LD block of OLR1 gene as the PCR template. The PCR products were cloned into pSPL3 vector (Invitrogen) and sequenced using the primer OLR1/SEQF (5’-GTT TCC TAT TCT TTG CTC AAC-3’) and OLR1/SEQR (5’-GTT GGG AGT GAT AAT TCT TCT GAG-3’).

To generate LOX-1-GFP and LOXIN-GFP constructs, the coding sequences of OLR1 gene and the splicing variant LOXIN were PCR-amplified from cDNA, which was derived from the human heart poly A+ RNA (Clontech) using selected oligos. For the amplification of OLR1, primers F1 (5’-CCGGCTGAGATGCTTTTGATGACCTAAG-3’) and F2 (5’-CCGGCATCTGTCGCTCTTAGTTTGC-3’) were used. For the amplification of LOXIN, primers F1 and F3 (5’-CCTCTGATCATCA GATCAGCTGCTATT-3’) were used. The PCR products were cloned into the Xhol/BamHI-digested pEGFP-N1 vector (Clontech) and sequenced using CEQ2000 (Beckman-Coulter).

Quantitative Real-Time PCR
Real-time RT-PCR was performed on a TaqMAN ABI 7000 Sequence Detection System (Applied Biosystems). By using the Primer Express 2.0 software (Applied Biosystems), we designed primers and MGB probes for the discrimination analysis of the 2 alternatively spliced isoforms (primers and MGB probes sequences are available on request, patent pending).

Commercially available predeveloped TaqMan endogenous reference GAPDH gene (Applied Biosystems) was used to normalize the amount of cdNA added per sample. A comparative Ct method was used to determine relative quantification of RNA expression. All PCR reactions were performed in triplicate.

Cell Culture and Transfection
Human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of subjects homozygous for the “risk” and “non-risk” haplotype. We promoted their transition to macrophages in vitro as previously described. Differentiation was determined by flow cytometry using anti-CD36 FITC monoclonal antibody (cell purity >95%). Simian COS-7 fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). Transient transfection of COS-7 was performed using the Nucleofector kit (Amaza Biosystems). 1 μg of plasmid DNA was added to 5×10^6 COS-7 suspended in 100 μL of human dermal fibroblast Nucleofector solution. The program A-24 was selected for a high density of transfection. All subjects gave informed consent, and the study protocol was approved by the Tor Vergata University Ethics Committee.

Evaluation of Apoptosis
Differentiated macrophages were cultured without serum for 15 hours and then incubated with oxLDL (100 μg/mL; Intracel) for 8 hours before detection of apoptosis. Apoptosis was evaluated by multiparameter flow cytometry using a method that distinguishes nuclei from apoptotic, necrotic, or viable lymphoid cells. Isolated nuclei were analyzed by fluorescence and by forward- and side-angle scatter multiparameter analysis using a FACScan flow cytometer (Becton Dickinson). A minimum of 5000 events was collected for each sample.

Apoptotic fibroblasts were visualized by staining with the blue fluorescent dye Hoechst 33342 (Sigma) and phosphatidylinerine assay as described previously. Annexin V and Hoechst 33342 positive cells were counted from cells transfected with GFP, LOX-1-GFP, and LOXIN-GFP recombinant proteins.

Immunofluorescence Staining
Immunofluorescence was performed as described. Affinity purified anti-rabbit LOX-1 (Santa Cruz), goat anti-calnexin (Santa Cruz), and mouse anti-Golgi 58K (Sigma) protein were used as primary antibodies. Texas Red goat anti-rabbit IgG (Calbiochem), Texas Red goat anti-mouse IgG (Calbiochem), and Rhodamine Red–X-conjugated affinipure rabbit anti-goat IgG (Jackson Immunoreasearch) were used as secondary antibodies. Hoechst 33342 dye was used at 1 μg/mL. Samples were examined with a DMRA Leica fluorescence microscope equipped with CCD camera. Acquired images were deconvolved using Leica Q-fluoro software and processed using Adobe Photoshop.

