In Vivo Low-Density Lipoprotein Exposure Induces Intercellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1 Correlated With Activator Protein-1 Expression

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Objective—We tested the hypothesis that direct native low-density lipoprotein (LDL) injection into LDL receptor–deficient (LDLR−/−) mice would induce the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in their aortic endothelial cells, and that transcriptional regulation of this pathway involved activator protein-1 (AP-1) but not nuclear factor κB (NF-κB).

Methods and Results—Using tail vein injection of LDL into LDLR−/− mice, we were able to maintain atherogenic LDL blood levels, which induced ICAM-1 and VCAM-1 expression in their aortic endothelial cells after 24 hours. We were able to visualize and quantify this expression using immunohistochemistry and confocal microscopy. Under conditions in which ICAM-1 and VCAM-1 were expressed, the regulatory AP-1 proteins c-Fos and c-Jun were also highly expressed in the endothelial cell cytoplasm and observed within the cell nucleus. The NF-κB protein P65, although expressed in the endothelial cell cytoplasm after LDL injection, was not observed within the cell nucleus.

Conclusions—Elevated LDL blood levels, maintained in vivo, increased the expression of the adhesion molecules ICAM-1 and VCAM-1 in aortic endothelial cells. This effect appeared to correlate with AP-1 but not NF-κB. (Arterioscler Thromb Vasc Biol. 2006;26:1344-1349.)

Key Words: atherosclerosis ■ endothelium ■ low-density lipoprotein ■ AP-1 ■ NF-κB

Expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) initiates atherosclerotic plaque formation by aiding monocyte adhesion to the endothelial cell (EC) layer. The adherent monocytes traverse the EC layer to enter the burgeoning plaque.1 In cell culture, low-density lipoprotein (LDL) exposure increases transcription, mRNA abundance, and surface expression of both ICAM-1 and VCAM-1, resulting in increased monocyte binding to the cell surface.2–5 The present study is aimed at determining whether the cell culture findings also occur in vivo. If so, this would demonstrate for the first time that native LDL can directly incite an EC inflammatory reaction in an intact animal.

Human umbilical vein EC (HUVEC) culture has produced considerable experimental data elucidating the effects of lipoproteins on the biologic function of large-vessel ECs. Exposure of HUVECs to pathophysiologic LDL concentrations has been shown to cause increased expression of ICAM-1 and VCAM-1, resulting in increased monocyte binding to the cell surface.2–5 The LDL-induced signaling pathway begins as LDL delivers cholesterol to the cells, where it initiates a signaling cascade involving mitogen-activated protein kinase, c-Jun N-terminal kinase, c-Jun and activator protein-1 (AP-1).6 AP-1 appears to be the transcriptional regulator that promotes transcription of the ICAM-1 and VCAM-1 proteins in this system.7 Recruitment of monocytes by the LDL-activated HUVECs was reversed by antibodies against ICAM-1 and VCAM-1, providing evidence that these adhesion molecules were responsible for the increased monocyte-binding properties of the cells.

In the adhesion molecule transcriptional regulatory pathway, the AP-1 dimer, comprising proteins from the Fos and Jun families, binds to the ICAM-1/VCAM-1 promoter consensus sequence TGA(C/G) trichloroacetic acid to initiate transcription. AP-1 binding activity in HUVECs was increased by LDL exposure; and it was shown that AP-1, but not nuclear factor κB (NF-κB), was the important activator of LDL-induced adhesion molecule signaling pathways.7–11 c-Jun was found to be the critical component of the AP-1 regulation of ICAM-1, assessed using the effect of a dominant-negative mutant c-Jun on HUVEC–ICAM-1 surface expression and monocyte adhesion.12 How well these in vitro effects translate into the in vivo setting has not been determined previously. Further, whether AP-1 or NF-κB would be found to be the relevant in vivo transcriptional activator for adhesion molecule activation has not been investigated. Also, studies to date have been unable...
to follow the course of LDL metabolism within an intact animal to determine whether adhesion molecule expression and signaling pathways change as LDL blood levels change.

