LDL from active SLE patients is more atherogenic to endothelial cells than LDL from the same patients during remission

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Abstract

Introduction. Systemic lupus erythematosus (SLE) patients have an enhanced risk of atherosclerosis and cardiovascular disease (CVD). However, the increased prevalence of CVD is not fully explained by traditional Framingham cardiovascular risk factors. Specific features of low-density lipoprotein (LDL) particles, other than plasma concentration, may induce accelerated atherosclerosis at early stages in these patients. Thus, we aimed to explore the impact of LDL from both active and inactive SLE patients on human aortic endothelial cells (HAECs)

Methods. Human aortic endothelial cells (HAECs) were stimulated with the same concentration of LDL particles isolated from pooled serum that was collected from 13 SLE patients during both active and inactive states. Gene expression and cell migration assays were performed.

Results. Circulating LDL particles were comparable among those obtained from healthy volunteers and those obtained from SLE patients in both remission and flare states in terms of number, cholesterol and triglyceride content, and net electrical charge. Stimulation of cells with LDL from active SLE patients induced the expression of VCAM1 (~2.0-fold, $p<0.05$), MCP1 (~2.0-fold, $p<0.05$) and MMP2 (~1.6-fold, $p<0.01$) compared with that of cells stimulated with LDL from inactive SLE patients. Additionally, LDL extracted from active patients increased cell migration in a wound-healing assay (1.4-fold, $p<0.05$).

Conclusions. Our data show that, at the same LDL concentration, LDL from active SLE patients had increased proatherogenic effects on endothelial cells compared with those of LDL from the same patients when in an inactive or remission state.

Key words: SLE, LDL, CVD, endothelial cells.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease that mainly affects women of childbearing age [1]. SLE is commonly characterized by periods of remission (inactive) and exacerbation (active or lupus flares) with prolonged periods of subclinical activity. Flares can be described as 'a measurable increase in disease activity in one or more organ systems involving new or worse clinical signs and symptoms and/or laboratory measurements' [2]. Disease flares often consist of episodes of arthritis or skin lesions but also can affect any vital organ causing serious damage and leading to an increased risk of premature death. Subclinical activity may also be present. Thus, the control of disease activity is important for the management of patients with SLE, mainly since the leading cause of mortality in SLE patients is cardiovascular disease (CVD) [3]. It is estimated that the overall risk of CVD in these patients ranges from 10- to 50-fold that of the general population [4, 5]. Increasing evidence points to accelerated atherosclerosis at early stages as the underlying cause of this augmented risk [6]. However, the plasma biochemical lipid profile in SLE patients is barely altered [7], and the increased prevalence of CVD cannot be fully explained by the traditional factors included in the Framingham risk score [4, 6, 8, 9]. Therefore, additional lipoprotein features, such as particle number, size, density, or net charge, among others, could explain this residual risk, as in the case of other conditions with significant residual risk, such as type 2 diabetes mellitus. Previous studies from our group found that small, medium-small and very small low-density lipoproteins (LDL) were positively associated with carotid intima-media thickness (cIMT) in normolipemic SLE patients [10]. Additionally, LDL net charge is inversely correlated with cIMT in these patients, regardless of its concentration or particle number [11]. However, the mechanisms by which these lipoproteins contribute to the development of atherosclerosis in SLE patients are not completely known.

Increasing evidence has indicated that impaired endothelial function leads to atherosclerosis progression in SLE patients [12]. SLE-associated autoantibodies directly promote an inflammatory response, increasing endothelial permeability. However, whether LDL directly impacts endothelial function in SLE patients is not fully known.

As results from different studies indicate that LDL from SLE patients has different size and charge characteristics, we hypothesize that LDL isolated from these patients may have a different effect on the endothelium depending on the activity of the disease, with a more pronounced effect in active SLE patients. To test this hypothesis, we studied the

impact of LDL from both inactive and active SLE patients on human aortic endothelial cell (HAEC) gene expression and migration.

