Carbamylated Low-Density Lipoprotein Induces Monocyte Adhesion to Endothelial Cells Through Intercellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1

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Objective—Carbamylated low-density lipoprotein (LDL), the most abundant modified LDL isofrom in human blood, has been recently implicated in causing the atherosclerosis-prone injuries to endothelial cells in vitro and atherosclerosis in humans. This study was aimed at testing the hypothesis that carbamylated LDL acts via inducing monocyte adhesion to endothelial cells and determining the adhesion molecules responsible for the recruitment of monocytes.

Methods and Results—Exposure of human coronary artery endothelial cells with carbamylated LDL but not native LDL caused U937 monocyte adhesion and the induction of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 adhesion molecules as measured by cell enzyme-linked immunosorbent assay. Silencing of intercellular adhesion molecule-1 by siRNA or its inhibition using neutralizing antibody resulted in decreased monocyte adhesion to the endothelial cells. Similar silencing or neutralizing of vascular cell adhesion molecule-1 alone did not have an effect but was shown to contribute to intercellular adhesion molecule-1 when tested simultaneously.

Conclusions—Taken together, these data provide evidence that intercellular adhesion molecule-1 in cooperation with vascular cell adhesion molecule-1 are essential for monocyte adhesion by carbamylated low-density lipoprotein-activated human vascular endothelial cells in vitro. (Arterioscler Thromb Vasc Biol. 2007;27:826-832.)

Key Words: atherosclerosis ■ carbamylated low-density lipoprotein ■ endothelial cells ■ intercellular adhesion molecule-1 ■ monocyte adhesion ■ vascular cell adhesion molecule-1

Monocyte (leukocyte) adhesion to activated vascular endothelial cells and their migration into the vessel wall is the critical event in the initiation of atherosclerosis. This process is caused by the upregulation of adhesion molecules on endothelial cells and an increased expression in the vascular wall of chemotactic factors to monocytes. Highly specific adhesive interactions between monocytes and endothelial cells are mediated by 3 main families of receptors: members of the immunoglobulin superfamily, selectins, and integrins. Recent studies indicate that intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), members of the immunoglobulin superfamily, are the most common participants in monocyte attraction induced by different stimuli. The expression of adhesion molecules and monocyte adhesion can be triggered by a variety of plasma components, such as interferon-γ, homocysteine, and lipoproteins, like oxidized low-density lipoprotein (oxLDL), or its derivatives, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine and lysophosphatidylcholine. The identification of new plasma components that are responsible for monocyte adhesion is important in light of their potential application as pathogenic atherosclerosis markers, predictors or therapeutic targets.

Carbamylated low-density lipoprotein (cLDL) is a recently identified type of modified low-density lipoprotein (LDL) that seems to be important in atherosclerosis in humans. It is generated by irreversible chemical modification of the protein component of the LDL particle, apolipoprotein B, and by urea-derived cyanate present in human blood plasma. Plasma levels of cLDL as determined by sandwich enzyme-linked immunosorbent assay (ELISA) methods vary in a higher range of concentrations than oxLDL, which makes it the most abundant LDL isofrom of human plasma. The elevation of blood urea in uremic patients causes a proportional increase of plasma cLDL. The LDL of chronic renal failure patients on dialysis was shown to induce greater monocyte–endothelial cell adhesion. A higher level of plasma cLDL correlates with atherosclerosis in uremic patients. Our recent study showed that cLDL induced a variety of effects on monocyte adhesion.
of damaging stimuli to endothelial and vascular smooth muscle cells, all of which are attributed to atherosclerosis.\textsuperscript{10,14} These include incorporation by endothelial cells, cytotoxicity toward endothelial cells, and the induction of vascular smooth muscle cell proliferation.

