Low-density lipoproteins impair migration of human coronary vascular smooth muscle cells and induce changes in the proteomic profile of myosin light chain

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Aims High-risk atheromatous plaques contain significant extra- and intracellular lipid deposits and very low smooth muscle cell numbers in the intima. However, the mechanisms inducing vessel wall remodelling and high-risk plaque composition are unknown. Low-density lipoproteins (LDLs) infiltrate the vessel wall and become retained and aggregated (agLDL) in the intima by binding to extracellular matrix proteoglycans. The cellular responses triggered by agLDL are not fully understood. This study was designed to investigate the effects of agLDL on vascular remodelling and repair, specifically studying human coronary vascular smooth muscle cell (VSMC) functions.

Methods and results Using a wound repair VSMC model system, we have shown that agLDL significantly impair cell migration. Proteomic analysis revealed a differential phenotypic pattern of myosin light chain and lower levels of phosphorylated myosin regulatory light chain (P-MRLC) in agLDL-exposed VSMC. LDL also induced changes in the subcellular localization of P-MRLC, with dephosphorylation strongly evident on the front edge of agLDL-treated migrating cells. PMA, a strong inducer of myosin light chain (MLC) phosphorylation, significantly reduced the effects of agLDL in VSMC migration. Inhibition of MLC kinase with ML9 did not affect MRLC dephosphorylation already induced by agLDL.

Conclusion Our results indicate that LDLs impair human VSMC migration and wound repair after injury. agLDL, and to a lesser extent nLDL, induce dephosphorylation of MRLC and striking changes in the subcellular localization of P-MRLC, a cytoskeleton protein involved in VSMC migration kinetics.

KEYWORDS
Lipoproteins; Smooth muscle cell; Migration; Myosin; Proteomics

1. Introduction

Migration of vascular smooth muscle cells (VSMCs) within the arterial wall is a crucial event in remodelling and progression of atherosclerotic plaques as well as in vascular repair.¹,² Elevated levels of low-density lipoproteins (LDLs) are an important risk factor for atherosclerosis.³ Accumulation of lipids within the arterial wall is associated with intimal disorganization and thickening, and often, in advanced atherosclerotic lesions, with complications such as rupture, haematoma, and thrombosis.²,⁴

Hypercholesterolemia induces changes in vascular cell gene expression and phenotype leading to alterations in vascular function.⁵,⁶ Indeed, LDLs are the most atherogenic type of lipoproteins both in plasma and in vessel wall, and retention and aggregation of LDL (agLDL) in the arterial intima, facilitated by the proteoglycans that conform the extracellular matrix, is a key event in atherosclerotic plaque formation.⁷,⁸ Our group has recently demonstrated that agLDLs induce high intracellular cholesteryl ester accumulation through LDL receptor-related protein (LRP-1) uptake in human VSMCs.⁹,¹⁰ Hypercholesterolemia has been associated with impaired vascular remodelling of balloon-treated porcine coronary arteries and the effect supposedly linked to hampering of VSMC migration and the decrease in VSMC accumulation and collagen deposition at the site of arterial injury.¹¹ Cell migration is a dynamic process that requires temporally and spatially coordinated regulation of intracellular events involving changes in the actin cytoskeleton.¹² Uptake of modified LDLs alters actin distribution and locomotor forces in macrophages.¹³ However to our knowledge, little is known about the effects of LDLs on the actin cytoskeleton of VSMC and their repercussion in migration kinetics and vascular remodelling.

In this study, we were interested in understanding the mechanisms elicited in VSMC by agLDL, as might be the case...
for cells in contact with lipoproteins within atherosclerotic lesions. Our results indicate that agLDLs impair VSMC attachment, migration, and hinder wound repair after injury.

2. Methods

2.1 Culture conditions of human coronary vascular smooth muscle cell

VSMCs were obtained from human non-atherosclerotic coronary arteries of hearts removed in transplant operations, by using a modification of the explant technique, and cultured as previously described. The investigation was approved by the Reviewer Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau that conforms to the Declaration of Helsinki. Cells used in the experiments were between the fourth and sixth passage. VSMC at these passages appeared as a relatively homogeneous cell population showing a hill-and-valley pattern at confluence and western blot analysis for specific differentiation markers revealed a clear positive band for α-actin (45 kDa) and calponin (33 kDa). Caldesmon (93 kDa) was only displayed as a very weak band. Smooth muscle MHC (227 kDa) and the vascular-specific isoform of smoothelin (110 kDa) were under the detection limits.