Methods

LDL origin and isolation

LDL was isolated from pooled serum from thirteen fasted SLE female patients (in both active and inactive status) who were recruited from the systemic autoimmune diseases unit of the Hospital Universitari Sant Joan de Reus and fulfilled at least four classification criteria from the American College of Rheumatology [13].

LDL from pooled serum of five fasted healthy female volunteers was obtained as a control. These controls did not present any cardiovascular risk factors.

None of the participants of the study were receiving lipid-lowering agents, and each provided informed consent to participate in the study. The study was approved by the local ethics committee (Hospital Universitari de Sant Joan de Reus) and was performed in full compliance with the Declaration of Helsinki.

LDL was obtained from pooled serum by sequential ultracentrifugation (170000 g, 8°C) in increasing density solutions (ranging from 1.019 to 1.063 g/mL) in an Optima XPN-100 ultracentrifuge (Beckman Coulter) with a Kontron 45.6 fixed-angle rotor as previously described [11]. The LDL fraction was dialyzed overnight against phosphate-buffered saline at a pH of 7 at 8°C [11]. LDL was sterilized by filtration through a low protein-binding durapore filter (Millex-GV, Millipore) and stored at 4°C in the dark.

LDL characterization

The level of cholesterol (cholesterol assay kit, Spinreact) and triglycerides (triglyceride assay kit, Spinreact) in LDL were determined by colorimetric assays. The number of LDL particles was estimated by quantifying apolipoprotein B100 (ApoB100) by using an immunoturbidimetric assay with specific antibodies (ApoB100 assay kit, Spinreact). These analyses were adapted to a Cobas Mira Plus auto-analyser (Roche Diagnostics). LDL net charge was measured by using a Zetasizer Nano-ZS (Malvern Instruments) as previously described [11].

Cell culture

Human aortic endothelial cells (HAECs) were obtained from Cascade Biologics™ (Invitrogen Life Technologies). The cells were cultured in M200 medium (Invitrogen Life Technologies) supplemented with low-serum growth factor supplement (LSGS) containing 2% foetal bovine serum, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor and 10 µg/ml heparin at 37 °C and 5% CO_2. The cells were seeded in multi-well plates and serum deprived for 24 hours in Medium 200 supplemented with 0.1% FBS in the absence of growth factors and

stimulated with increasing concentrations of LDL (expressed in ng/mL of ApoB100) from SLE patients (in regression or flare states) for 4 (RNA) or 24 (wound healing) hours. Cellular toxicity was assessed by analysing lactate dehydrogenase (LDH) release using a cytotoxicity detection kit (Roche Diagnostics). After incubation, RNA was extracted as described below.

Wound-healing assay

Confluent plates were injured with a single scratch by removing the attached cells with a sterile pipette tip. Then, the cells were stimulated for 24 hours with 100 mg/dL LDL from SLE patients (in regression and in flare states) to allow cell migration to repair the wound. Pictures were taken at 0, 1, 2, 3, 4, 6, 8, and 24 hours using a microscope (Olympus IX71), and the migration rate was calculated using Cell^F imaging software (Olympus). The area under the curve (AUC) was determined as the measure of wound closure.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

mRNA levels were assessed by real-time RT-PCR as previously described [14, 15]. Total RNA was isolated using the PureLink® RNA mini kit (Invitrogen, Life Technologies) according to the manufacturer's recommendations. RNA (0.5 µg) was reverse-transcribed using random hexamers and SuperScript II (Invitrogen Life Technologies, UK) according to the manufacturer's protocol. mRNA levels were assessed by real-time PCR on a LightCycler® 96 System (Roche Life Science). TaqMan predesigned assays-on-demand (Applied Biosystems) were used for human matrix metallopeptidase 2 (MMP2) (Hs00234422_m1), human endothelial nitric oxide synthase (eNOS) (Hs00167166_m1), human vascular cell adhesion molecule 1 (VCAM1) (Hs00365485_m1) and human monocyte chemoattractant protein 1 (MCP1) (Hs00234140_m1). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) was used as the endogenous control.