The present study was undertaken to determine whether monocyte adhesion to endothelial cells can be induced by cLDL and to identify the adhesion molecules involved in this process. This in vitro study utilizes U937 monocyte cells and human coronary artery endothelial cells (HCAECs), commonly used for studies of monocyte adhesion.\textsuperscript{3,16} We demonstrated that cLDL induces ICAM-1 and VCAM-1 on endothelial cells, and the role of these adhesion molecules in the attraction of monocytes in vitro. Inactivation of these pathways by RNA interference or inhibiting the antibody strongly protected against the adhesion of monocytes to endothelial cells, thus providing evidence for the potential importance of these mechanisms in cLDL-mediated atherosclerosis.

**Materials and Methods**

An expanded materials and methods section is available online (please see http://atvb.ahajournals.org).

**Native, cLDL, and oxLDLs**

Native human LDL (nLDL) and all chemicals were purchased from Sigma (St. Louis, Mo) unless designated otherwise. The cLDL was prepared as previously described by us.\textsuperscript{10,11} The oxLDL was prepared as described by Kume et al\textsuperscript{15} and used as a positive control.

**Monocyte Adhesion**

The U937 cells were chosen because they have the phenotype of the monocytes and are commonly used in monocyte adhesion studies.\textsuperscript{3,5,6} Monocyte adhesion was determined as described by Koga et al.\textsuperscript{16}

Monocyte adhesion to cLDL-activated HCAECs under flow conditions was performed similarly to methods described elsewhere.\textsuperscript{17}

**Cell ELISA**

Cell ELISA was performed as described by Frahm et al.\textsuperscript{18} Antibody titers and optimal reaction conditions were elaborated before the experiment.

**Immunocytochemistry**

Immunocytochemical staining of LDL-treated cells with polyclonal anti–ICAM-1 (1:100) or anti–VCAM-1 (1:100) antibody (Santa Cruz Biotechnology) was performed similarly as that described by Langer et al.\textsuperscript{19}

**ICAM-1 and VCAM-1 siRNA Silencing**

For the study of protection from monocyte adhesion, HCAECs were transfected with siRNA to ICAM-1, VCAM-1, or both for 48 hours, then the transfection medium was removed, and the cells were treated with cLDL or vehicle in serum-free medium for 24 hours.

**RNA Extraction and Real-Time Reverse-Transcription Polymerase Chain Reaction**

The total RNA was extracted using a RNeasy Mini kit from Qiagen (Valencia, Calif) as suggested by the manufacturer. The reverse-transcription reaction was performed using the GeneAmp Gold RNA PCR core kit (Applied Biosystems, Foster City, Calif) using Oligo (d)\textsubscript{T}16. The reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen Corporation, Carlsbad, Calif) according to manufacturer recommendations.

**Statistical Analysis**

The results were expressed as mean±SEM. The statistical analysis was performed using SPSS software (SPSS Inc, Chicago, Ill). To evaluate the significance of differences between the 2 groups of experiments, the ANOVA and Student t test were used. Additionally, to evaluate the significance of several time points in comparison to one control point, the Bonferroni adjustment of the t test was used. A value of \( P<0.05 \) was considered statistically significant.

**Results**

**Acceleration of Monocyte Adhesion to Endothelial Cells by cLDL**

To determine whether cLDL causes monocyte adhesion to endothelial cells, cLDL (200 \( \mu \)g/mL) was applied to the
endothelial cells for different periods of time and BCECF-AM–labeled U937 cells were allowed to adhere for 30 minutes. We found a significant increase of monocyte adhesion to the endothelial cells treated for 12 hours or longer with cLDL, whereas nLDL and the vehicle control did not cause any monocyte adhesion (Figure 1). The oxLDL that was previously described to induce monocyte adhesion,6,20,21 was used as a positive control. A 6-hour or longer treatment with oxLDL (200 μg/mL) caused a 4-fold increase of monocyte adhesion to the endothelial cells. It is interesting that once monocyte adhesion to oxLDL-treated endothelial cells reached the maximum, no further increase of adhesion was observed. Contrary to oxLDL, cLDL caused significant monocyte adhesion versus nLDL and vehicle control only after a 12-hour course, followed then by a further increase of monocyte adhesion over the level of oxLDL. At the end of the 24-hour course, the adhesion induced by cLDL was higher than the one induced by oxLDL.