To explore how LDLs affect cell attachment, migration, and the cytoskeleton proteome in human coronary VSMC, cells were incubated in the presence or absence of 100 μg/mL nLDL and agLDL for a time period up to 24 h. This concentration was chosen due to the increased levels of intracellular esterified cholesterol depicted by previous dose–response experiments.

In a set of experiments, cells were incubated with or without agLDL for 24 h and inhibitors of myosin regulatory light chain (MRLC)–phosphorylation, 50 μM ML9 (1-[5-cloronaphthalene-1-sulfonyl]-1H-hexahydro-1,4-diazepine-hydrochloride, Sigma), or 10 μM Y-27632 (cyclohexanecarboxamide-dihydrochloride-monohydrate, Sigma) were added for the last 2 h.

For proteomic studies, cells were arrested at subconfluency with medium containing 0.4% FCS for 24 h. Thereafter, VSMCs were incubated for 24 h in serum-free M199 media, containing phenol red acting as an antioxidant in the presence or absence of native or aggregated LDL (nLDL and agLDL). Cells were harvested in 5 mM EDTA and stored at −80 °C.

2.2 Preparation of human low-density lipoproteins

LDLs (density 1.019–1.063 g/mL) were obtained from pooled sera of normocholesterolemic volunteers by sequential ultracentrifugation. agLDLs were prepared by vortexing LDL (1 mg/mL), as previously described. The obtained vortexed particles are similar in size to those obtained by aggregation due to versican fusion. agLDLs were prepared by vortexing LDL (1 mg/mL), as previously described. For proteomic studies, cells were arrested at subconfluency with medium containing 0.4% FCS for 24 h. Thereafter, VSMCs were incubated for 24 h in serum-free M199 media, containing phenol red acting as an antioxidant in the presence or absence of native or aggregated LDL (nLDL and agLDL). Cells were harvested in 5 mM EDTA and stored at −80 °C.

2.2.1 Two-dimensional gel electrophoresis

For analytical and preparative gels, respectively, a protein load of 120 and 300 μg protein of the urea/chaps soluble extracts was applied to 17 cm dry strips (pH 3–10 linear range, Bio-Rad). Gels were developed by silver staining or Coomassie blue (preparative gels). For each independent experiment, two-dimensional gel electrophoresis (2-DE) for protein extracts from the control, nLDL and agLDL groups were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PDQuest software (Bio-Rad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared with the volume of all spots in the gel, following background extraction and normalization between gels.

2.2.2 Gels for protein identification

Protein spots of interest were excised from 2-DE gels, destained (15 mM potassium ferricyanide and 50 mM thioulate solution), dehydrated, dried, and enzymatic digested with sequence-grade modified porcine trypsin (Promega). Peptides from gel digestion were mixed 1:1 with 5 mg/mL a-cyano-4-hydroxy-cinnamic acid and spotted on a stainless steel mass spectrometry slide. Protein identification was performed by peptide mass fingerprinting using an Ettan MALDI-ToF Pro matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (GE-Healthcare) operated in delayed extraction/reflector mode. MALDI-generated mass spectra were internally calibrated using trypsin autolysis products, Ang III (angiotensin III), and ACTH (adrenocorticotropic hormone) peaks. The peptide masses were searched against the National Center for Biotechnology Information non-redundant mammalian database using ProFound and confirmed using a Mascot search from Matrixscience selecting the SwissProt database. For the present study, protein identification was based on the measurement of at least 10 peptides with a minimum of 50% matched peptides and coverage higher than 35%. Minimal expectation for valid identification was 0.0001 and P < 0.05.

2.5 Western blot analysis and glycerol/urea-PAGE for detection of phosphorylated proteins

Sample extracts from the urea/chaps-soluble fraction were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes,
Results are presented as means ± 2.9.

