A novel chemokine, Leukotactin-1, induces chemotaxis, pro-atherogenic cytokines, and tissue factor expression in atherosclerosis

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Abstract

Chemokines such as monocyte chemoattractant protein (MCP) -1 and interleukin (IL)-8 are known to be involved in various processes in atherosclerosis such as plaque formation, plaque rupture, and thrombus formation. We investigated whether a new chemokine, Leukotactin (LKN)-1, is involved in atherosclerosis. We tested the expression of LKN-1 by immunohistochemical methods in carotid atherosclerotic plaque specimen. Induction of pro-inflammatory cytokines, transmigration, and tissue factor (TF) expression were tested in THP-1 cells and human peripheral blood monocytes treated with recombinant human LKN-1. Immunohistochemical analyses revealed that expression of LKN-1 occurs in regions of plaques rich in foam cells. In a Boyden chamber assay, THP-1 cells treated with 0.01–10 nM of LKN-1 transmigrated through gelatin coated filters in a dose dependent manner. LKN-1 also induced the transient expression of TNF-α, IL-8, and MCP-1 within 15 min of the treatment of the THP-1 cells. When peripheral blood monocytes were treated with LKN-1, expression levels of TF and TF-mediated procoagulating activity were induced in a time- and dose-dependent manner. These results raise the possibility that LKN-1 is another chemokine that is involved in the atherogenesis. LKN-1 may chemoattract immune cells into the plaque, induce pro-inflammatory cytokines, and produce thrombi by inducing TF expression. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Chemokines have been seen to be involved at many stages of atherosclerosis. Chemokines such as monocyte chemoattractant protein (MCP) -1 and interleukin (IL) -8 are expressed by foam cells, smooth muscle cells (SMCs), and endothelial cells [1–3] after stimulation with various atherogenic stimuli such as oxidized-LDL, thrombin, pro-inflammatory cytokines, complement, mechanical strain, and nitric oxide [3–8]. Chemokines induce adhesion of circulating peripheral blood mononuclear cells to the endothelium by expression of adhesion molecules such as selectins (E, L and P), ICAM-1, and VCAM-1, and the beta 2 and beta 1
integrins (e.g. LFA-1 and VLA-4) by the endothelium and leukocytes [9]. MCP-1 also activates human monocytes to express pro-inflammatory cytokines such as IL-1 and IL-6, which mediate various processes involved in the formation of fatty streaks and advanced complicated lesions [10].

The stability of atherosclerotic plaques depends greatly on the integrity of the fibrous cap, which in turn depends on its content of extracellular matrix protein. Dysregulation between matrix degrading enzymes, matrix metalloproteinases (MMPs), and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs), is believed to be responsible for the rupture of atherosclerotic plaques [11]. IL-8, a member of CXC (α-type) chemokines, was reported to activate MMP expression [12] and to suppress TIMP expression [13]. Tissue factor (TF) is expressed on the surface of activated monocytes and SMCs in the plaque. When the atherosclerotic plaque ruptures, TF may initiate coagulation protease cascades, which generate thrombin and lead to fibrin deposition [14,15]. MCP-1, a member of CC (β-type) chemokines, was reported to induce the expression of TF in human SMCs and THP-1 cells [16], as well as peripheral blood monocytes [17].

Identification and characterization of a novel chemokine, named leukotactin-1 (LKN-1), was reported by Youn et al. [18]. LKN-1 is a CC chemokine and is composed of 92 amino acids. A recombinant LKN-1 is a potent chemotactant for neutrophils, monocytes, and lymphocytes. It exerts its activity through the chemokine receptors CCR1 and CCR3. Although the in vitro biological activity of LKN-1 has been tested, the role of LKN-1 under normal or pathological conditions remains unknown. We hypothesize that the potent chemotactic activity of LKN-1 might play a role in atherogenesis. To investigate this possible role of LKN-1 in atherosclerosis, we looked at the expression of LKN-1 in atherosclerotic plaques. We also studied the activity of human recombinant LKN-1 in the induction of various responses associated with the development of atherosclerosis. Analyses revealed that LKN-1 is expressed in atherosclerotic plaques and induces transmigration of monocytic cells. LKN-1 also induced the expression TF in human peripheral blood monocytes. These data indicate that LKN-1 may be a chemokine with multiple roles in atherosclerosis.