In the current study, we examined these critical questions by infusing LDL into LDL receptor–deficient (LDLR−/−) mice. This is the first time that such a system has been used, and it allows injected LDL to circulate for extended periods of time in contact with the EC layer in these animals. This allows us to evaluate and quantify ICAM-1 and VCAM-1 as well as the transcriptional regulatory proteins AP-1 and NF-κB using immunohistochemistry and confocal microscopy of the mouse aorta EC layer. Using this method, we explored the hypothesis that elevated levels of serum LDL can activate ICAM-1 and VCAM-1 in aortic ECs in vivo via an AP-1 mechanism, distinct from NF-κB activation.

Materials and Methods

LDL, High-Density Lipoprotein, and Mice

LDL and high-density lipoprotein (HDL) are prepared in our laboratory from fresh human plasma, and each batch is tested for oxidation, using thiobarbituric acid reaction substances, and for endotoxin contamination. The cholesterol content of each batch is determined as described previously. Homozygous LDLR−/− mice and C57BL/6J mice, which are the background strain for LDLR−/−, were purchased from Jackson Laboratories (Bar Harbor, Me), and are bred in-house. Breeding pairs are replaced yearly. The mice are maintained on standard Purina rodent Chow with a 12-hour light/dark cycle and are cared for according to accepted laboratory methods.

LDL and HDL Injection

To test the activation of each adhesion molecule or transcription factor, we injected LDL into the tail veins of LDLR−/− mice (4 to 5 months of age) and injected the same volume of PBS into another group of LDLR−/− mice as controls. As a second set of controls, C57BL/6J mice were injected using the same protocol. To control for the possibility that the human protein in LDL was the activating factor, we injected LDL into the tail veins of LDLR−/− mice as controls. As a second set of controls, C57BL/6J mice were injected using the same protocol. To control for the possibility that LDL might be acting through an inflammatory pathway that would involve Fc receptors, ICAM-1 and VCAM-1 activation by LDL injection was repeated using nonimmunized LDLR−/− mice.

Aorta Preparation and Blood Cholesterol

After tail vein injection of LDL, PBS, or HDL and a 4-, 24-, or 48-hour waiting period, mice were euthanized by CO2 inhalation, and blood was removed from the right ventricle for lipid determination. Blood was assayed for total cholesterol concentration, LDL, HDL, very low–density lipoprotein, and triglycerides. Immediately after blood removal, the aorta was cut ≥2 cm. Below the heart, the left ventricle was cannulated and the aorta perfused with PBS until the fluid ran clear. Then it was perfused for an additional 20 minutes with cold 4% paraformaldehyde. Aortas were dissected out, attached to the heart, and placed in cold PBS. After removing fat and adventitial tissue, aortas were cut into 3 segments. The segment proximal to the heart has shown greater atherosclerosis and was used as the positive portion for immunohistochemistry and confocal microscopy. The distal portion served to determine the baseline for quantification of the confocal microscopy results.

ICAM-1/VCAM-1 Immunohistochemistry

Using a method described previously, staining of the proximal aorta portion consisted of incubation with 20% mouse serum for 20 minutes; rat, anti-mouse ICAM-1, or VCAM-1 primary antibody (1:100 in PBS; Jackson ImmunoResearch) overnight at 4°C; mouse, anti-rat CY3-labeled secondary antibody (1:200 in PBS; Jackson ImmunoResearch) for 30 minutes at room temperature; and SYTOX green nuclear stain (1:50 000 in HEPES; Molecular Probes) for 15 minutes at room temperature, with 3 5-minute washes between each incubation. To test the possibility that LDL might be acting through an inflammatory pathway that would involve Fc receptors, ICAM-1 and VCAM-1 primary antibody was stained in the same way as the proximal segment except that nonimmune rat IgG (Jackson Immuno Research) replaced the ICAM-1 or VCAM-1 primary antibody. After staining, each aorta section was cut lengthwise and placed endothelium side up on a glass slide coated with Vectabond and covered with glycerol and a plastic coverslip. Slides were protected from light and placed at 4°C to await confocal microscopy within 24 hours.