Data analysis

agLDL (100 mGPL) were incubated with or without PBS. Immunolabelling of smooth muscle cells was performed with antibodies against total MRLC (mouse-MAb, clone MY 21, dilution 1:100, Abcam), phosphorylated-MRLC (P-MRLC) [rabbit polyclonal to myosin light chain (MLC)-ps20; dilution 1:100, Abcam], and human actin (dilution 1:10,000, Dako). Band densities were determined with the ChemiDoc™XR system in chemiluminescence detection modus and Quantity-One software (Bio-Rad).

Phosphorylated and unphosphorylated forms of the MRLC were analysed by electrophoresis in glycerol/urea-PAGE gels. Sample extracts in urea/glycine buffer (7 M urea, 2 M thiourea, 200 mM Trizma-Base, 220 mM glycine, 10 mM DTT, 600 mM potassium iodide, 10 mM EGTA, and 1 mM EDTA, pH 7.0) were separated in 10% acrylamide gels containing 40% glycerol at 6 mA/gel. The different phosphorylated forms of MRLC were identified by immunoblotting using the MAb against total MRLC, MY-21 (Abcam). Detection, imaging, and quantification were performed as described for western blot analysis. Data were expressed as the ratio of P-MRLC over unphosphorylated MRLC.

2.6 Immunohistochemistry

Human coronary specimens fixed in 4% paraformaldehyde were tested. Cross-sectional consecutive sections (5 μm thick) were stained with Masson’s Trichromic to identify cellular areas and immunohistochemical analysis for apoB (mouse MAb anti-human Apo B, 1:200, Biodesign), total MRLC (mouse MAb MY 21 for human MRLC, dilution 1:50), and P-MRLC (mouse anti-human P-MRLC, Abcam dilution 1:50) was performed as previously described.

2.7 Immunofluorescence labelling and confocal microscopy

Cells were fixed with 3.5% paraformaldehyde and permeabilized with 0.5% Tween-PBS and blocked with 1% BSA in 0.1% Tween-PBS. Immunolabelling of smooth muscle α-actin was performed with a mouse anti-human monoclonal antibody (clone 1A4, DAKO, dilution 1:100), MRLC was detected with the MAB MY 21 (dilution 1:50), and its phosphorylated form (P-MRLC) with a rabbit polyclonal antibody against the human P-MRLC (SC-12896, dilution 1:50). The secondary antibodies were Alexa Fluor-488 conjugated-goat anti-mouse or anti-rabbit antibodies (Molecular Probes). F-actin was labelled with Alexa Fluor-568 phallolidin (Molecular Probes) and nuclear counterstain with Hoechst (Molecular Probes). Immunolabelled cells were examined in an inverted fluorescence confocal microscope (Leica TCS SP2-AOBS) and processed with the LCS-AOBS software (Leica). Controls with no primary antibody showed no fluorescence labelling.

2.8 Attachment and spreading assay

Subconfluent cultures of VSMC were incubated with or without agLDL (100 mGPL) for 18 h, harvested by trypsin/EDTA, and suspended in 5% FCS/5% human serum-containing medium. Afterwards, cells (1.5 × 10^5) were seeded on FCS-coated glass bottom dishes in the absence or presence of 100 mGPL agLDL. After 1, 3, and 6 h, the non-adherent cells were removed by washing with PBS and attached cells were released by trypsinization, stained with trypan blue for determination of cell viability, and counted in a Neubauer-chamber. At these time periods, cells were fixed with 3.5% paraformaldehyde and labelled for smooth muscle cell α-actin and F-actin, as described above.

2.9 Data analysis

Results are presented as means ± SD and the number of experiments is shown in every case (see figure legends). Statistical differences between control- and LDL-treated groups were analysed by the non-parametric Mann-Whitney test for unpaired data. A P-value of 0.05 or less was considered significant.