Our next step experiment showed that freshly isolated human monocytes also have higher rate of adherence to endothelial cells pretreated with both cLDL and oxLDL (Figure 2A, 2B). Further, to see whether modified LDL-activated endothelial cells still attract monocytes under flow conditions, the adhesion experiments using laminar flow chambers were performed (Figure 2C, 2D). Our results demonstrate that similar to static adhesion experiments with both U937 cells and freshly isolated monocytes, HCAECs treated with modified LDLs attract more monocytes than vehicle-treated or nLDL-treated cells.

cLDL Induces ICAM-1 and VCAM-1 Expression in Endothelial Cells

We studied the expression of adhesion molecules, ICAM-1, VCAM-1, and P-selectin, and a chemokine, MCP-1, which could potentially mediate cLDL-induced monocyte adhesion to endothelial cells. HCAECs were treated with varying concentrations of cLDL or nLDL for 24 hours, and the expressions of adhesion molecules were determined using cell ELISA (Figure 3). The data showed that the expression of MCP-1 and P-selectin was not induced by the treatment with cLDL in comparison to nLDL. However, the expression of ICAM-1 and VCAM-1 was significantly increased by cLDL. ICAM-1 was induced to a higher degree than VCAM-1. Contrary to cLDL, oxLDL did not affect ICAM-1 and VCAM-1 expression, whereas expressions of P-selectin and, to a lesser extent, MCP-1 molecules were increased. The
endothelial cells, 2 approaches were applied. In the first, we abolished the function of ICAM-1 and VCAM-1 with antibodies, and in the second, the expression of these adhesion molecules was silenced by using siRNA.

Because of the superficial location of adhesion molecules in the endothelium, the antibodies to these proteins are widely used to determine the role of adhesion molecules in endothelial cells.23-27 Our experiments showed that the inhibition of ICAM-1 caused a significant ~30% reduction of monocyte adhesion to endothelial cells, whereas the inhibition of VCAM-1 had only a minor and nonsignificant effect (Figure 5). In the same experiment, simultaneous pretreatment of endothelial cells with both anti–ICAM-1 and anti–VCAM-1 antibodies caused the most significant inhibition of monocyte adhesion (~60%). Rabbit γ-immunoglobulins used as a negative control did not have a significant effect on monocyte adhesion regardless of the treatment.

Before using RNA interference, we determined the optimal conditions for siRNA transfection using siRNA–fluorescein isothiocyanate and specific siRNA, because HCAECs are notoriously difficult cells to transfect.23 These experiments showed that the optimal conditions for siRNA transfection to the endothelial cells are 60% to 70% confluence with a transfection time of no more than 48 hours. These conditions allowed reaching a transfection level of up to 70% to 80% (supplemental Figure IIA). The introduction of the specific siRNA resulted in a significant inhibition of ICAM-1 or VCAM-1 expression after cLDL treatment as measured by real-time reverse-transcription polymerase chain reaction. HCAECs were treated with 200 µg/mL cLDL for 16 hours in 6-well plates. Control cells were treated with vehicle or 200 µg/mL nLDL. Absolute C(t) data varied in the ranges of 17.2 to 17.8 cycles and 18.1 to 21.5 cycles for 18s and ICAM-1 or VCAM-1, respectively. n = 3 per point, *P < 0.05, **P < 0.01, ***P < 0.001 vs. same treatment at 0 hour; #P < 0.05, ##P < 0.01 vs. nLDL-treated cells at the same time point.

ICAM-1 and VCAM-1 Mediate cLDL-Induced Monocyte Adhesion to Endothelial Cells

To determine whether cLDL-induced ICAM-1 and/or VCAM-1 overexpression cause the adhesion of monocytes to

nLDL did not cause any significant change of either ICAM-1 or VCAM-1 expression.