c-Jun, c-Fos, and P65 Immunohistochemistry

Harvested proximal aorta segments were exposed to 20% mouse serum for 20 minutes and incubated overnight at 4°C with rabbit c-Jun, (1:100 in PBS; Santa Cruz Biotechnology) or rabbit c-Fos (1:100 in PBS; Santa Cruz Biotechnology). Nonimmune rat IgG served as the negative control for the distal segment. After 3 5-minute washes, both proximal and distal segments were incubated with rhodamine-labeled anti-rabbit IgG (1:200 in PBS; Santa Cruz Biotechnology) for 30 minutes at room temperature. SYTOX was used to stain the nuclei. After staining, the aorta segments were processed as described above.

To examine NF-κB activation, the prepared proximal segments were incubated with rabbit P65 primary antibody (1:100 in PBS; Santa Cruz Biotechnology), with rat IgG serving as the negative control on the distal aorta portion. The secondary antibody, rhodamine-labeled anti-rabbit IgG (1:200 in PBS; Santa Cruz Biotechnology), was used for both aorta pieces, with incubation at room temperature for 30 minutes. The aorta segments were stained with SYTOX and processed as described above.

Confocal Microscopy

Images of the EC monolayer were obtained on a Zeiss 510 confocal microscope using the fluorescein isothiocyanate/rhodamine channels using the 63× water objective. Pixels were calculated in the rhodamine channel as a means of quantitation. The rhodamine channel detects CY3-tagged ICAM-1/VCAM-1 and rhodamine-tagged c-Jun, c-Fos and P65, whereas the fluorescein isothiocyanate channel detects nuclei stained with SYTOX. The distal segment, initially incubated with rat IgG, was observed and quantified in exactly the same way. Confocal settings were adjusted for the proximal segment and left unchanged for the distal segment. Pixels generated from the distal segment were subtracted from pixels generated in the proximal segment, allowing quantification of the results, using the histogram function on the confocal microscope.

For each aorta segment, ≥6 measurements were taken. For the proximal segment, 3 to 4 measurements were taken in the area close to the heart, known as the area of high probability, and 3 to 4 measurements were taken in other regions, known as the area of low probability. Only the measurements from the high probability area were used for computation.

Statistical Analysis

Statistical analyses were performed using the unpaired Student t test. Comparing aortas from LDL-exposed mice with aortas from non–LDL-exposed mice, data (mean±SE) were considered statistically significant at a value of P<0.05. Each experiment was performed 4
period, LDLR cles, and VCAM-1 data are graphed as green squares.

Figure 2. Blood cholesterol (mg/dL) levels in LDLR−/− mice before LDL injection (−24 hours) and after LDL injection (4, 24, and 48 hours). n = 4 to 5 for each time period.

to 5 times, depending on the calculated $P$ values. There was no variation in any of the values according to sex or age.

Results

LDL Induced ICAM-1 and VCAM-1 In Vivo

Blood cholesterol levels were examined in all mice before LDL injection and 4, 24, and 48 hours after injection. After injection, serum cholesterol levels in LDLR−/− mice remained elevated for 24 hours but began to diminish after 48 hours, as shown in Figure 1. Serum cholesterol levels were not appreciably altered in C57BL/6J mice after injection with LDL at any time period (data not shown).

Maximal adhesion molecule response, after LDL injection in LDLR−/− mice, was established at 24 hours. At this time period, LDLR−/− mice were observed to have strongly increased expression of ICAM-1 and VCAM-1 in their aortic ECs related to their elevated blood cholesterol levels. These data, relating ICAM-1 and VCAM-1 expression to cholesterol levels measured when the mouse was euthanized. There was no ICAM-1 or VCAM-1 change at any time period measured in the aortas of C57BL/6J mice exposed to LDL. Repeating the LDL injection protocol using nonimmune primary antibodies for ICAM-1 and VCAM-1 resulted in no increased expression over the PBS-injected baseline results.