2. Methods

2.1. Histological analysis

For immunohistochemical analysis, specimen were obtained from 15 patients, aged 63–81, who underwent carotid endarterectomy at Samsung Seoul Hospital, Seoul, South Korea. The study was approved by an institutional review committee and the subjects gave informed consent. The atherosclerotic plaque specimen were washed with saline solution and then either fixed with 4% paraformaldehyde or embedded in OCT compound within 1 h after removal to make paraffin or frozen blocks, respectively. Normal artery samples were obtained from remaining coronary arteries of the donor after cardiac transplantation. Standard 5-μm sections of the tissues were stained using the LSAB kit (DAKO, Copenhagen, Denmark) according to the protocol provided by the manufacturer. Rabbit polyclonal antibody against recombinant human LKN-1 was used for the detection of LKN-1 in plaques [18]. Monoclonal antibodies to CD68 (KP1), and SMC α-actin (1A4) were purchased from DAKO (Glostrup, Denmark). Monoclonal antibody to HLA-DR (G46-6 (L243)) was purchased from Parmingen (San Diego, CA, USA). Monoclonal antibody to oxidized-LDL (4E6) was generously provided by Dr P. Holvoet (University of Leuven, Belgium).

2.2. Cell culture

Human monocytic leukemia THP-1 cells [19] were obtained from the American Type Culture Collection (Rockville, MD). Whole blood was collected either in heparin vacutainer or CTAD Diatubes (Becton Dickinson/Diagnostica Stago) containing dipyridamole and theophylline to prevent in vitro platelet activation. Peripheral blood monocytes were obtained as described previously [18].

2.3. Production and purification of rLKN-1

Recombinant LKN-1 (rLKN-1) was produced in the High Five insect cell line (Invitrogen, San Diego, CA) grown in EX-cell 400-medium (JRH Biosciences, Lenexa, KS). rLKN-1 was purified from culture media by heparin and HiTrap-SP columns (Pharmacia). Fractions that showed a single band of ~9 kDa on SDS-PAGE were pooled. Purified rLKN-1 were dialyzed against PBS (Life Technologies, Grand Island, NY) then analyzed by immunoblotting with rabbit polyclonal anti-LKN-1 [18]. Endotoxins in the rLKN-1 were removed by Affi-prep polymixin matrix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

2.4. Flow cytometric analysis

Flow cytometric analysis of peripheral blood monocytes was performed on FACS-vantage (Becton-Dickinson, Mountain View, CA). Whole blood was diluted 10-fold with RPMI medium without serum and incubated in a CO₂ incubator during the treatment with rLKN-1. After incubation, 1 ml of cell suspension was
pelleted and the cells were double stained with FITC labeled anti-TF antibody and PE labeled anti-CD14 antibody (purchased from American Diagnostica Inc (Greenwich, CT, USA) and Caltag Laboratory (Burlingame, CA, USA), respectively), following a procedure that has been described previously [20]. Upon flow cytometric analysis, the data obtained from 30,000 cells were collected and analyzed. The monocytes were electronically isolated by recording the dual-histogram of a 90° light scatter versus a forward angle light scatter. CD 14 positive cells were then analyzed for TF expression levels, which are presented as the mean fluorescence intensity (MFI) for each measurement.

