Interleukin-8 Secretion by Fibroblasts Induced by Low Density Lipoproteins Is p38 MAPK-dependent and Leads to Cell Spreading and Wound Closure*5

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We have previously reported (Dobreva, I., Waeber, G., Mooser, V., James, R. W., and Widmann, C. (2003) J. Lipid Res. 44, 2382–2390) that low density lipoproteins (LDLs) induce activation of the p38 MAPK pathway, resulting in fibroblast spreading and lamellipodia formation. Here, we show that LDL-stimulated fibroblast spreading and wound sealing are due to secretion of a soluble factor. Using an antibody-based human protein array, interleukin-8 (IL-8) was identified as the main cytokine whose concentration was increased in supernatants from LDL-stimulated cells. Incubation of supernatants from LDL-treated cells with an anti-IL-8 blocking antibody completely abolished their ability to induce cell spreading and mediate wound closure. In addition, fibroblasts treated with recombinant IL-8 spread to the same extent as cells incubated with LDL or supernatants from LDL-treated cells. The ability of LDL and IL-8 to induce fibroblast spreading was mediated by the IL-8 receptor type II (CXCR-2). Furthermore, LDL-induced IL-8 production and subsequent wound closure required the activation of the p38 MAPK pathway, because both processes were abrogated by a specific p38 inhibitor. Therefore, the capacity of LDLs to induce fibroblast spreading and accelerate wound closure relies on their ability to stimulate IL-8 secretion in a p38 MAPK-dependent manner. Regulation of fibroblast shape and migration by lipoproteins may be relevant to atherosclerosis that is characterized by increased LDL cholesterol levels, IL-8 production, and extensive remodeling of the vessel wall.

Abnormal activation of endothelial and smooth muscle cells plays a critical role in atherosclerosis progression (1). Recent studies also report the active participation of a third vascular wall cell type, the adventitial fibroblasts, in the remodeling process observed in blood vessels during the development of the disease (2). In fact, adventitia is no longer viewed as a supporting tissue but rather as an important mediator of vascular remodeling (3, 4) making it a potential therapeutic target. Moreover, the adventitia appears to play a significant role in arterial repair (5–7). It has been shown in experimental models of blood vessel injury that the adventitia goes through rapid transformations. Furthermore, the residing fibroblasts undergo phenotypic changes that contribute to tissue repair (8, 9). In this case, fibroblast activation has beneficial effects. In contrast, if there is a continuous activation of the cells in the adventitia, aberrant tissue remodeling might participate in thickening of the blood vessels. Therefore, it is important to investigate the molecular mechanisms involved in fibroblast activation.

Lipoproteins, initially characterized as the main cholesterol cargos for cells, are now known to modulate various mitogen-activated protein kinase (MAPK)7 pathways in different cell types of the vessel wall (10–12). However, the cellular responses resulting from the activation of a given MAPK cascade induced by lipoproteins vary considerably from one cell type to another. For example the p38 MAPK pathway mediates proliferation in vascular smooth muscle cell (13) and lamellipodia formation and cell spreading in fibroblasts in response to LDLs (10). The latter phenomenon might be relevant to vascular remodeling, as recent evidence indicates that activation of MAPKs in fibroblasts is involved in this process (reviewed in Ref. 6).

In the present study, we have investigated how lipoproteins induce fibroblast spreading. Our results show that fibroblasts secrete IL-8 in response to LDLs in a p38 MAPK-dependent manner. This, in turn, induces cell spreading and facilitates wound closure.