3. Results

3.1 Wound repair and migration of human coronary vascular smooth muscle cell

We have studied the effect of nLDL and agLDL on wound repair and VSMC migration using an in vitro scrape-injury model. Microscopic analysis of the cell monolayer within 5 min after injury depicted a wound (350 ± 70 μm wide) with a distinct demarcation between the cell-free area and the remaining intact cell layer (0 h, Figure 1A). nLDL and agLDL markedly retarded repair of the injured area compared with control VSMC (Figure 1A). Based in a cell depleted area of 338 × 10^3 ± 69 × 10^3 μm² at time of injury, control VSMC had covered 81 × 10^3 ± 8 × 10^3 μm² of the wounded area after 3 h, whereas nLDL and agLDL reduced it to 39 × 10^3 ± 7 × 10^3 μm² (P < 0.05) and 36 × 10^3 ± 7 × 10^3 μm² (P < 0.05), respectively (Figure 1B). After 7 h, control VSMC from both sides of the wound had already reached contact at different sites (Figure 1A). This was not achieved in the LDL groups, even after 15 h. At this time period, an extensive area (~75% surface) of the wound in control cultures was already repaired (Figure 1A), whereas the cell repopulated area was 72 × 10^3 ± 7 × 10^3 μm² in the nLDL group and 60 × 10^3 ± 7 × 10^3 μm² in the agLDL group.
To directly measure the effect of LDL on VSMC migration, we used a digital time-lapse video microscopy to follow the movements of individual VSMC. Figure 1B shows that the mean migration rate (given as μm displacement/h) decreased ~two-fold in nLDL- and agLDL-treated cells compared with controls (P < 0.05 for nLDL and agLDL vs. control group).

3.2 Cytoskeleton proteome changes in LDL-treated cells

Differential proteomic analysis was used to identify changes in cytoskeleton related proteins of nLDL- and agLDL-treated VSMCs. Average gels were obtained from at least three independent experiments. Using a broad range pH gradient (pH 3–10), of a total of ~850 protein features detected in each cell extract, 52 spots were identified by fingerprinting analysis as cytoskeleton related proteins. The most significant changes induced by LDL in the cytoskeleton related proteins appear to be in the proteomic profile of the motor protein, MLC (squared regions in Figure 2A).

3.3 Identification of myosin light chain and changes induced by low-density lipoprotein

MLC was identified as two series of major spots with relative molecular mass of ~18 and 21 kDa, clustered around pH 4.5 and 5.2, and identified by MALDI-TOF analysis as myosin alkali light chain (alkali-MLC; region 1 in Figure 2A) and myosin regulatory light chain (MRLC, region 2 in Figure 2A), respectively.

By MALDI-TOF analysis, we identified three series of discrete spots as MRLC (region 2 in Figure 2B), which referred to the smooth muscle isoform (sm-MLC, spot 1116) and the non-sarcomeric isoform-2 and isoform-3 (spots 1013 and 1014). Identification of the three spots as MRLC was confirmed by western blot analysis from 2-DE gels (data not shown). Non-sarcomeric MRLC isoforms were apparently down-regulated (~two-fold decrease) in VSMC treated with either nLDL or agLDL (Table 1). Besides, they were detected as a double spot of different pH. The most acidic spots were strongly weakened or either none detected after treatment with nLDL or agLDL (white arrowheads in Figure 2B). As MRLC is regulated by phosphorylation of Thr18 and Ser19, the 'doubled-spot' might indicate phosphorylation of the protein.

As shown in Figure 2C, western blot analysis from 1-DE gels of protein extracts from control- and LDL-treated VSMC also demonstrated that the non-sarcomeric MRLC lower molecular mass isoform was down-regulated by LDL (see arrowhead in Figure 2C).

3.4 Myosin regulatory light chain phosphorylation in response to low-density lipoprotein

Analysis by glycerol/urea gel-electrophoresis demonstrated two independent bands of MRLC that presumably corresponded to the unphosphorylated and monophosphorylated forms (bands marked as MRLC and P-MRLC in Figure 3, insert, respectively). Traces of a third band with higher mobility (diphosphorylated MRLC) were occasionally, but not consistently, detected (data not shown). VSMC exposed to LDL showed a significant decrease in P-MRLC band, the reduction in the level of P-MRLC, calculated as a ratio of the total MRLC content for each cell extract, was more evident in the agLDL-group (control: 2.1 ± 0.8; nLDL-group: 1.1 ± 0.2, P < 0.05 vs. control; agLDL-group: 0.8 ± 0.4, P < 0.05, n = 3).