2.5. Transmigration assay

Migration of cells was assessed in a 48-well microchamber Neuroprobe, Cabin John, MD, USA) as previously described [18]. Briefly, the lower wells were filled with 27 µl serum-free RPM11640 containing LKN-1, IL-8, or MCP-1 (R&D systems, McKinley Place, NE, USA), and the upper wells were filled with 56 µl of THP-1 cells at a concentration of 2 × 10⁶ cells per ml. The two compartments were separated by a gelatin-coated polyvinylpyrrolidone-free filter (Nucleopore, Neuro Probe Inc, Gaithersburg, MD, USA) with 8-µm pores. After incubation for 8 h at 37 °C, the number of cells that migrated was determined using a hemocytometer. For gelatin-coating, the filters were incubated in 0.01% gelatin and 0.1% acetic acid at 4 °C overnight.

2.6. Clotting test

Peripheral blood monocytes were isolated, seeded in 7.5 × 10⁴ cells per well in 96-well plates in triplicate, and stimulated with 1 µg/ml LPS, 10 nM MCP-1 or 10–100 nM LKN-1 for 4 h. Procoagulating activity was assayed by determining the acceleration of clotting of recalcified normal human citrated platelet-poor plasma in a one-stage clotting test [21]. The clotting time measured after addition of a homogenate obtained from unstimulated cells was used as a reference point, and coagulating activity represents the clotting time shortened by the addition of a homogenate obtained from stimulated cells. For the blocking experiment, 0.1 µg of anti-TF or anti-CD14 monoclonal antibody was added to the cell homogenate and the sample incubated for 5 min before performing the clotting assay.

3. Results

3.1. Expression of LKN-1 in activated monocytes and atherosclerotic plaques

The immunohistological analysis revealed the expression of LKN-1 in foam cell-rich regions in 14 out of 15 specimen tested. The LKN-1 positive foam cells were found mainly in the shoulder region of the plaque (Fig. 1A and B, F and G) or the boundary of the necrotic core. The expression pattern of LKN-1 overlapped with the presence of oxidized-LDL and HLA-DR (Fig. 1C–E). When normal coronary arteries were analyzed, LKN-1 expression was not detected at all (Fig. 1H–J).

When peripheral blood monocytes were stimulated with LPS, an upregulation of the LKN-1 mRNA level was observed within 3 h after activation along with an upregulation of IL-8 and MCP-1 mRNA levels (Fig. 2).

3.2. LKN-1 induces transmigration

LKN-1 was shown to have a potent chemotactic activity when compared with macrophage inflammatory protein (MIP)-1α and IL-8 [18]. Since MCP-1 and IL-8 are the chemokines known to be involved in atherosclerosis, we tested the chemotactic activity of LKN-1 in THP-1 cells and compared it with IL-8 and MCP-1 in a boyden chamber assay. THP-1 cells transmigrated the gelatin coated filter in a dose dependent manner at concentration of 0.1–10 nM (Fig. 3).

Fig. 1. Immunohistochemical analyses reveal expression of LKN-1 in atherosclerotic plaques. Human carotid endoarterectomy specimen were stained for foam cell specific marker, CD68 (A); LKN-1 (B); smooth muscle cell specific marker, α-actin (C); HLA-DR (D); oxidized-LDL (E); in frozen sections as described in Section 2 (× 400). Consecutive paraffin sections of human carotid endoarterectomy specimen were stained for CD68 (F); and LKN-1 (G); (× 2000). Consecutive sections of normal coronary artery were stained with antibodies specific to CD68 (H); LKN-1 (I); and α-actin (J); (× 1000). M, media; L, lumen.
Peripheral blood monocytes were isolated and activated with 1 µg/ml LPS. Expression levels of cytokine mRNA were analyzed at various time points by Northern blot analysis. For comparison of the amounts of total RNA loaded in each lane, the 28S RNA bands are shown.

THP-1 cells were treated with 100 nM of LKN-1, expression of MCP-1, TNF-α, and IL-8 was detected within 15 min after treatment and the level then returned to its original level within 1 h (Fig. 4). Expression of IL-1β was not induced by the treatment with LKN-1. When THP-1 cells were treated with LPS, the maximum cytokine mRNA levels were reached in 2 h and the high expression levels were sustained even after 4 h.