EXPERIMENTAL PROCEDURES

Materials—Anti-human IL-8 mouse monoclonal antibody (catalog number 554726), recombinant human IL-8 (catalog number 554609), and the BD OptEIA human anti-IL-8 ELISA kit (catalog number 550999) were from BD Biosciences. Anti-hemagglutinin-tagged mouse monoclonal antibody (HA11; catalog number AFC-101P) was from Covance. 2′, 7′-bis(2-carboxyethyl)-5′(6)-carboxyfluorescein tetrakis(acetoxyethyl) ester (BCECF-AM) was purchased from Fluka (catalog number 14562). SB 239063 (trans-4-[4-(4-fluorophenyl)-5-(2-methoxy-4-pyrimidinyl)-1H-imidazol-1-yl] cyclohexanol was from Tocris Bioscience (catalog number 1962). Anti-human CXCR-1 and CXCR-2 monoclonal antibodies were from R&D Systems (catalog numbers MAB330 and MAB331).

Cell Culture—GM00316B human skin fibroblasts were purchased from Coriell Cell Repositories and maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 15% non-decomplemented fetal calf serum (FCS; Amied) at 37 °C and 5% CO2.

Lipoprotein and Lipoprotein-free Serum Preparation and Purification—LDLs were isolated from human serum by sequential density ultracentrifugation as described previously (14, 15), dialyzed for 48 h against PBS

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2 The abbreviations used are: MAPK, mitogen-activated protein kinase; BCECF-AM, 2′, 7′-bis(2-carboxyethyl)-5′(6)-carboxyfluorescein tetrakis(acetoxyethyl) ester; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; IL, interleukin; LDL, low density lipoprotein; PBS, phosphate-buffered saline.

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containing 100 μM EDTA, and stored under nitrogen. Human lipoprotein-free serum was prepared by the removal of lipoproteins via ultracentrifugation at a density of 1.23 g/liter (15). The protein concentration was measured by Bradford assay using BSA as a standard. The cholesterol concentration was measured by an enzymatic in vitro test kit from Roche Applied Science (catalog number 2016630).

Cell Spreading Measurements—Human fibroblasts were plated on uncoated 12-mm-diameter glass coverslips (VWR International; catalog number 6012401) in 6-well plates supplemented with DMEM and 15% FCS. On the following day, the medium was replaced with serum-free DMEM. The cells were starved for 24 h, and cell surface was measured as follows. The cells were stained with the fluorescent dye BCECF-AM (green staining) or fixed cells stained with rhodamine-phalloidin (red staining) to visualize the actin cytoskeleton (see “Experimental Procedures”). If human fibroblasts were starved as described above and treated with increasing concentrations of LDLs for 24 h. Images were taken on living cells stained with BCECF-AM, and cell surface was measured on digital pictures with ImageJ software (see “Experimental Procedures”). The control value was set to 1. Results are expressed as the mean ± S.E. The significance of the differences with the control condition was assessed by Student’s t test (NS, not significant; **, p < 0.01; ***, p < 0.001).

Preparation of Cell Culture Supernatants—Human fibroblasts were plated in 10-cm plates in DMEM with 15% FCS and grown until confluence. The cells were then starved in DMEM containing 2.5 mg/ml human lipoprotein-free serum for 24 h and stimulated with vehicle (PBS containing 100 μM EDTA) or 200 μg/ml LDL for 30 min. The cells were then washed three times with PBS and incubated in serum-free DMEM for 24 h. Cell culture supernatants were then collected and passed through 0.22-μm sterile Millipore filters. Aliquots of the supernatants were frozen at −20 °C or dialyzed against membranes with 3.5- or 100-kDa molecular mass cutoff in DMEM for 8 h (2 × 4 h). The dialyzed supernatants were then filtered and incubated with naïve cells for 24 h.