The results of cell ELISA regarding ICAM-1 and VCAM-1 expression after cLDL treatment was confirmed by immunocytochemistry: cLDL-treated HCAECs expressed significantly more ICAM-1 and VCAM-1 in comparison to the vehicle control or nLDL-treated cells (Figure 4). To determine whether the observed induction of ICAM-1 and VCAM-1 is present at the level of transcription, real-time PCR was performed with RNA extracted from the vehicle-treated, cLDL-treated (200 µg/mL), or nLDL-treated (200 µg/mL) endothelial cells. Our results suggest that the transcription of both studied molecules was upregulated at 4 and 8 hours (supplemental Figure I, available online at http://www.atvb.ahajournals.org). The mRNA expression of both molecules was decreasing at later time points.

ICAM-1 and VCAM-1 Mediate cLDL-Induced Monocyte Adhesion to Endothelial Cells

To determine whether cLDL-induced ICAM-1 and/or VCAM-1 overexpression cause the adhesion of monocytes to
VCAM-1 expression in modified LDL-treated cells as determined using real-time reverse-transcription polymerase chain reaction (supplemental Figure IIB).

After the application of siRNAs to the HCAECs for 48 hours, the cells were treated for an additional 16 hours with vehicle, cLDL, or nLDL and monocyte adhesion was measured as described in the Methods section. The data presented in Figure 6 show that cLDL caused accelerated monocyte adhesion to endothelial cells, and it was significantly suppressed by anti-ICAM-1 siRNA. Anti–VCAM-1 siRNA had only a partial effect while simultaneously using both anti–ICAM-1 and anti–VCAM-1 siRNAs caused the most prominent and significant suppression of monocyte adhesion. It did not reach the level of cells treated with the vehicle or nLDL, but it was consistent with the transfection efficiency observed in this experiment. Although nLDL did not accelerate monocyte adhesion, it was slightly suppressed by specific siRNAs. The control siRNA did not protect the endothelial cells from monocyte adhesion. These experiments provide evidence that ICAM-1 in cooperation with VCAM-1 is involved in monocyte adhesion by cLDL-activated human endothelial cells in vitro.

Discussion

This study for the first time to our knowledge determined that cLDL induces the adhesion of monocytes to human vascular endothelial cells in vitro and identified the adhesion molecules, which are important in this process. The cLDL was demonstrated to induce ICAM-1 and VCAM-1 and had no effect on MCP-1 or P-selectin. These 4 molecules and some others were described before to be involved in the monocyte adhesion to the endothelium.24 As opposed to cLDL, oxLDL, the most studied modified LDL, was shown to induce the expression of P-selectin25 and MCP-1.26 There is conflicting evidence regarding the involvement of ICAM-1 and VCAM-1 in oxLDL-induced monocyte–endothelial adhesion. While some studies showed that oxLDL may cause the
induction of ICAM-1 and VCAM-1 expression and monocyte adhesion,\(^2^1,2^7\) others suggested that oxLDL does not induce VCAM-1\(^2^8\) or either one.\(^2^6,2^9\) In support of this, Khan et al observed no change in ICAM-1 or VCAM-1 expression as measured by ELISA of cultured HAECS or human umbilical vein endothelial cells that were incubated with oxLDL or glycated LDL.\(^3^0\) Our data suggest that oxLDL action is not mediated by ICAM-1 or VCAM-1. Therefore, our subsequent experiments were aimed to study cause–effect relationship between cLDL-induced expression of ICAM-1 and VCAM-1 and monocyte adhesion. We have shown that unlike the other modified LDLs, cLDL induces ICAM-1 and VCAM-1 in a dose-dependent manner. The protein increase of ICAM-1 and VCAM-1 was strongly associated with the mRNA increase and monocyte adhesion. Therefore, there is a possibility that cLDL is a more potent inducer of monocyte adhesion than other modified LDLs.

