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 atherosclerosis 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 age. However, newly defined nontraditional risk factors are emerging as being equally important.2 Among these are

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

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Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/01.RES.0000174563.62625.8e

Original received May 17, 2005; revision received June 9, 2005; accepted June 10, 2005.

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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’-TGTGAAATGGCTGATCGCTT-3’), reverse OLR1-RW primer (5’-TTCTGAGCCAGCTAATTGA-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 GAT CTG ATT CTA TGG-3’) and OLR1-EcorIR (5’-TGT GTG GAT ATC CTG CAG CTA GGA AAA ACA AAA-3’). We used human genomic DNA homoyzogous with respect to “risk” or “non-risk” haplotype at LD block of OLR1 gene as the PCR template. The PCR products were cloned into Sph1-L vector (Invitrogen) and sequenced using the primer OLR1-SEGQ (5’-GTT TCC TAT TCT TCTT CTG AAC-3’) and OLR1-SEQR (5’-GGT GGG AGT GAT ACT 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’-CGGAGATGGATATTTTGATGAC-3’) and F2 (5’-CGGAGATTCCTGCTGCTTAGGTG-3’) were used. For the amplification of LOXIN, primers F1 and F3 (5’-CCGGGATCCATCA GATCCGTGCTTATT-3’) were used. The PCR products were cloned into the Xhol/BamHI-digested pEGFP-N1 vector (Clontech) and sequenced using CEQ2000 (Beckman-Coulard).

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 C_b 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 homoyzogous 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 5×10⁶ 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 Flowcytometer (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 phosphatidylinerse 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 antibody (Santa Cruz), goat anti-calcxin (Santa Cruz), and mouse anti-Gal (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 affiprime 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 0.3 mmol/L aprotinin). Nuclei and large debris were removed by centrifugation at 290g 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 4× 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 version13 software. Data are presented as means ± SD. Normal distribution of continuous variables has been verified by Kolmogorov–Smirnov with Lillefors correction. Differences in means of continuous variables were analyzed by 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 (OLR1/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

Figure 1. OLR1 from gene to protein. A, Genomic organization of the OLR1 gene. The exon-intron structure of OLR1 gene is shown along with SNPs position, comprised in a LD block. The top box represents the “risk” and “non-risk” haplotype at the LD block. B, Identification of alternatively spliced forms of OLR1 gene and expression profile in different cell types and tissues as indicated. C, Predicted protein schematic representation.

Figure 2. SNPs located in the LD block modulate the levels of the OLR1 mRNA isoforms. A, Quantification of the OLR1 and LOXIN transcripts using real-time quantitative PCR. Total RNA was isolated from monocyte-derived macrophages of 10 subjects homozygous for the “risk” haplotype and 10 for the “non-risk” haplotype and 10 heterozygotes. The 2 isoforms were quantified using isoform specific primers and probes. Bar graphs show the relative amount of the 2 isoforms expressed as a ratio of OLR1 isoform (containing exon 5) to the LOXIN isoform (lacking exon 5). B, Map of the 2 minigene plasmids P1 and P2. The plasmids P1 and P2 contain the genomic sequence homozygous for the “risk” haplotype and 10 heterozygotes. The 2 isoforms were quantified using isoform specific primers and probes. Bar graphs show the relative amount of the 2 isoforms expressed as a ratio of OLR1 isoform (containing exon 5) to the LOXIN isoform (lacking exon 5). C, Quantification of the P1 and P2 minigenes expression. Total RNA was isolated from COS-7 cells at 36 and 48 hours after transfection with P1 or P2 plasmids. The corresponding cDNA were quantified using an isoform-specific real-time PCR. Bar graphs show the ratio of the transcript containing exon 5 to the transcript lacking exon 5.
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–GFP showed a typical punctuate plasma membrane–associated fluorescence (Figure 3B, panel C). Remarkably, <10% of COS-7 cells transfected with LOXIN-GFP showed surface staining (Figure 3B, panel F). The very low expression of LOXIN at the plasma membrane demonstrates that truncation of the C-terminal portion in LOXIN protein leads to a profound effect on cellular trafficking of the protein to the plasma membrane.

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” and “non-risk” haplotypes incubated for 3 hours with PNGase F (1000 U), as indicated.

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). At least 100 positively transfected cells for each plasmid were counted and checked for their positive reaction to phosphatidylserine and Hoechst 33342. The average of the percentage of apoptotic cells in cells transfected with GFP alone (6%) was subtracted to all experimental points. The results shown are the average of 3 different experiments.

Discussion

During genetic screening, it is a common experience to end up with a large fraction of orphan variants with an unclear
pathogenetic role. 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. Macrophages are expressed at a similar level as the full-length receptor relative abundance of the 2 transcripts. Both in vivo and 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.

Acknowledgments

Funding for this work was through the Italian Ministry of University and Research (PRIN 2002, FIRB 2001, CofinLab 2000), the Italian Ministry of Health (to G.N.) and a MIUR-FIRB Grant RBNE01ZK8F_002 (to S.B.). We thank A. Cardinale for helpful discussions, L. Vecchione, and B. Raso for genetic screening of subjects, as well as P. Borgiani for statistical analysis and G. Bonelli for graphical support.

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Circ Res. 2005;97:152-158; originally published online June 23, 2005;
doi: 10.1161/01.RES.0000174563.62625.8e

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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