LDL Induced c-Jun, c-Fos, and P65 In Vivo

The AP-1 proteins c-Jun and c-Fos and the NF-kB protein P65 seen in the LDLR−/− mouse aorta after LDL exposure and a 24-hour waiting period are shown in Figure 4. c-Jun, in Figure 4A, was expressed in PBS-exposed aortas at a somewhat higher level than the other transcription factor proteins, indicating a certain general presence in the cytoplasm even without added LDL. After LDL injection and a 24-hour waiting period, c-Jun expression was greatly increased in both EC cytosol and nucleus. It can be seen as the yellow color within the nuclei of the ECs, indicating its potential for gene activation. In Figure 4B, c-Fos, for the most part, was not evident in PBS-exposed mouse aortas, but its expression was increased in both cytosol and nucleus after LDL exposure, although less abundantly than c-Jun. Nonetheless, it can be seen within the EC as the yellow color within the nuclei, indicating its potential for gene activation. As shown in Figure 4C, P65 was not evident in PBS-exposed mouse aortas, although its expression was increased in the cytoplasm after 24-hour LDL exposure. However, it was not observed within the nucleus after this exposure period, indicating that it was not available to activate adhesion molecule genes under these conditions.

LDLR−/− mice were also injected with HDL in an amount that would provide HDL protein equal to the LDL protein in injected animals. Very little ICAM-1 or VCAM-1 was evident in the HDL-injected mice, although there was a slight decrease in the aortas after both 24 and 48 hours. ICAM-1 expression in the LDLR−/− mouse aorta, after 24 and 48 hours, comparing PBS, HDL, and LDL injection, is shown in Figure 5. VCAM-1 expression in the LDLR−/− mouse aorta, after 24 and 48 hours, comparing PBS, HDL, and LDL injection, is shown in Figure 6.

Discussion

These experiments convincingly support the hypothesis of vascular EC activation by native LDL. Injection of LDL into LDLR−/− mice has caused a time-dependent increase in the expression of both endothelial ICAM-1 and VCAM-1. This
activation appears to be associated with AP-1 but not NF-κB regulatory pathways. Furthermore, HDL, injected into LDLR−/− mice, did not produce such an effect (as shown in Figures 5 and 6), indicating the specificity of LDL as an EC-activating lipoprotein particle and strongly supporting the notion that native LDL can induce inflammatory changes in ECs. Repeating the ICAM-1 and VCAM-1 experiments using injected LDL and nonimmune primary antibodies yielded no fluorescence, ruling out the involvement of Fc receptors in these LDL-induced ICAM-1 and VCAM-1 expression pathways.

Although injection of LDL into the tail veins of LDLR−/− mice and a 24-hour waiting period produced greatly increased ICAM-1 and VCAM-1 in the ECs of the aortas, their appearance varied. In all of the aortas in which it was evaluated, ICAM-1 expression was much more pervasive, whereas VCAM-1 expression appeared more focal and was somewhat less elevated. These expression patterns of ICAM-1 and VCAM-1

Figure 3. Quantification of ICAM-1 and VCAM-1 expression in mouse aortic ECs 24 hours after injection with LDL or PBS, along with representative confocal images. Each image is 22,500 μm². ICAM-1, graphed in A, shows a large increase in expression after injection of LDL compared with PBS, which is highly statistically significant at P=0.0056; n=4 to 5. Representative confocal images are shown beneath the graph. The red color shows CY-3 staining of the ICAM-1 protein, and the green color shows the EC nuclei stained with SYTOX. The bar graph above each image shows the average ICAM-1 immunofluorescent quantification. The size bar represents 50 μm. Pixel numbers are per confocal image. VCAM-1, graphed in B, shows a large increase in expression after injection of LDL compared with PBS, which is highly statistically significant at P=0.00043; n=4 to 5. Representative confocal images are shown beneath the graph. The red color shows CY-3 staining of the VCAM-1 protein, and the green color shows the EC nuclei stained with SYTOX. The bar graph above each image shows the average ICAM-1 immunofluorescent quantification. The size bar represents 50 μm. Pixel numbers are per confocal image.