To determine whether the LDL effect on MRLC-phosphorylation was dose-dependent, VSMCs were incubated with 0, 50, 100, and 150 μg/mL nLDL or agLDL for 24 h. Western blot analysis using specific antibodies against phosphorylated forms of the MRLC and total MRLC (Figure 3B) revealed that treatment of the cells with increasing doses nLDL (50 and 100 μg/mL) reduced phosphorylation rates to 63 and 41%, respectively, compared with control-untreated cell. No further dephosphorylation was found when cells were incubated with 150 μg/mL (49% phosphorylation level vs. 100% in controls). However, the lowest concentration of

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Figure 2  LDL-induced modifications in the MLC-proteomic profile of human coronary vascular smooth muscle cell (VSMC). (A) Protein spots in 2-DE gels of the urea/detergent subproteome. Spots within the dashed boxes correspond to different variants of alkali-MLC (region 1) and MRLC (region 2). (B) Enlarged images of region 2 from three independent experiments. Arrows denote a double spot for myosin regulatory light chain (MRLC), suggesting differences in the phosphorylation state of MRLC. Left spots (open arrowheads) were less apparent or not detected in nLDL- and agLDL-treated groups. (C) Representative western blot for total MRLC and α-actin expression in control, nLDL- and agLDL-treated VSMC.
agLDL (50 μg/mL) already reduced phosphorylation rate of MRLC to a maximum dephosphorylation (20% of the phosphorylation level in controls § Figure 3B).

### Table 1: Protein identification by MALDI-TOF MS

<table>
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<tr>
<th>Spot ID</th>
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<th>SWISS-PROT accession number</th>
<th>Observed Mr (kDa)</th>
<th>Intensity (relative values)§ ratio vs. control</th>
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</thead>
<tbody>
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<td>1116</td>
<td>sm-MRLC</td>
<td>NP_006088.2</td>
<td>5.3</td>
<td>Y 1.16 ± 0.83 0.94 ± 0.31</td>
</tr>
<tr>
<td>1013</td>
<td>MRLC-isoform 3</td>
<td>NP_006462.1</td>
<td>5.0</td>
<td>Y 0.66 ± 0.35 0.50 ± 0.08*</td>
</tr>
<tr>
<td>1014</td>
<td>MRLC-isoform 2</td>
<td>NP_291024.1</td>
<td>5.1</td>
<td>Y 0.49 ± 0.09* 0.62 ± 0.14*</td>
</tr>
</tbody>
</table>

Effect of LDL on myosin regulatory light chain (MRLC) proteomic profile.
§ Ratios are calculated in basis of the normalized intensity value (see Section 2) obtained for the referred spots (spot ID) in the nLDL- or agLDL-treated groups and the intensity value of the matching spots in the control group for each independent experiment. Results are given as means ± SD of three independent experiments.
* P < 0.05.
Y, protein spots detected in the gel.

![Figure 3](https://example.com/fig3.png)

**Figure 3.** Effect of nLDL- and agLDL treatment on myosin regulatory light chain (MRLC) phosphorylation in human coronary vascular smooth muscle cell (VSMC). (A) Unphosphorylated and phosphorylated forms of MRLC in extracts of VSMC were separated by glycerol/urea gel-electrophoresis and identified by western blot (Insert). Bars represent means ± SD of the P-MRLC/MRLC ratios obtained from three independent experiments. MRLC phosphorylation is markedly decreased in nLDL or agLDL groups compared with controls. P < 0.05 (Mann-Whitney test): § vs. no LDL-exposed VSMC (control). (B) Western blot of total- and phosphorylated-MRLC in protein extracts of VSMC, untreated or treated with 50, 100, and 150 μg/mL nLDL or agLDL for 24 h. Equal protein loadings were controlled by staining the nitrocellulose membrane with Ponceau’s red. P-MRLC (pSer, upper bands) and total-MRLC (lower bands) were sequentially determined in the same membranes, using a rabbit-polyclonal anti-pSer-MRLC-antibody and a mouse monoclonal anti-MRLC-antibody. Dehybridation of membranes (Pierce kit) between labelling was performed. Negative controls to exclude cross-over between antibodies were performed simultaneously. Western blots are representative of three independent cell culture experiments.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Myosin regulatory light chain (MRLC) distribution pattern in intermediate atherosclerotic lesions of human coronary arteries. Representative photomicrographs for Masson’s Trichromic staining (A) and immunostaining for apo-B (B), MRLC (C), and P-MRLC (D). In (A), note that SMC stain in red. (B through D) Positive signal for immunohistochemistry depicted as brown colour (diaminobenzimide, DAB). lu, lumen; i, intima; Magnification ×400.