Statistical analysis

The data are expressed as the mean ± standard deviation (SD) of 3 separate experiments performed in duplicate. Significant differences were established by Student's t-test or one-way ANOVA, according to the number of groups compared. Differences were considered statistically significant at $p < 0.05$. The data were analysed by using SPSS software (IBM SPSS Statistics, version 22.0).

Results

Baseline characteristics of participants

We separated the LDL particles from 13 inactive SLE patients, from the same 13 patients during a flare, and from 5 healthy controls.

We first compared anthropometric data at baseline and the characteristics of the pooled lipoproteins obtained from SLE patients in both inactive and active status with those from healthy volunteers. Healthy participants tended to be younger. LDL pools from healthy volunteers and both inactive and active SLE patients were comparable regarding the cholesterol content, triglycerides and the net charge. Serum from active patients tended to have more LDL particles, as assessed by apolipoprotein B100.

LDL from SLE patients does not induce cytotoxicity in HAECs

HAECs were stimulated with increasing concentrations of LDL particles (calculated as increasing ApoB100 concentrations) from both inactive and active SLE patients, and cellular toxicity was assessed by measuring LDH in cell supernatants. No changes were found in LDH release in cells stimulated with LDL from the different origins (Supplementary Figure 1).

LDL isolated from SLE patients during a flare enhances the effects of LDL-mediated regulation of gene expression in endothelial cells.

Next, we explored the effect of LDL in regulating the expression of several genes that are involved in endothelial dysfunction (Supplementary Figure 2). LDL particles from inactive SLE patients (A) reduced eNOS expression in endothelial cells at both concentrations (-66%, $p<0.001$ at 50 ng/mL; and -69%, $p<0.001$ at 100 ng/mL). LDL from inactive SLE patients induced VCAM1 and MCP1 expression but only at 100 ng/mL (4.8-fold, $p<0.05$ and 3.3-fold, $p<0.05$, respectively). LDL from active patients (B) did not affect eNOS expression, and only LDL at 100 ng/mL induced VCAM1 and MCP1 expression (4.7-fold, $p<0.01$, and 4.1-fold, $p<0.001$, respectively).

Because of the increased expression of some genes (i.e., VCAM1 and MCP1) in response to 100 ng/mL LDL, we chose this concentration for further experiments.

To explore whether a flare contributes to the enhanced risk of CVD, we compared the effect of LDL from inactive and active SLE patients. LDL from active SLE patients induced the expression of VCAM1 (~2.0-fold, $p<0.05$; Figure 1B), MCP1 (~2.0-fold, $p<0.05$; Figure 1C) and MMP2 (~1.6-fold, $p<0.01$; Figure 1D), without altering the mRNA levels of eNOS (Figure 1A), compared to those of LDL from inactive SLE patients.

LDL from active SLE patients exacerbates LDL-induced HAEC migration in a wound-healing assay.

To explore whether the changes in gene expression induced by LDL from active SLE patients were related to disturbances in endothelial cell migration, a wound-healing assay was performed. As shown in Figure 2, LDL from active SLE patients exacerbated LDL-induced HAEC migration (~1.4-fold, $p<0.05$; Figure 2).

Discussion

Despite the evidence supporting accelerated and premature atherosclerosis as the main cause for increased CVD risk in SLE patients [6], little is known about the molecular basis underlying these processes in these patients. To better understand this phenomenon, we hypothesized that LDL isolated from SLE patients may have a different effect on the endothelium depending on the active or inactive status of the disease. In the present study, we provide evidence that the same concentration of LDL, with comparable particle concentration, size and electrical charge, produces an increased atherogenic response in endothelial cells when extracted from active SLE patients.

Since endothelial dysfunction is one of the most relevant participants in the atheromatous process [16], we first challenged endothelial cells with equal amounts of LDL from inactive and active SLE patients, as determined by the ApoB100 concentration.

We analysed the impact of LDL from our experimental groups on the expression of key genes involved in endothelial dysfunction: eNOS, MCP1, VCAM1 and MMP2. LDL from both inactive and active SLE patients reduced the mRNA levels of eNOS and induced the expression of both VCAM1 and MCP1 compared to those of unstimulated cells. Interestingly, there were no changes in the expression of MMP2, suggesting that LDL acts in a specific manner on certain endothelial processes.