Western Blot Analysis and Enzymatic Digestion
PBMCs and COS-7 were lysed for 30 minutes in ice-cold cell extraction buffer (EB) (100 mmol/L NaCl, 10 mmol/L EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L PMSF, 10 μg/mL pepstatin A, 10 μg/mL leupeptin and 0.03 mmol/L aprotinin). Nuclei and large debris were removed by centrifugation at 2400g for 10 minutes at 4°C. The supernatants were then precipitated with 5 volumes of MeOH at −20°C for 2 hours. After centrifugation (16 000g, 10 minutes, 4°C), protein pellets were dissolved in 4X sample buffer (500 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 40 mmol/L DTT and 0.02% bromophenol blue) and heated at 95°C for 5 minutes.
Blots were probed with rabbit anti-LOX-1 antibody (Santa Cruz). Immunoreactive bands were detected with sheep anti-rabbit IgG horseradish peroxidase (Amersham) and visualized by ECL (Sigma). Enzymatic deglycosylation was performed as previously described.18

**Statistical Analysis**

All statistical analyses were performed using SPSS version 13 software. Data are presented as means ± 1 SD. Normal distribution of continuous variables has been verified by Kolmogorov–Smirnov with Lilliefors correction. Differences in means of continuous variables were analyzed by impaired t test or ANOVA as needed. Bonferroni correction was used for multiple comparison.

**Results**

**Identification of an Alternatively Spliced Forms of OLR1 Gene**

We started from total or poly (A) RNA of human monocyte-derived macrophages and subjected it to reverse transcription to test the potential effect of the SNPs located in the LD block on RNA splicing of the human OLR1 gene. We performed PCR amplification using primers located in the 5’UTR and 3’UTR of the OLR1 gene and sequenced the amplified fragments. This analysis identified a reproducible pattern of alternative splicing around exon 5. In particular we identified 2 OLR1 transcripts in both RNA fractions. One of these products corresponded to the full-length transcript (OLR1), while the other lacked exon 5; we named it LOXIN (Figure 1B). The newly spliced mRNA has a stop codon in the open reading frame that leads to a premature termination of the translation product and generates a predicted protein that lacks 2/3 of the lectin-like domain (Figure 1C). Both isoforms were detected in several cell types (endothelial cells, fibroblasts, and smooth muscle cells), and tissues (heart, kidney, and brain), suggesting that they reflect a physiological pattern of expression of the OLR1 gene (Figure 1B).

**SNPs Located in the LD Block Modulate the Levels of the mRNA Isoforms**

To find in vivo evidence that SNPs located in the LD block modulate the level of the two mRNA isoforms, we performed an isoform-specific real-time PCR starting from total RNA of human monocyte-derived macrophages of selected patients carrying the “risk” and “non-risk” haplotype at OLR1 gene. By this analysis we noted a marked difference in the mRNA ratio (OLRI/LOXIN) according to the haplotype. In particular, the OLR1/LOXIN mRNA ratio was 33% higher in human monocyte-derived macrophages of subjects homozygous for the “risk” haplotype compared with homozygous for the “non-risk” haplotype (Figure 2A). The finding that the relative amount of the LOXIN transcript is significantly
greater in subjects carrying the “non-risk” haplotype strongly suggests a negative link between levels of LOXIN mRNA and the incidence of MI in humans.

To further confirm the regulatory role of the intronic polymorphism, we extended these studies by constructing minigenes carrying the “risk” and “non-risk” haplotypes with genomic sequences containing intron 4, exon 5, and intron 5 (Figure 2B). These constructs were transfected in COS-7 fibroblasts and the ratio of unspliced (exon 5+) to spliced (exon 5−) transcript was analyzed by real time isoform-specific PCR. As can be seen in Figure 2C, the ratio was 27% higher in RNA extracted from cells transfected with minigenes carrying the “risk” haplotype. These in vitro experiments not only suggest that the relative abundance of the 2 isoforms is modulated by the intronic SNPs mapping within the LD block, but also confirm the previously described in vivo results (Figure 2A).

Subcellular and Membrane Distribution of LOX-1 and the LOXIN Splice Variant
To investigate the cellular localization of the full-length and the splice variant, we constructed two plasmids that allowed the efficient expression of LOX-1 and LOXIN in mammalian cells fused, C-terminally, to the green fluorescent protein (GFP). Immunofluorescence analysis of transfected COS-7 fibroblasts revealed that the intracellular expression of the full-length LOX-1–GFP causes a cell-lethal phenotype. Many transfected cells are roundly shaped and tend to detach from the dish. On the contrary, LOXIN-GFP isoform is efficiently expressed, and its expression does not result in cytotoxicity.