Figure 4. Quantification of c-Jun, c-Fos, and P65 expression in mouse aortic ECs 24 hours after injection with LDL or PBS, along with representative confocal images of the PBS- and LDL-injected aortas. Each image is 22,500 μm². C-Jun, graphed in A, shows a large increase in expression after injection of LDL compared with PBS, which is highly statistically significant at P=0.000723; n=4 to 5. Representative confocal images of the PBS- and LDL-injected aortas are shown beneath the graph. The red color shows CY-3 staining of the c-Jun protein, the green color shows the EC nuclei stained with SYTOX, and the yellow color shows penetration of the c-Jun protein into the nucleus. The bar graph above the image shows the average c-Jun immunofluorescent quantification. The size bar represents 50 μm. Pixel numbers are per confocal image. C-Fos, graphed in B, shows a large increase in expression after injection of LDL compared with PBS, which is highly statistically significant at P=0.00035; n=4 to 5. Representative confocal images of the PBS- and LDL-injected aortas are shown beneath the graph. The red color shows CY-3 staining of the c-Fos protein, the green color shows the EC nuclei stained with SYTOX, and the yellow color shows the penetration of the c-Fos protein into the nucleus. The bar graph above the image shows the average c-Fos immunofluorescent quantification. The size bar represents 50 μm. Pixel numbers are per confocal image. P65, graphed in C, shows a large increase in expression after injection of LDL compared with PBS, which is highly statistically significant at P=0.00099; n=4 to 5. Representative confocal images of the PBS- and LDL-injected aortas are shown beneath the graph. The red color shows CY-3 staining of the P65 protein, the green color shows the EC nuclei stained with SYTOX, and the lack of yellow color within the nucleus indicates that P65 has not penetrated into the nucleus. The bar graph above the image shows the average P65 immunofluorescent quantification. The size bar represents 50 μm. Pixel numbers are per confocal image.
are similar to those seen in the aortas of LDLR−/− mice on a high-fat diet.16 Although many blood parameters were measured in our study, total cholesterol, HDL, LDL, very low-density lipoprotein, and triglycerides, adhesion molecule expression was most closely related to the value for total blood cholesterol.

Both AP-1 and NF-κB have been implicated as transcriptional regulators for adhesion molecule synthesis.18,19 Because the c-Jun and c-Fos protein subunits of AP-1 were elevated in the EC cytosol by LDL injection and were also able to penetrate the nucleus, they were thus in position to affect gene regulation of adhesion molecules under these LDL exposure conditions. It was apparent the expression of c-Fos was less robust than c-Jun in these experiments. The P65 protein subunit of NF-κB was elevated in the EC cytosol by LDL injection but was unable to penetrate the EC nucleus under these LDL exposure conditions and therefore unable to affect gene regulation. We conclude that the in vivo LDL-induced adhesion molecule mechanism for ICAM-1 and VCAM-1 expression is correlated with AP-1 but not with NF-κB, which is also true of the many in vitro findings. The findings of the current study substantiate our cell culture observations and indicate that LDL cholesterol is likely involved in an AP-1–related mechanism for ICAM-1 and VCAM-1 expression.6–8,10,12

It could be argued that because atherosclerosis has an inflammatory component,1,20 the presence of nonspecific human protein might be sufficient to induce adhesion molecule expression in the mouse aorta. We tested this possibility by injection of human HDL into the LDLR−/− mouse tail vein and examining ICAM-1 and VCAM-1 using the described confocal procedure. Using an HDL injection containing the same amount of protein as the injected LDL, we found very little increased ICAM-1 or VCAM-1 over basal level in the LDLR−/− mouse aorta after 4, 24, or 48 hours. This finding indicated that there was probably no inflammatory component attributable to nonspecific human protein in the LDL-induced ICAM-1 or VCAM-1 elevation by in vivo exposure to human LDL.