It is well known that endothelial dysfunction is closely related to reduced eNOS activity and a loss of nitric oxide production [17]. Nitric oxide regulates several atheroprotective processes, including the inhibition of adhesion molecule expression and monocyte adhesion to the endothelial monolayer [16, 17]. Therefore, downregulation of eNOS expression during endothelial injury may be related to overexpression of chemokines and adhesion molecules, such as MCP1 and VCAM1, thereby promoting inflammatory cell attraction to the endothelium [18]. Interestingly, vascular inflammation has been shown to promote MMP expression, directly contributing to vascular remodelling during atherosclerotic lesion formation [19].

Furthermore, since LDL from active SLE patients induced the expression of MCP1 and VCAM1 compared to that of LDL from inactive SLE patients, our results suggest an augmented inflammatory response and enhanced monocyte and lipoprotein recruitment. Additionally, MMP2 expression was further induced in cells that were challenged with

LDL from active SLE patients, indicating augmented vascular remodelling during flare status.

Endothelial cells play a pivotal role in regulating angiogenesis in atherosclerotic plaques. This process is associated with the activation of endothelial cell migration and proliferation to create new vessels inside the injured areas. Interestingly, MMP expression has been found to be increased in wound healing processes [20]. According to the role of LDL from active SLE patients upregulating MMP2 expression, these particles exacerbated endothelial cell migration compared to LDL from inactive SLE patients in a wound-healing assay, thereby showing an enhanced physiological response of these lipoproteins aimed to neo-vascularize the atherosclerotic lesions under active disease conditions.

Taken together, our data suggest that LDL from active SLE patients favours atherosclerotic processes and CVD in these patients. Specifically, our data suggest that accelerated atherosclerosis is enhanced during flare episodes. Previous studies suggested that the relevant role of LDL in atherosclerotic pathology in SLE patients is explained by characteristics other than its blood concentration, namely, size, density [8] or net charge [13], that have been associated with cIMT in SLE patients. However, the observed effect does not seem to be due to the LDL lipid content, number of particles (assessed by ApoB100 content), or net electrical charge, and so there are obviously other features that may help explain the increased atherogenicity of LDL from SLE patients.

Although this study shows for the first time that there is an effect of LDL from active SLE patients on endothelial cell activation, further studies are necessary to fully elucidate the features of LDL derived from active SLE patients that contribute to the atheromatous process.

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Conflict of interest

No conflicts of interest have been declared.

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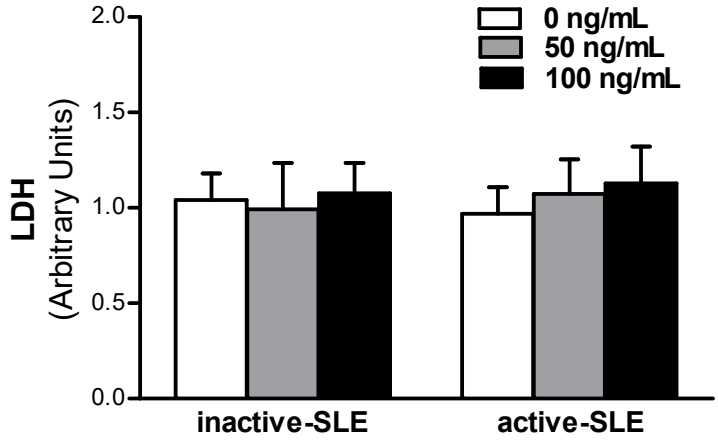
Supplementary Figure 1. LDL from SLE patients does not induce cytotoxicity in HAECs.
HAECs were stimulated with 50 and 100 ng/mL LDL from both inactive and active SLE patients for 4 hours. The absence of cellular toxicity was confirmed by the LDH levels in cell supernatants.