Both ICAM-1 and VCAM-1 protein expressions were increased after cLDL treatment in 16 to 24 hours, whereas the mRNAs of both molecules reached a maximum at the 8-hour point. In endothelial cells treated with lipoproteins, ICAM-1 and VCAM-1 mRNAs were previously shown to be induced within 2 to 8 hours after the impact and then to be downregulated, whereas the proteins are usually induced later, at 8 to 48 hours after treatments.\(^3^1,3^2\) Our results suggested that after cLDL treatment, the VCAM-1 expression was increased to a lesser degree than the expression of ICAM-1. These data are in agreement with other studies suggesting higher reactivity of ICAM-1 after exposure to exogenous factors, including LDL.\(^6,3^0,3^1\) Monocyte recruiting is a multistep process that consists of capture, rolling, activation, adhesion, and transmigration. Although the roles of ICAM-1 and VCAM-1 are known to be mainly in adhesion and transmigration, their inactivation may in some cases also affect rolling.\(^2^4\) We can speculate that cLDL is involved in all of these processes.

Using cause–effect relationship approaches by using specific siRNA or neutralizing antibodies, we found that ICAM-1 and VCAM-1 are the adhesion molecules responsible for the endothelial cell attraction of monocytes induced by cLDL. Our studies also indicate that ICAM-1 is more important for cLDL-induced monocyte adhesion than VCAM-1. Both siRNA and the utilization of neutralizing antibodies determined that silencing/neutralizing of ICAM-1 alone provided inhibition of monocyte adhesion to endothelial cells. VCAM-1 contributes to this process because the silencing or neutralizing of both molecules caused the maximal effect. To the best of our knowledge, reports on the use of specific anti-ICAM-1 or anti-VCAM-1 siRNA to study monocyte adhesion to endothelial cells are not available. Our observation of the predominance of ICAM-1 is in agreement with at least one previous study. Using the pretreatment of endothelium with anti-ICAM-1 and anti-VCAM-1 antibodies in a human ex vivo experiment, Crook et al reported that ICAM-1 participates in monocyte adhesion while the role of VCAM-1 is rather secondary and dependent on the degree of its expression in the endothelium.\(^3^3\) It is likely that cooperation between the molecules promotes monocyte adhesion. For example, monocyte adhesion induced by a high glucose concentration was inhibited by simultaneous use of anti-ICAM-1, anti–VCAM-1, and anti–CD-18 antibodies.\(^3^4\) Taken together with the previous study of cLDL cytotoxicity to HCAECs in vitro,\(^1^0\) it can be concluded that cLDL produces a variety of atherosclerotic-prone signals to endothelial cells. Some of these signals may be different from the effects of other modified LDLs.

Several experiments in this study use high concentrations of cLDL, 50 to 400 mg/L, which may raise a question whether these conditions are physiologically relevant. While several LDL modifications have been identified, only 2 of them, oxLDL and cLDL, can be precisely measured in human plasma using sandwich ELISAs. Malondialdehyde-modified LDL, an isoform of oxLDL, was detected in healthy individuals at 1.9±0.2 \(\mu g/mL\),\(^3^5\) 3.1±1.6 \(\mu g/mL\),\(^3^6\) and 17.1±50.2 \(\mu g/mL\).\(^3^7\) The oxLDL concentration was 0.5±0.3 U/\(\mu g\) or 10.8±2.8 U/mL\(^3^9\) LDL protein (1 U was 1 \(\mu g\) of mildly oxLDL). The cLDL concentration determined in our recent study showed a much higher value of 86.0±29.7 \(\mu g/mL\).\(^1^1\) It reached \(\approx 300 \mu g/mL\) in uremic patients with chronic renal failure, who are known to be predisposed to atherosclerosis. Therefore, the cLDL concentrations used in this study are physiologically meaningful.

Future studies may be focused on determining whether cLDL-induced adhesion results in increased rolling, arrest, and transmigration of monocytes.\(^4^0\) If observations from this study are confirmed in vivo, future therapeutic approaches may be aimed at the inhibition of ICAM-1 and VCAM-1 as protective measures to attenuate the progression of atherosclerosis. In this regard, using siRNA that we have tested or inhibiting the signal transduction pathways leading to induction of the adhesion molecules, for example, NF-\(\kappa B\)-activating signaling pathways,\(^4^1\) would be highly appropriate.

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Disclosure

None.