Notwithstanding the toxic effect, many LOX-1–GFP transfected cells retain normal morphology, and this has allowed us to study its intracellular distribution. As shown in Figure 3A, (panels A through C), LOX-1 distributed in the ER and in the Golgi apparatus. At 24 hours after transfection, LOX-1 colocalized almost exclusively with the Golgi 58K protein, indicating that the protein initially traffics along the secretory pathway. In contrast, LOXIN-GFP was not detectable in the Golgi patches of permeabilized COS-7 cells. In these cells we observed a more widespread staining, characteristic of typical ER distribution. In 40% to 50% of transfected cells, an accumulation of fluorescence in the perinuclear area was also detected (Figure 3A, panels D through F).

The 2 GFP-tagged isoforms were also labeled for surface receptors in live cells (Figure 3B and 4). At 24 hours after transfection, over 90% of cells expressing LOX-1 and LOXIN in mammalian cells fused, C-terminally, to the green fluorescent protein (GFP). Immunofluorescence analysis of transfected COS-7 fibroblasts revealed that the intracellular expression of the full-length LOX-1–GFP causes a cell-lethal phenotype. Many transfected cells are roundly shaped and tend to detach from the dish. On the contrary, LOXIN-GFP isoform is efficiently expressed, and its expression does not result in cytotoxicity.

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The expression level of the 2 isoforms in transfected fibroblasts was also confirmed in Western blot. A single band corresponding to LOX-1–GFP and LOXIN-GFP fusion proteins were detected (Figure 4A, lanes 1 and 2). The molecular weight of the bands corresponded to the predicted molecular weight of nonglycosylated proteins. The LOX-1–GFP band was, however, much fainter, probably because of the previously mentioned cytotoxic effect of this construct (Figure 4A, lane 2).

In Vivo Expression of LOX-1 and LOXIN Isoforms
To verify whether LOXIN transcript is indeed translated in vivo and to analyze the level of its expression, we used Western blot to examine the relative amounts of the 2 isoforms in PBMCs derived from selected subjects with different haplotypes. As shown in Figure 4B, immunoreaction of cell lysates showed 2 major bands of 34 and 22 kDa, corresponding to the predicted molecular weight of the 2 isoforms. Interestingly, we noticed a relative increase in the amount of LOXIN in cells derived from subjects homozygous for the “non-risk” haplotype (Figure 4B). Removal of N-linked glycans by PNGase digestion (Figure 4B) resulted in the disappearance of few faint bands, indicating that the two bands, 34 and 22 kDa, correspond to the unglycosylated LOX-1 and LOXIN proteins. In many gels, including the one shown in Figure 4B, a third band of 26 kDa was also observed. This band may represent a degradation product of the LOX-1 protein that we are currently studying.
In Vivo Proapoptotic Effect of LOX-1 and Rescue By LOXIN

We considered that an altered balance between the 2 isoforms could be related to the increased susceptibility to apoptosis. To test this notion, we analyzed oxLDL-induced apoptosis in a monocytes/macrophages in vivo assay. Human monocytes were isolated from buffy coats of healthy donors carrying the “risk” and “non-risk” haplotype at LD block of OLR1 gene. After Ficoll gradient centrifugation, monocytes were separated to induce differentiation in macrophages. 100 μg/mL of oxLDL was added to these cells to induce apoptosis. Remarkably, flow cytometric analysis of macrophages derived from subjects carrying the “risk” (lanes 1 and 3) and “non-risk” (lanes 2 and 4) haplotypes incubated for 3 hours with PNGase F (1000 U), as indicated.

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Increased LOXIN levels relate to the “non-risk” haplotype, we explored the possibility that LOXIN isoform may have a protective effect versus apoptosis. To test this hypothesis, we transfected COS-7 fibroblasts with DNA encoding for LOXIN-GFP and LOX-1–GFP alone or cotransfected with a fixed amount of LOX-1 and increasing concentration of LOXIN-GFP plasmids (as indicated in Figure 5B). The phenotype of transfected cells was analyzed using 2 markers of apoptosis. First, we used Annexin V, which detects alteration at the level of the plasma membrane. Next, we examined the uptake of blue-fluorescent Hoechst 33342 dye, which stains the condensed chromatin of apoptotic cells more brightly than the chromatin of nonapoptotic cells. On the basis of the combined staining patterns of these dyes, we were able to distinguish between normal, apoptotic, and dead cells.