Supplementary Figure 2. LDL regulates the expression of several genes that are involved in endothelial dysfunction.
HAECs were stimulated with 50 and 100 ng/mL LDL from inactive (A) and active (B) SLE patients, and the mRNA levels of eNOS, VCAM1, MCP1 and MMP2 were assessed by real-time RT-PCR. The data were normalized to GAPDH mRNA levels and are expressed as the mean ± SD. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs*. 0 ng/mL).

Figure 1. LDL from active SLE patients has an enhanced capacity to regulate gene expression in endothelial cells compared to that of LDL from inactive SLE patients.
HAECs were stimulated with 100 ng/mL LDL from SLE patients in inactive or active states, and the mRNA levels of eNOS (A), VCAM1 (B), MCP1 (C) and MMP2 (D) were assessed by real-time RT-PCR. The data were normalized to GAPDH mRNA levels and are expressed as the mean ± SD. (*$p < 0.05$, **$p < 0.01$ *vs*. inactive SLE).

Figure 2. LDL from active SLE patients exacerbates LDL-induced HAEC migration in a wound-healing assay.
Confluent cultures of HAECs were wounded by scraping with a sterile pipette tip and then stimulated with 100 ng/mL LDL from inactive and active SLE patients. (A) Representative pictures of cell migration to close the wound. (B) Quantification of wound closure at different times. (C) Area under the curve (AUC) of wound closure is expressed as the mean ± SD. (*$p < 0.05$ *vs*. inactive SLE).

Supplementary Figure 1.



Supplementary Figure 2.

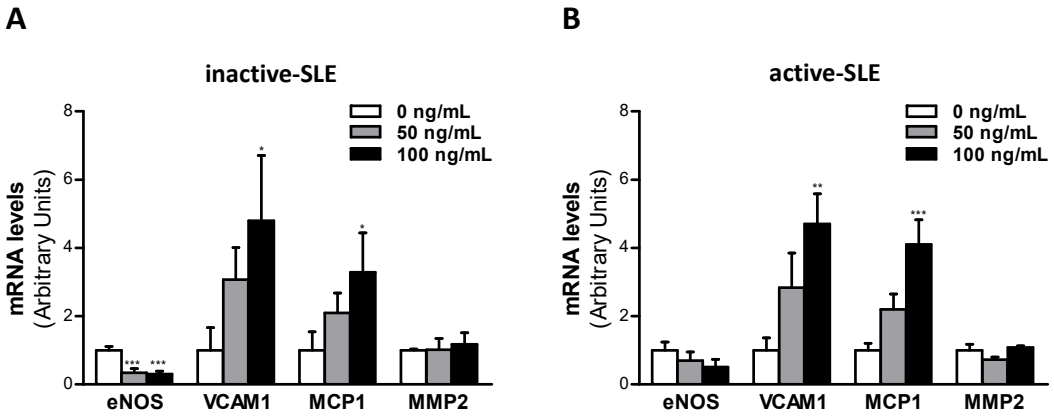


Figure 1.