References


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Supplemental data for Apostolov et al. Carbamylated LDL induces monocyte adhesion to endothelial cells through ICAM-1 and VCAM-1

Materials and Methods

Native, carbamylated and oxidized LDLs

Native human LDL (nLDL) and all chemicals were purchased from Sigma (St. Louis, MO) unless designated otherwise. Carbamylated LDL was prepared as previously described by us \(^1\) – \(^2\). Oxidized LDL was prepared as described by Kume et al \(^3\) and used as a positive control. After modification, all LDLs were dialyzed separately against phosphate buffered saline (PBS). Carbamylation of LDL was evaluated using diacetyl monoxime as described elsewhere \(^2\). The oxidation of LDL was assessed by the thiobarbituric acid reactive substances (TBARS) assay \(^4\). The electrophoretic mobility of nLDL, cLDL and oxLDL was determined in 0.5% agarose gel supplemented with 0.2% bovine serum albumin (w/v) as described by Noble \(^5\). The LDLs were adjusted to 1 mg protein/mL with PBS containing 200 µmol/L EDTA, kept at 4°C away from light, and used within 2 weeks after preparation. If a sediment appeared during storage, it was removed by low-speed centrifugation, and only soluble fractions of the modified LDL were used for experiments. The concentration of cLDL protein did not change after the removal of the precipitate.

Cell cultures

HCAECs were supplied by Cambrex (Walkersville, MD) in passage 3 and used between passages 4 and 6. In the experiments, cells were treated with 200 mg/L of modified or native LDL in serum-free EGM-2-MV medium (Cambrex) for 1 to 24 h. The vehicle solution in the same medium was used as a control. For adhesion molecule cell ELISA, the cells were exposed to either cLDL or nLDL from 0 to 400 µg/mL for 24 hours. U937
(ATCC number CRL-1593.2) cells (ATCC, Manassas, VA) were seeded at density 50 x 10^5 cell/mL in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and cultured until they reached the density of 1-2 x 10^6 cells/mL. In some experiments, prior to the application of monocytes, HCAECs after exposure to LDLs were treated additionally with specific antibody to ICAM-1, VCAM-1 or both ICAM-1 and VCAM-1 at a final concentration of 10 ng/mL. Non-specific IgGs were used for antibody treatment control.

Human peripheral monocytes were isolated similarly to described method which is giving monocyte enrich cell population (>90%) \(^6\). Briefly, 10 ml of heparinized fresh blood from fasting normolipemic subjects was diluted 1:2 with PBS and subjected for discontinuous gradient centrifugation on Ficoll-Paque and Percoll (GE Healthcare, Uppsala, Sweden).

**Monocyte adhesion**

The U937 cells were chosen because they have the phenotype of the monocytes and are commonly used in monocyte adhesion studies \(^7-9\). Monocyte adhesion was determined as described by Koga et al. \(^10\). Briefly, endothelial cells were seeded using EBM-2-MV medium in either 96- or 6-well plates at a density of 10,000 or 200,000 cells per well, respectively. Twenty four hours later HCAECs were treated with LDL for a designated time. Immediately prior to the end of the HCAECs treatment with LDL, U937 cells were labeled with 5 μmol/l 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxy methyl ester (BCECF-AM) and the excess of the BCECF-AM was washed out with PBS containing 1% FBS (PBS-FBS). Monocytes were diluted with basal EBM-2 medium to 5x10^5 cells/mL and applied to the gently washed HCAECs (150 μL/well in a 96-well plate or 2 mL/well in a 6-well plate). The total fluorescence was measured using a Synergy-HT-I plate reader (BioTek, Winooski, VT) at 485/530 nm. Monocytes were allowed to adhere for 30 min at 37°C, washed 3 times with PBS-FBS, and the remaining
fluorescence was measured again. The background of LDL-treated or control HCAECs without monocytes was measured in separate wells and then was subtracted from the values of the experimental wells. The relative value of the remaining fluorescence was evaluated for every well.