### 3.5 Myosin regulatory light chain distribution pattern in human atherosclerotic coronary arteries

Immunohistochemistry (Figure 4) of atherosclerotic human coronary lesions rich in VSMC (α-actin positive, data not shown) showed that total MRLC and P-MRLC were consistently detected in areas with low content in apo-B (filled arrowheads, Figure 4B and D), whereas areas with high content in apo-B were positive stained for total MRLC, but they depicted a very low or non-staining signal for the phosphorylated form (open arrowheads in Figure 4B and D).

### 3.6 Effect of nLDL and agLDL on myosin regulatory light chain distribution pattern in migrating vascular smooth muscle cell

The distribution pattern of total MRLC, its phosphorylated forms, and the co-localization with F-actin was analysed...
by confocal microscopy in *in vitro* migrating VSMC after inducing a scrape-wound on cell monolayer (Figure 5).

MRLC-fluorescent signal was strongly detected in control migrating cells and cells at the wound edge (Figure 5A). The cell-positive signals particularly localized close to the leading front of migration. MRLC positive signal was also detected, although with less intensity, throughout the posterior region of the cells in the control group (white arrows in Figure 5A). Compared with controls, the level of MRLC-fluorescence was decreased in migrating LDL-treated cells (Figure 5C and E). MRLC-labelling was primarily detected in the anterior region of migrating cells in the LDL-group, but MRLC-signal detection along the fibre structures in these cells was low.

Phalloidin-labelling visualized numerous filopodia emerging from control cells entering in the wound area (Figure 5A and B). Filopodia were less apparent in migrating VSMC in the presence of nLDL and agLDL (Figure 5C–F). MRLC was detectable at the base, but not at the tip of filopodia neither in control VSMC nor in LDL-treated VSMC (yellow arrowheads in Figure 5A).

### 3.7 Low-density lipoprotein modify subcellular localization of phosphorylated myosin regulatory light chain in migrating vascular smooth muscle cell

Control VSMC express strong positive signals for P-MRLC throughout the cell anterior region and traces of positive immunolabelling at the rear of the cells, with a distribution pattern very similar to that observed for the total MRLC-immunolabelling in the control group (compare Figure 5A and B). In addition, it co-localized with F-actin in part at the base of the filopodia in the migrating cells. Confocal analysis of serial stack sections (0.1 μm distance) also displayed P-MRLC-positive signals around the nuclei in these cells, but the significance of that is unclear.

LDL induced severe changes in the subcellular distribution of P-MRLC. Thus, LDL-treated VSMC showed a weak positive labelling for P-MRLC that distributed all through the cell without any specific localization, neither within cells migrating into the wounded area (Figure 5D and F). The decrease of P-MRLC-positive signals was especially evident in the agLDL group (Figure 5F). Compared with controls, a

![Figure 5](https://academic.oup.com/cardiovascres/article-abstract/77/1/211/463103)
low degree of co-localization was detected between P-MRLC and F-actin in all the LDL-treated cells (compare Figure 5D and F with B).

3.8 Involvement of myosin regulatory light chain-phosphorylation on the migrating response of vascular smooth muscle cell to agLDL

We further investigate whether changes in the MRLC-phosphorylation pattern might account for the impairment in wound repair and VSMC migration in the presence of LDL. To this end, we performed a set of experiments using the in vitro cell injury-model as described above with or without exposure to 100 nM PMA (phorbol 12-myristate 13-acetate, Sigma), an activator of MRLC-phosphorylation,24 or to 50 μM ML9, an inhibitor of MRLC phosphorylation25 (Figure 6B). PMA consistently increased and ML9 decreased the migration rate in control VSMC (P < 0.05). In parallel experiments, co-treatment of VSMC with 100 nM PMA and 100 μg/mL agLDL increased in two-fold the VSMC migration rate compared with values obtained in the presence of agLDL alone (P < 0.05). The MRLC-phosphorylation rate in the agLDL-treated group was 34% of the control group. MRLC-phosphorylation rate was increased up to 60% when the cells were treated with agLDL in the presence of 100 nM PMA (P < 0.05; western blot in Figure 6A).