As mentioned above, cells expressing the LOX-1–GFP fusion protein did not thrive, with many of the cells exhibiting cell shrinkage, which is a feature of apoptosis. Thus, LOX-1–GFP alone resulted in 36% of cells developing apoptosis. In contrast, LOXIN expression was not toxic and the number of apoptotic cells was comparable to the mock-transfected cells. Interestingly, the coexpression of LOXIN-GFP resulted in a complete dose-dependent rescue of the LOX-1–GFP–induced phenotype. It is worth noting that a very low dose of LOXIN, corresponding to a ratio of 1:4 with LOX-1, resulted in a 72% reduction in the number of apoptotic cells, and a 1:1 ratio of the 2 plasmids used for transfection completely prevented the phenotype. This finding suggests that small differences in LOX-1/LOXIN balance and, especially, a small increase in LOXIN expression, may have a very profound effect on the cytotoxic phenotype also in vivo.

Discussion

During genetic screening, it is a common experience to end up with a large fraction of orphan variants with an unclear
pathogenetic role.20 It is therefore essential to find a plausible biological basis for each such association to strengthen the genetic significance of SNPs in prediction of complex diseases. This article reports, for the first time, the identification and characterization of a new functional isoform of the ORL1 gene and provides a functional explanation of the genetic association between SNPs within ORL1 gene and myocardial infarction.

We show that SNPs located in the LD block modulate the relative abundance of the 2 transcripts. Both in vivo and in vitro experiments indicate that the new splice variant LOXIN is expressed at a similar level as the full-length receptor LOX-1. Interestingly, macrophages from subjects carrying the “non-risk” haplotype express more LOXIN and result in fewer cells undergoing apoptosis on oxLDL induction. Macrophages play an important role in all phases of atherosclerosis, from the development of the fatty streak to the process that ultimately contribute to plaque rupture and myocardial infarction.21–23 Indeed, evidence from pathological studies link apoptosis of plaque resident macrophages with rupture and thrombosis of atherosclerotic lesions and subsequent development of acute vascular complication. In particular, it has been proposed that extensive apoptosis of macrophages occurs only at sites of plaque rupture and possibly contributes to the process of rupture and thrombosis.24 Our finding that increased levels of LOXIN in macrophages relate to reduced levels of apoptosis suggests that this in turn could result in plaque stabilization by influencing the nature of the vulnerable plaque.

We report that C-terminal splice variants of LOX-1 receptor differentially traffic from the ER to the plasma membrane. In cell lines we show that LOXIN isoform displays no surface expression in 90% of transfected cells and lower expression in 1/10 of the cells. This is attributable to retention of LOXIN in the ER and accumulation of the protein in the perinuclear region. Regulation of the trafficking of LOX-1 receptors to the plasma membrane may provide an essential mechanism for the control of its function in vivo. In this context, different splice variant isoforms in the C-terminal domain have been reported for other receptors, such as glutamate receptors. In this case, the domain has been shown to be the site of interactions of proteins involved in the trafficking and stabilization of glutamate receptors in the synaptic membrane and, in turn, to play a role in synaptic strength and plasticity.25,26 Whether LOXIN forms heteromeric receptors with LOX-1 and regulates its intracellular traffic in vivo is under study.

LOX-1 receptor, when ectopically expressed in fibroblasts, is toxic and results in a high percentage of cells undergoing apoptosis. We demonstrate here that LOXIN molecules, when coexpressed with LOX-1 receptors, have a protective role and are able to rescue the apoptotic phenotype. The mechanism by which LOXIN exerts its protective role is not known but we propose different hypotheses. First, LOXIN may act on LOX-1 receptors by forming inactive heterodimers. Because the LOXIN splice variant lacks exon 5, it misses the binding region of ox-LDL. By increasing the intracellular LOXIN relative amount, the number of functional receptors able to bind oxLDL may be reduced. Because oxLDL induces apoptosis in macrophages, thereby reducing oxLDL binding sites, fewer cells will go through apoptosis. Secondly, LOXIN may exert its action on the transport of LOX-1 receptors toward the cellular membrane, blocking them into the ER and downregulating their membrane expression. Thirdly, the LOXIN itself may even have an ant apoptotic activity. These different hypotheses warrant further studies.

It is important to note that the expression level of both isoforms in vivo is similar. Subjects homozygous for “non-risk” haplotype show a small increase in LOXIN expression compared with homozygous for “risk” haplotype. In vitro experiments confirmed this fine balance between the 2 isoforms. A small amount of LOXIN is sufficient to rescue the lethal phenotype.

We conclude that SNPs located in the LD block of ORL1 gene, with their functional role, may represent an important genetic risk factor for prediction of susceptibility to myocardial infarction. Moreover LOXIN could represent a new target for prevention of progression and destabilization of atherosclerotic plaque. Therefore, new research or therapeutic strategies favoring increasing expression of LOXIN could be effective for preventing plaque instability.

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References


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