Monocyte adhesion to cLDL-activated HCAECs under flow conditions was performed similarly to described elsewhere. Briefly, 100% confluent endothelial cells were treated with vehicle and LDLs at concentration 200 µg/mL for 16h and then mounted in the parallel plate flow chamber (Glycotech, Rockville, MD). U937 cells were prepared at density 10⁶ cell/ml in EMB-2 medium and applied to the endothelial cells at a shear stress of 1.0 dyne/cm² (4 minutes, 37°C). Experiments were visualized by phase-contrast microscopy and recorded using an Olympus IX-81 microscope (Olympus America Inc., Center Valley, PA) and HAMAMATSU ORCA-ER (Hamamatsu Photonics K.K., Hamamatsu City, Japan) camera. During the last 30 seconds of perfusion session the series of images (500 images with 8-ms interval) were grabbed from the four fields of view (0.57 mm²) and cells which remained stationary were count as adherent.

**Cell ELISA**

Cell ELISA was performed as described by Frahm et al. Antibody titers and optimal reaction conditions were elaborated prior to the experiment. The cells were seeded in a 96-well plate (10,000 cells/well) and grown in a complete media for 24 h. The cells were washed with serum-free media, permeabilized and treated with fixative (4% w/v paraformaldehyde, 0.012% saponin, PBS) for 10 min at room temperature. After fixation, the cells were washed and rehydrated in PBS and the endogenous peroxidase activity was inhibited by exposure to 0.5% hydrogen peroxide for 30 min at room temperature. Then the cells were probed with one of the following antibodies: anti-ICAM-1 (1:250), anti-VCAM-1 (1:250), anti-monocyte chemoattractant protein-1 (anti-MCP-1) (1:500) or
anti-P-selectin (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer (2% bovine serum albumin, PBS) for 2 h. After triple washing with PBST (0.05% Tween-20 in PBS), the primary antibody was detected with anti-rabbit IgG antibody (1:1000) conjugated with horse raddish peroxidase (HRP) (Santa Cruz Biotechnology). The HRP activity was measured with 3,3’,5,5’-tetramethylbenzidine substrate solution (Sigma) at delta 450-540 nm using a microplate reader. All measurements were done in quadruplicates per one marker per one cell line and were repeated at least three times in different plates. The negative controls of the primary antibody were done by their substitution with blocking buffer.

**Immunocytochemistry**

Immunocytochemical staining of LDL-treated cells with polyclonal anti-ICAM-1 (1:100) or anti-VCAM-1 (1:100) antibody (Santa Cruz Biotechnology) was performed as similarly to described by Langer et al. 13. Anti-rabbit-AlexaFluor 488 conjugate at dilution 1:500 (Invitrogen, Carlsbad, CA) was used for the detection of the primary antibodies. Slides were analyzed under an Olympus IX-81 microscope; images and acquisitions were made with a digital camera HAMAMATSU ORCA-ER and software Slidebook 4.1 (SciTech Pty Ltd., Australia).

**ICAM-1 and VCAM-1 siRNA silencing**

HCAECs were seeded in 6- or 96-well plates and grown to 60-70% confluence. To knock-down ICAM-1 and VCAM-1 mRNA, cells were transfected with siRNA duplexes or Control Non-Targeting siRNA #1 (Santa Cruz Biotechnology). The following siRNA sequences were used to silence ICAM-1: sense, 5’-GCCUCAGCAGCUGACGUGUCCUAdTdT-3’, and antisense, 3’-UAGAGGUACGUGCUCCGAGGcdTdT-5’. For VCAM-1, the cocktail of 3 siRNAs was applied: (A) sense, 5’-CCCUACCUUGAUGAGAUACUdTdT-3’,
antisense, 3'-AGUAUCUUCAUUGUAGGdTdT-5'; (B) sense, 5'-CAUGGAUAUCUGAAGAdTdT-3',
antisense, 3'-UUCUUCAGGAUUAUCAGdTdT-5'; and (C) sense, 5'-
GCAACACUCUUAUUGAdTdT-3',
antisense, 3'-UCUAAAUAUGAGUGUUGCdTdT-5'. The cells were treated with 50 nmol/L siRNA
mixed with DeliverX transfection reagent (Panomics, Fremont, CA) according to
manufacturer recommendations for 48 h. ICAM-1 and VCAM-1 mRNA expression was
measured using real-time RT-PCR of the extracted total RNA from the cells grown in 6-
well plates. For the study of protection from monocyte adhesion, HCAECs were
transfected with siRNA to ICAM-1, VCAM-1 or both for 48 h, then the transfection
medium was removed, and the cells were treated with cLDL or vehicle in serum-free
medium for 24 h.