3.9 Role of MLCK and MLC-phosphatase on agLDL induced myosin regulatory light chain-dephosphorylation

To further analyse the mechanism underlying MRLC dephosphorylation in agLDL-treated human VSMC, effects of the MLCK-inhibitor, ML-9, were compared with those of the Rho-kinase inhibitor, Y-27632.26 As shown in Figure 7, treatment of control VSMC with 50 μM ML-9 or 10 μM Y-27632 for 2 h inhibited MRLC-phosphorylation by ~85 and 70%, respectively (phosphorylation ratio, C:1.59 ± 0.18, C + ML9:0.24 ± 0.11, C + Y-27632:0.49 ± 0.08). After pre-incubation of VSMC with 100 μg/mL agLDL (22 h), addition of ML-9 did not show any further effect on MRLC-phosphorylation (agLDL:0.64 ± 0.10 vs. agLDL + ML9:0.70 ± 0.08). On the contrary, Y-7632 induced a further 20% MRLC-dephosphorylation in the agLDL-treated VSMC (agLDL + Y-27632:0.39 ± 0.14).

3.10 Vascular smooth muscle cell attachment and spreading

Cells were seeded and allowed to attach on glass plates for time periods up to 6 h. Incubation with agLDL significantly impaired attachment of VSMC compared with untreated cells (Table 2). Thus, 6 h after seeding, 87 ± 7% of the plated VSMC were attached in the control group and 46 ± 5% in the agLDL-treated group (P < 0.05). Organization of actin during cell attachment and attachment was examined by confocal microscopy of VSMC incubated with or without LDL. Figure 6 shows that cells treated with agLDL depicted a more expanded shape and formed F-actin rich ruffles compared with untreated cells that mainly developed numerous small filopodia with positive signals for either F-actin and α-actin (compare Figure 8A and B). Three hours after plating, relative F-actin labelling was more intense in the control VSMC, an extended and well formed net of actin fibrils (F-actin positive) was already detected throughout the cytoplasm of these cells (compare Figure 8C), whereas this was not apparent in agLDL-treated VSMC neither at this time period nor 6 h after plating (Figure 8E and F).
4. Discussion

A key pathogenic event in the development of atherosclerosis is the retention of lipoproteins in the intima, which bind extracellular proteoglycans and undergo aggregation and oxidative modification. The present study demonstrates that agLDL significantly impair migration of human coronary VSMC and hinder repair of mechanically induced wounds on VSMC monolayers.

We have identified by means of 2-DE analysis that native and aggregated LDLs induce significant changes in proteins directly involved in the assembly/disassembly and stabilization of the VSMC cytoskeleton network. More specifically, LDL both in their native form or modified by aggregation, alter the expression and proteomic pattern of different isoforms of MRLC in human coronary VSMC.

Cell migration has been associated with changes in the subcellular localization of the myosin-actin cytoskeleton. In migrating VSMC, LDL does not significantly alter MRLC subcellular distribution when comparing it with LDL non-treated cells. However, MRLC-fluorescent signals are less evident in the LDL-treated VSMC.

The MRLC active form, which interacts with actin, is generated by phosphorylation of MRLC and Regulation of MRLC phosphorylation is proposed to play an important role in controlling morphological changes during cell attachment, spreading, and migration. The present study shows that native and aggregated LDLs induce MRLC dephosphorylation in VSMC. In addition, dose–response studies demonstrate that LDL when modified by aggregation

<table>
<thead>
<tr>
<th>Time period (h) after plating</th>
<th>Control ( \times 10^5 )</th>
<th>agLDL ( \times 10^5 )</th>
<th>( P )-value</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.08 ± 0.10</td>
<td>0.36 ± 0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>6</td>
<td>1.30 ± 0.10</td>
<td>0.69 ± 0.07</td>
<td>&lt;0.05</td>
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Results refer to the number of adherent cells from a total of 1.5 \( \times 10^5 \) seeded cells. Cell viability was in all cases >95% as determined by trypan blue staining. Results are given as means ± SD of three independent experiments in triplicates.

\( P \)-value refers to comparison for statistical significance (Mann–Whitney test) between control- and agLDL-groups at each time period.