FIGURE 1. LDLs induce fibroblast spreading in a dose-dependent manner. A, human fibroblasts plated on coverslips were starved in DMEM for 24 h and treated with increasing concentrations of LDLs for 24 h. Shown are pictures of living cells stained with the fluorescent dye BCECF-AM (green staining) or fixed cells stained with rhodamine-phalloidin (red staining) to visualize the actin cytoskeleton (see “Experimental Procedures”). B, human fibroblasts were starved as described above and treated with increasing concentrations of LDLs for 24 h. Images were taken on living cells stained with BCECF-AM, and cell surface was measured on digital pictures with ImageJ software (see “Experimental Procedures”). The control value was set to 1. Results are expressed as the mean ± S.E. The significance of the differences with the control condition was assessed by Student’s t test (NS, not significant; **, p < 0.01; ***, p < 0.001).
Cytokine Detection and Quantitation—Cell culture supernatants were collected from human fibroblasts that were incubated or not incubated with 200 μg/ml LDL for 24 h. Cell debris was removed by centrifugation of the supernatants at 500 g for 5 min. The supernatants were analyzed for the presence of cytokines by an antibody-based human cytokine array (Novagen ProteoPlex 16-well human cytokine array, catalog number 71414-3) as per the manufacturer’s instructions. The concentration of the cytokines was determined according to standard curves provided for each cytokine. The microarray plates were scanned with a Packard Biochip Technologies scanner (PerkinElmer Life Science) using the ScanArray 4000 software. Quantitation of the individual spots was done by using the Imagen software. Each cytokine concentration is presented as the mean of the intensity of four independent spots ± S.D. In addition, the amount of IL-8 in cell culture supernatants was measured with a human anti-IL-8 ELISA kit from BD Biosciences, and its concentration was determined according to a standard curve with a standard provided by the manufacturer.

Time-lapse Microscopy—Cells were grown to confluence in a time-lapse chamber and starved in DMEM containing 0.1% FCS for 24 h. The cells were then wounded as described previously (16) and preincubated or not preincubated with either 3 μM SB 239063 or 400 ng/ml anti-IL8 antibody for 30 min, and 200 μg/ml LDL were then added. The chamber was placed in a Nikon TE-2000-E inverted time-lapse microscope system, and pictures were taken with a 10× objective every 10 min for 2–3 days. Images and movies were analyzed with MetaMorph software (Universal Imaging).

Statistics—Comparisons between groups were made by homoscedastic two-tailed t analysis using the Excel 2000 software.

RESULTS

LDLs Induce Fibroblast Spreading and Facilitate Wound Closure—LDL stimulation of fibroblasts results in lamellipodia formation and cell spreading (10). To determine at which LDL concentration cell spreading occurs, fibroblasts were incubated with increasing concentrations of LDLs, and their surface and changes of the actin cytoskeleton were assessed. Fig. 1 shows that LDLs start to initiate spreading at concentrations of 50–100 μg/ml (but this did not reach statistical significance). Maximal spreading was achieved at LDL concentrations of 200 μg/ml and above. Spreading of fibroblasts induced by LDLs was associated with enlargement of the actin cytoskeleton (Fig. 1A).

To determine whether LDLs could promote wound repair, human fibroblast monolayers were wounded and stimulated or not with LDLs. The process of wound closure was followed by time-lapse video microscopy. As shown in Fig. 2 (see also the supplemental movie available with the on-line version of this article), LDL-stimulated cells populated the wounded area faster than control cells. LDLs can therefore induce cell spreading and migration into wounds, two processes that rely on modulation of the actin cytoskeleton (17).

LDLs Induce Cell Spreading Indirectly by Stimulating Secretion of an Autocrine Factor—Because fibroblast lamellipodia formation and cell spreading in response to LDLs is initiated after a lag phase of ~8 h (10), we assessed whether a continuous presence of LDLs is required for cell spreading to occur. Human fibroblasts were stimulated with vehicle or with 200 μg/ml LDLs for 30 min. LDLs were then removed by extensive washings. The cells stimulated with LDLs for only 30 min spread to the same extent as cells incubated with LDLs for 24 h (Fig. 3A). LDL removal by washing was effective, because stimulation of naïve cells with medium from washed cells did not induce cell spreading (Fig. 3, A and B). These results are compatible with the hypothesis that LDLs do not induce cell spreading directly but rather stimulate signal transduction pathways, such as the p38 MAPK pathway (10), to produce a factor that causes cell spreading. To test this hypothesis, cells were stimulated with LDLs for 30 min, washed extensively, and incubated in serum-free
medium for 24 h. This medium was then collected and placed on naive cells. Fig. 3B shows that supernatants from cells transiently stimulated with LDLs, although not containing residual amounts of LDLs (see Fig. 3A, condition e), efficiently induced cell spreading. This finding indicates that LDL-stimulated cells secrete an autocrine factor able to induce spreading.