RNA extraction and real-time RT-PCR
The total RNA was extracted using a RNeasy Mini kit from Qiagen (Valencia, CA) as
suggested by the manufacturer. The quality of RNA was determined in 1.2%
formaldehyde-agarose gel. The reverse transcription reaction was performed using the
GeneAmp Gold RNA PCR core kit (Applied Biosystems, Foster City, CA) using Oligo
d(T)16. In general, 0.5 µg of total RNA was reverse-transcribed in a 50-µL reaction
followed by real-time PCR in a 25-µL reaction using SmartCycler (Cepheid, Sunnyvale,
CA). The reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG
(Invitrogen Corporation, Carlsbad, CA) according to manufacturer recommendations and
the following primers: ICAM-1: 5'-TGCAGACAGTGACCATCTACAGC-3' and 5'-
TCTGAGACCTCTGGCTTCGTC-3'; VCAM-1: 5'-GAGATTCATTTGAGTGGCCCTC-3'
and 5'-CTGTCAAATGGGTATACATCAGCAA-3'. 18s ribosomal subunit RNA was
amplified in parallel reaction using primers: 5'-TTCGAACGTCTGCCCTATCAA-3' and 5'-
ATGGTAGGCACGCGACTA-3'. Two-temperature cycles with annealing/extension temperature at 62°C for ICAM-1 and 64°C for VCAM-1 and 18s were used. The fluorescence was measured at the end of the annealing step. The melting curve analyses were performed at the end of the reaction (after the 45th cycle) between 60°C and 95°C to assess the quality of the final PCR products. C(t) values were calculated by fixing the basal fluorescence at 15 units. Three replicate reactions were performed for each sample and the average C(t) was calculated. The standard curve of the reaction effectiveness was performed using the serially diluted (5 points) mixture of experimental cDNA samples for ICAM-1, VCAM-1 and 18s separately. The calculation of the relative RNA concentration was performed using Cepheid SmartCycle software (version 2.0d). Data are presented as the ratio of EndoG/18s mRNA.

**Statistical analysis**

The results were expressed as mean ± standard error of mean (SEM). The statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL USA). To evaluate the significance of differences between the two groups of experiments, the analysis of variance (ANOVA) and Student's t test were used. Additionally, to evaluate the significance of several time points in comparison to one control point, the Bonferroni adjustment of the t-test was used. A value of P < 0.05 was considered statistically significant.
REFERENCES


Figure I. Expression of ICAM-1 and VCAM-1 proteins after cLDL treatment determined by immunocytochemistry. Cells were treated with 200 µg/mL cLDL for 16 h. Control cells were treated with either vehicle or 200 µg/mL nLDL. Scale bar, 20 µm.
Figure II. Apostolov et al. Carbamylated LDL induces monocyte adhesion to endothelial cells through ICAM-1 and VCAM-1.

Figure II. Transfection of HCAECs with siRNA to ICAM-1 or VCAM-1. HCAECs were transfected with siRNA-FITC (A) or with anti-ICAM-1 or anti-VCAM-1 siRNA (B, C) for 48 h and then treated with 200 µg/mL cLDL for 8 h. At the end of the experiment, the cells were harvested, total RNA was extracted and real-time PCR performed. Control cells were treated with vehicle or nLDL. n = 4-5 per point, *P<0.05, **P<0.01 vs. cells at “before treatment” point, #P<0.05, ##P<0.01, ###P<0.001 vs. control siRNA-pre-treated cells at the same treatment point.