3.4. LKN-1 induces tissue factor expression in monocytic cells

When peripheral blood cells were treated with 100 nM LKN-1 and expression levels of TF were measured at various times after treatment, the highest expression level was detected 24 h after treatment (Fig. 5A). When peripheral blood cells were treated with 10 and 100 nM LKN-1, monocytic TF expression levels increased in a dose dependent manner (Fig. 5B). Since TF is responsible for the initiation of the cell mediated coagulation cascade, we tested the pro-coagulating activity of peripheral blood monocytes after treatment with LKN-1. LKN-1 treatment resulted in a dose-dependent increase in pro-coagulating activity, which could be blocked with anti-TF antibody but not with anti-CD14 antibody (Fig. 5C).

4. Discussion

4.1. Expression of LKN-1 in atherosclerosis

Activation of peripheral blood monocytes with LPS induced expression of LKN-1 along with other
Fig. 5. LKN-1 induces expression of TF and procoagulating activity. (A) Whole blood samples were incubated with 100 nM rhLKN-1 for the indicated times, and monocytic TF expression levels were measured by flow cytometry as described in Section 2. As a control, measured value for a blood sample treated with 1 μg/ml LPS for 6 h is shown. In the inset, the fluorescence profiles of background staining (BG) and a sample treated for 6 h with LKN-1 are compared. (B) Whole blood samples were incubated with 10 and 100 nM concentrations of rhLKN-1 or with 1 μg/ml LPS for 6 h, and monocytic TF expression levels were measured. In the inset, the fluorescence profiles obtained from the background staining (BG) and a sample treated with 100 nM LKN-1 are compared. (C) Human peripheral blood monocytes were isolated and treated with 10–100 nM rhLKN-1, 10 nM MCP-1, or 1 μg/ml LPS for 4 h, and procoagulating activity was measured as described in Section 2. The clotting time measurement for the unstimulated monocytic cells was 427 s (mean of triplicate measurements). Coagulating activity was blocked by pre-treatment with monoclonal antibody specific to TF (black bar) but not by pre-treatment with monoclonal antibody specific to CD14 (empty bar).

4.2. Role of LKN-1 in atherosclerotic plaque formation

Atherosclerosis is now considered an inflammatory disease. The initial process of atherogenesis is the transmigration of circulating immune cells such as monocytes and T-lymphocytes through the endothelial layer into the intimal area in the vessel wall [22]. Our data showing that LKN-1 is an efficient chemoattractant (Fig. 3) strongly support the conclusion that LKN-1 expression by monocytic cells in the plaque attract more monocytic cells which will then further contribute to inflammatory processes.

4.3. Role of LKN-1 in thrombus formation after the rupture of an atherosclerotic plaque

TF plays an important role during the acute phase of atherosclerotic coronary artery disease. Expression of TF was detected in atherosclerotic plaque, and the number of TF expressing monocytes in patients with acute coronary syndrome (ACS) was increased. TF expressed on the surface of activated monocytes and macrophages aggravates thrombus formation when the plaque ruptures [23–25]. The observation that the treatment of peripheral blood monocytes with LKN-1 can induce TF expression and TF-dependent pro-coagulating activity suggests that the expression of LKN-1 could be one of the factors responsible for the formation of thrombi upon the rupture of plaques (Fig. 5). Our data indicate that LKN-1 could be a novel mediator of atherosclerosis by inducing chemotaxis and the expression of pro-atherogenic cytokines and TF. The multifunctional role of LKN-1, along with that of other chemokines such as MCP-1 and IL-8, suggests that the manipulation or inhibition of functions of these chemokines or the inhibition of processes that lead to the activation of these chemokines could be beneficial in the treatment of atherosclerosis and coronary artery diseases.

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