Table 2  Effect of the treatment with agLDL in the number of adherent cells

![Figure 8](https://academic.oup.com/cardiovascres/article-abstract/77/1/211/463103)
have inhibitory effects at lower concentrations than LDL in their native form. Besides, P-MRLC that is localized on some fibre-like structures close to the leading edge on migrating control VSMC depicts a clearly different distribution pattern in the native- and aggregated-LDL-treated cells. Delocalization of the P-MRLC signal is more apparent in cells of the agLDL group that also demonstrates the lowest wound repair capability. The finding that PMA significantly reverts both the effect of agLDL on MRLC-phosphorylation levels and their inhibitory effect on VSMC migration (50%) strongly emphasizes the relevance of MRLC-desphosphorylation in the impairment of cell migration induced by LDL. In agreement with the in vitro studies, weak or undetectable P-MRLC staining was observed in areas of coronary atherosclerotic lesions that otherwise depicted clear positive signals for total MRLC and high content in apo-B.

MRLC-phosphorylation is mainly influenced by the opposing activities of MLCK-kinase (MLCK) and Rho-kinase (ROCK), and the corresponding phosphatases. In VSMC, agLDL completely preclude the effect of ML-9 (inhibitor of MLCK) on MRLC-phosphorylation, whereas Rho-kinase inhibition reduces MRLC-phosphorylation either in the absence or in the presence of agLDL. Taken together, the present results suggest that agLDL induce MRLC-desphosphorylation mainly interfering with the MLCK-pathway.

In the actin cytoskeleton, MRLC generates motive forces required for cell attachment, spreading, and locomotion. We show here that incubation of VSMC with agLDL affects F-actin organization and decreases cell attachment, which might account in part for the lower migration rate in the LDL-treated VSMC.

Both nLDL and agLDL can affect MRLC expression and subcellular distribution. However, the modified LDL forms are usually found in the vessel wall in close contact with smooth muscle cells. We here report a significant effect of agLDL on MRLC phosphorylation and wound repair suggesting that agLDL may likely have a major functional effect on the formation of the atherosclerotic plaque. Indeed, in VSMC, LDL up-regulate the expression of LR1P1, receptor for agLDL, and down-regulate the expression of LDLR. Therefore, LR1P1 internalizes significant amounts of cholesteryl esters from agLDL contributing to the transformation of VSMC into lipid loaded cells. Interestingly LR1P1 is both up-regulated by high-circulating levels of plasma LDL and by extracellular matrix retained LDL in human atherosclerotic lesions.

From a mechanistic standpoint, the fact that both nLDL and agLDL exert inhibitory effects on human VSMC migration, while they are internalized by different receptors (LDLR and LR1P1, respectively) and internalization pathways, seems to indicate that part of the observed functional effects are due to accumulation of cholesteryl esters. Indeed, oxidatively modified LDL, which are internalized through the scavenger receptor in macrophages, strongly inhibit the chemotactic responses of mouse resident peritoneal macrophages and either oxidized or aggregated LDL have been described to change the organization of the F-actin cytoskeleton and decrease the ability of J774A.1 macrophage-like cells to generate locomotor forces. Recent studies in inflammatory cell lines relate cellular responses to increases in membrane cholesterol levels and cholesterol loading to a decrease on phosphorylation of the myosin light chain.

In our study, particular care was taken for the preparation of LDL to avoid pathway crossovers. By ELISA-based assay, we excluded oxidative modification of LDL. In a similar experimental setting, Locher et al. did not observe oxidation-dependent changes in LDL mobility using a gel electrophoresis assay after 24 h of incubation with human VSMC.

From the present study, we cannot discriminate whether some cell proliferation occurring at the wound edge could also contribute to the repair process. However, previous studies on the mitogenic effect of LDL in VSMC would suggest that migration rather than proliferation is inhibited by LDL in the wound repair assay. As a necessary further step in the validation of these data we need, and we are presently working on it, the proof of concept of intervening in an animal model of atherosclerosis and vascular remodelling.

In summary, human LDLs inhibit migration and impair wound repair capacity of human coronary VSMC by modulating effects on phosphorylation and subcellular localization of MRLC. Our present findings provide new insights to explain mechanisms by which lipid deposits and more specifically LDL retained in the intima, which becomes aggregated, contribute to the development of soft-high-risk plaques with decreased VSMC accumulation. Actomyosin activity in VSMCs seems to be a key element in remodelling, progression of atherosclerosis, and vascular repair.

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