To determine the molecular mass of this soluble factor, we compared the ability of supernatants from LDL-stimulated cells to induce the spreading of naive cells after dialyses against 3.5- and 100-kDa cutoff membranes. The supernatants dialyzed against the 3.5-kDa cutoff membranes retained their ability to induce spreading, whereas the supernatants dialyzed against the 100-kDa cutoff membranes totally lost their spreading activity (Fig. 3C). These results indicate that LDL-treated cells secrete a soluble factor, the molecular mass of which is comprised between 3.5 and 100 kDa. Moreover, this confirms that the soluble factor does not correspond to LDLs (if any were left after the extensive washing and medium replacement), as the LDLs weigh more than $2 \times 10^6$ Da and would not be dialyzed off the 100-kDa cutoff membrane.

**LDL-stimulated Cells Secrete IL-8** —Because LDLs induce p38 MAPK activation (10, 18) and because the p38 MAPK pathway is known to regulate the production of various autocrine factors such as members of the IL-8 family, we hypothesized that LDL-stimulated cells secrete IL-8. To test this hypothesis, we incubated cell culture supernatants from control or LDL-treated cells with a ProteoPlex human cytokine array and visualized the results using a microarray scanner. As shown in Fig. 4A, both control and LDL-treated supernatants contained IL-8, indicating that LDL-stimulated cells secrete this factor.

We then quantified the concentrations of various cytokines in the supernatants of control or LDL-treated cells using ProteoPlex human cytokine arrays. As shown in Table 1, LDL-treated supernatants contained significantly higher levels of IL-8 than control supernatants, while the concentrations of other cytokines were similar in both conditions. These results confirm that LDL-stimulated cells secrete IL-8.

Finally, we investigated the role of IL-8 in LDL-mediated cell spreading and wound repair. As shown in Fig. 5A, human fibroblasts treated with LDL-supernatants for 24 h and preincubated with anti-human IL-8 blocking antibody showed significantly less spreading than cells treated with LDL-supernatants and isotype-matched control antibody. These results indicate that IL-8 is required for LDL-mediated cell spreading.

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the cytokine family (19), we determined whether specific cytokines were secreted after LDL stimulation. Using an antibody-based cytokine array, supernatants from control- or LDL-stimulated cells were analyzed for the presence of various human cytokines. Fig. 4 shows that IL-8 concentration in the culture medium is increased upon LDL stimulation. IL-6 release was also slightly induced in supernatants from these cells, whereas tumor necrosis factor concentrations were slightly decreased in comparison to control cells (Fig. 4B). However, after determination of the concentrations of these cytokines under different experimental conditions, we concluded that IL-8 is the only cytokine whose release is systematically and markedly increased in supernatants of LDL-treated cells.