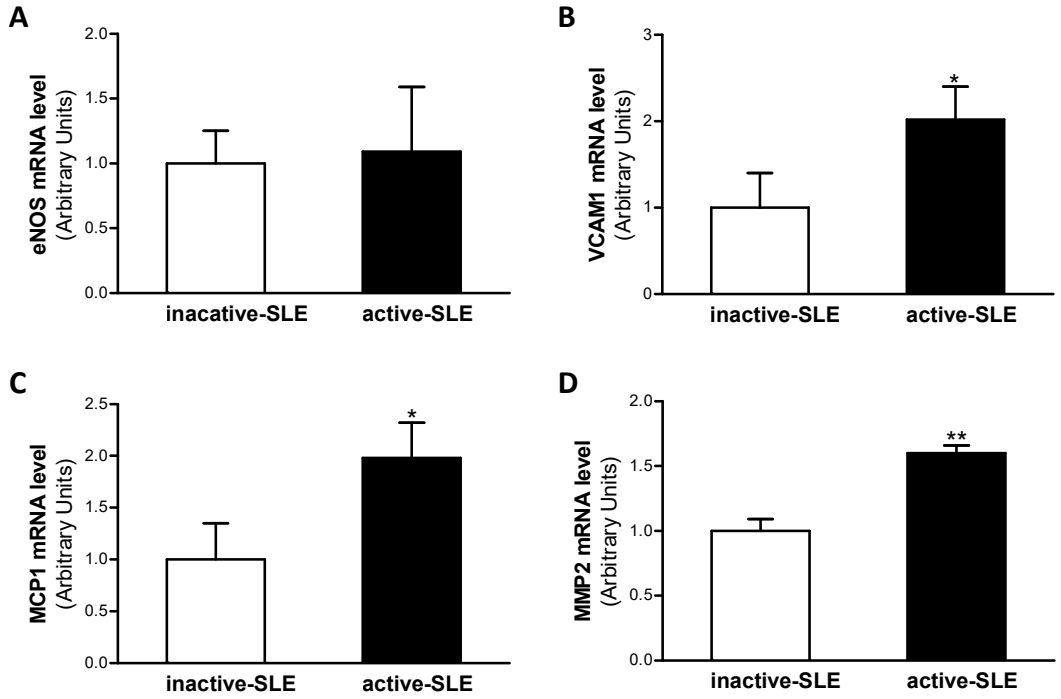

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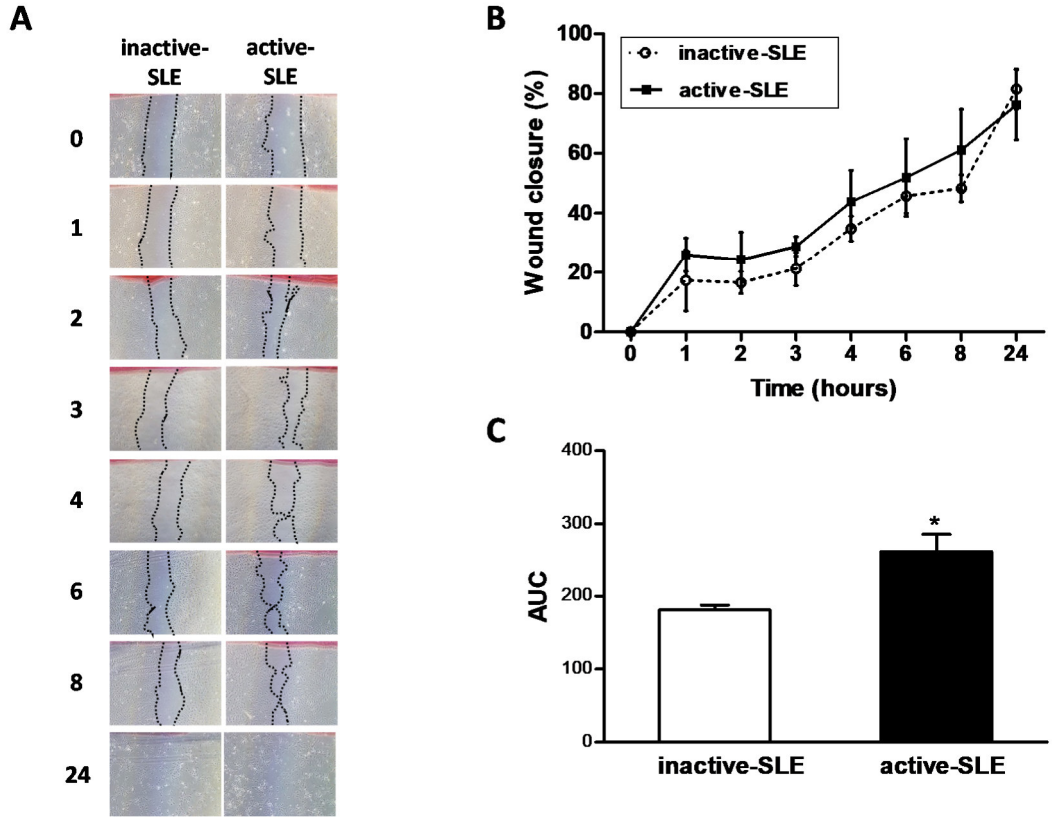
Figure 2.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Wound closure (%)
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Time (hours)
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