It is interesting to note that the adhesion molecule expression does not appear immediately after LDL injection, but is seen maximally at 24 hours. This delay is also seen in EC culture and indicates a similarity between the in vivo and in vitro activation pathways. Two possibilities for the 24-hour time lag are that a sufficient amount of LDL cholesterol must enter and accumulate in the cells before activation of the adhesion molecule pathways can begin, and a certain amount of signaling intermediate such as c-Jun must be formed before sufficient adhesion molecule protein can be produced. Of course, both of these possibilities may be true.

It is also interesting to note that the adhesion molecule activation effect appears to be dose dependent. Although, for our study, we compared PBS-injected with LDL-injected mice, and the ICAM-1 and VCAM-1 activation corresponded to the achieved total blood cholesterol at 24 hours. Note the straight-line dose-response in Figure 2 for both ICAM-1 and VCAM-1 as a function of blood cholesterol. Further, adhesion molecule expression, as a result of LDL injection, did not depend on age or sex of the mice and thus appeared to be a universal outcome of EC exposure to elevated cholesterol. These observations indicate that in an intact animal, cholesterol exposure or accumulation within the ECs is critical for ICAM-1 and VCAM-1 expression and does not appear to be influenced by age or gender.

The significance of this inquiry lies in the establishment, in vivo, that elevated LDL induces signals that result in elevation of ICAM-1/VCAM-1, which has never before been shown in a living animal model. Our data implicate LDL as an EC activator in vivo, the effect of which is dependent on the achieved dose after an induction period. Tail vein injection of LDL, into LDLR−/− mice, allows us a unique opportunity to observe in vivo adhesion molecule signaling pathways, uncomplicated by contributions from the solvents and growth factors, which may be used with in vitro models. It also bypasses the side effects of a long-term high-fat diet. Although ICAM-1 and VCAM-1 were observed in atherosclerosis-prone areas of the aortas of LDLR−/− mice after an extended period on a high-fat diet,16 the added complications of accumulated abdominal fat and oxidized LDL may be arbiters of adhesion molecule expression and eventual atherosclerosis in the high-fat chow mouse model.
In depth knowledge of in vivo lipoprotein-induced EC signaling should allow us to target specific signaling intermediates for therapeutic intervention against atherosclerosis. It is critical to explain how high blood LDL or cholesterol levels can induce a proinflammatory, proatherogenic state and how this state can be reversed or prevented in an intact animal. It has been shown that when human LDL levels are precipitously reduced, rapid correction of vascular change occurs,21,22 indicating that certain LDL- or cholesterol-induced effects are likely to be reversible. This reversibility effect appears to have been corroborated in our study because there is almost no ICAM-1 or VCAM-1 after 48 hours, when cholesterol levels are again approaching baseline. The reversibility of adhesion molecule expression seen in this study, as blood cholesterol returns to baseline, corroborates the recent evidence that atherosclerosis can be reversed as cholesterol is removed from circulation.23–27 Further, it appears likely that evidence that atherosclerosis can be reversed as cholesterol is removed from circulation,23–27 indicating that certain LDL- or cholesterol-induced effects are likely to be reversible. This reversibility effect appears to have been corroborated in our study because there is almost no ICAM-1 or VCAM-1 after 48 hours, when cholesterol levels are again approaching baseline. The reversibility of adhesion molecule expression seen in this study, as blood cholesterol returns to baseline, corroborates the recent evidence that atherosclerosis can be reversed as cholesterol is removed from circulation.23–27

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References

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