**IL-8 Is Responsible for LDL-mediated Cell Spreading and Wound Closure**—Two different approaches were used to assess whether increased secretion of IL-8 in supernatants of LDL-stimulated cells leads to changes in cell morphology. First, supernatants from LDL-treated cells were pre-incubated with an anti-human IL-8 blocking antibody. As shown in Fig. 5A, supernatants from LDL-treated cells that were incubated with the anti-IL-8 antibody completely lost their ability to stimulate the spreading of naive cells. In contrast, supernatants from LDL-treated cells pre-incubated with the same amount of an unrelated isotype-matched control antibody (anti-HA11) did not lose their ability to induce cell spreading. To confirm that the presence of IL-8 is sufficient to trigger spreading of the cells, human fibroblasts were incubated with increasing concentrations of recombinant human IL-8. As shown in Fig. 5B, this resulted in an enlargement of their surface. This increase was initiated at 100 pg/ml IL-8 and reached its maximum at 200 pg/ml, which corresponds to the amounts of IL-8 measured in the supernatants of LDL-stimulated cells (see Fig. 4B). In addition, cells treated with recombinant human IL-8 spread to the same extent as cells incubated with LDL or supernatants from LDL-treated cells (Fig. 5B). Moreover, neutralization of IL-8 by the addition of an anti-IL8 blocking antibody abrogated the ability of fibroblasts to mediate wound closure in response to LDL (Fig. 5C; see also supplemental movie).

**CXCR-2, but Not CXCR-1, Mediates Fibroblast Spreading Induced by LDL and IL-8**—To determine which one of the IL-8 receptors mediates the ability of LDL and IL-8 to induce fibroblast spreading, we neutralized the two IL-8 receptors by the addition of blocking antibodies. As shown in Fig. 6, the anti-CXCR-2 antibody completely inhibited LDL- and IL-8-induced fibroblast spreading. In contrast, the antibody directed at CXCR-1 or an unrelated isotype-matched control antibody...
**LDLs Induce IL-8 Secretion**

(HA11) did not affect cell spreading. This indicates that modulation of cell shape as a result of LDL-induced IL-8 secretion requires activation of the IL-8 receptor type II in fibroblasts.

**LDL-stimulated IL-8 Secretion and Wound Closure Are Mediated by the p38 MAPK Pathway**—Because LDL-induced p38 MAPK activation is required for fibroblast lamellipodia formation and cell spreading (10), we assessed whether activation of this signaling cascade mediates IL-8 production in response to LDLs. Fig. 7A shows that the increase in IL-8 concentration in supernatants from LDL-treated cells is blocked by SB 239063, a specific p38 inhibitor. The concentration of the inhibitor used here (3 μM) has been shown to specifically block the p38 MAPK pathway but not the related extracellular signal-regulated kinase and c-Jun NH2-terminal kinase MAPK pathways in fibroblasts (18). The results presented in Fig. 7A indicate therefore that the p38 MAPK pathway mediates the induction of IL-8 production in response to LDLs. In addition, inhibition of the p38 MAPK pathway abrogates fibroblast wound healing induced by LDLs (Fig. 7B; see also supplemental movie). Taken together, these data show that LDLs mediate cell spreading and wound repair indirectly by stimulating IL-8 secretion in a p38 MAPK-dependent manner.

**DISCUSSION**

We have recently shown that, in fibroblasts, LDL-induced p38 MAPK activation results in cell spreading and lamellipodia formation (10). We have determined now that LDLs also contribute to accelerated wound healing. Analysis of supernatants from LDL-stimulated fibroblasts demonstrated that these cells produce a soluble factor able to induce cell spreading. Using an antibody-based human cytokine array, IL-8 was identified as the main cytokine secreted from LDL-stimulated cells. Incubation of these cells with purified IL-8 was sufficient to induce their spreading. As we have shown earlier that LDLs induce cell spreading in a p38 MAPK-dependent manner (10), we propose that LDLs stimulate p38 MAPK activation, which promotes the IL-8 secretion that, in turn, induces cell spreading. In support of this notion is the fact that LDL-induced IL-8 production and subsequent wound closure are abrogated in the presence of a specific p38 inhibitor (Fig. 7, A and B).

Activation of the p38 MAPK pathway has been shown to regulate the stability of mRNAs encoding various cytokines (tumor necrosis factor-α, IL-1β, IL-6, IL-8, monocyte chemoattractant protein-MCP-1, and granulocyte/macrophage-colony-stimulating factor), metalloproteinases (MMP-1 and MMP-3), and adhesion molecules (P-selectin, vascular cell adhesion molecule 1, and intercellular cell adhesion molecule 1) (20). LDL-induced p38 MAPK activation might therefore allow fibroblasts to stabilize the IL-8 mRNA and consequently lead to an increased secretion of the cytokine.

LDL oxidation, which can occur in various pathophysiological situations (elevated LDL plasma levels, increased retention of LDLs in the circulation, or trapping of LDL particles in the sub-endothelial space of blood vessels), may contribute to the induction of IL-8 as was previously reported in macrophages (21). However, although oxidation may increase the ability of LDLs to activate the p38 MAPK pathway in fibroblasts (10), we have found that activation of this pathway does not correlate with the accumulation of oxidized products but with the amount of cholesterol present in the lipoprotein particles (18). We therefore suggest that the ability of LDLs to stimulate IL-8 secretion and cell spreading does not require their oxidation.

It has been proposed that enzymatic, nonoxidative modifications could render LDLs more atherogenic. These enzymatically degraded, nonoxidized LDLs (E-LDLs), which were found in early atherosclerotic lesions, could stimulate the expression of gp130, the signal-transducing chain of the IL-6 receptor as well as IL-6 secretion (22). Interestingly, there is an increase in the amount of free cholesterol in the E-LDLs in comparison to native LDLs. In addition, we have shown that the rate of p38 MAPK activation is proportional to the free cholesterol concentration in lipoproteins (18). Therefore the production of cytokines such as IL-6 and IL-8, which is mediated by the p38 MAPK pathway, may be induced by an increase in lipoprotein cholesterol levels.

IL-8 is believed to be a pro-atherogenic factor, because it can increase monocyte adhesion to the endothelium and exert a mitogenic effect on endothelial and vascular smooth muscle cells (23, 24). Consistent with the pro-atherogenic role of IL-8 are the observations that the expression of IL-8 is up-regulated in human atherosclerotic arterial walls (25) and that IL-8 concentration is increased in patients with hypercholesterolemia (26). Various hematopoietic cell lineages secrete IL-8 in response to elevated lipoprotein concentrations. For example, peripheral blood mononuclear cells produce IL-8 in hypercholesterolemic patients (26), and natural killer cells secrete IL-8 in response to LDL (27). Lipoproteins also stimulate endothelial cells to produce IL-8 (28), which favors their proliferation and migration (29). Together with the results presented here, these studies indicate that a great variety of the blood vessel cell types and cells able to infiltrate blood vessels have the capacity to secrete IL-8 in response to lipoproteins.

It has been demonstrated that under hypercholesterolemic conditions there is an induction of angiogenesis in the adventitia, which may be a part of the early atherosclerotic remodeling (30). This is accompanied by an increase in the vessel wall area of hypercholesterolemic coronary arteries. The observed changes in the vascular wall in response to hypercholesterolemia may therefore involve IL-8 production from several distinct cell types.

IL-8-mediated spreading of fibroblasts (and possibly other cell types) in blood vessel walls could have different outcomes. The increase in cell surface, which facilitates wound healing, is considered as positive remodeling. However, chronic production of IL-8, as a result of hyperlipidemia for example, would trigger hyperplasia that can contribute to vessel wall thickening and accelerate the atherogenic process. This, along with the fact that thickening of blood vessels can be inhibited by p38 MAPK inhibitors (31), supports the notion that activation of the p38 MAPK pathway could participate in the thickening of the arterial walls observed during atherogenesis.

It will be important in the future to determine the molecular mechanisms underlying adventitia transformation in order to better understand its role in vascular remodeling in respect to pathological situations such as atherosclerosis. Our results highlight the possibility that adventitial fibroblasts, by secreting IL-8 and modulating cell shape changes, may play important roles in the development of atherosclerosis.

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