Immunohistochemical localization of endothelial cell-derived lipase in atherosclerotic human coronary arteries

Hiroshi Azumi, Ken-ichi Hirata, Tatsuro Ishida, Yoko Kojima, Yoshiyuki Rikitake, Shigeto Takeuchi, Nobutaka Inoue, Seinosuke Kawashima, Yoshitake Hayashi, Hiroshi Itoh, Thomas Quertermous, Mitsuhiro Yokoyama

A Division of Cardiovascular and Respiratory Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
B Division of Surgical Pathology, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
C Donald W. Reynolds Cardiovascular Clinical Research Center, Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, CT, USA

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Abstract

Objective: A novel lipoprotein lipase (LPL)-like gene, endothelial cell-derived lipase (EDL), was recently cloned from vascular endothelial cells. The presence of LPL in the vascular wall has been implicated in the progression of atherosclerosis through the bridging function between lipoprotein particles and matrix proteoglycans to enhance lipoprotein uptake into the vascular wall. The aim of this study was to investigate the local expression of EDL in human coronary arteries. Methods and Results: Human coronary arterial specimens from 10 autopsied cases were examined by immunohistochemistry with polyclonal antibodies against specific synthetic EDL peptides. Immunohistochemical analysis revealed that EDL was expressed in endothelial cells and medial smooth muscle cells in non-atherosclerotic coronary arteries. In addition, EDL was expressed in infiltrating cells within atheromatous plaques as well as endothelial and smooth muscle cells. Double labeling immunofluorescence confirmed EDL positive-cells were endothelial cells, smooth muscle cells and macrophages. EDL immunoreactivity was also detected in neovasculature within atheromatous plaques in atherosclerotic coronary arteries. Conclusions: These results suggest that EDL may have unique functional roles in the pathogenesis of coronary artery diseases such as atherosclerosis as well as in lipid metabolism in the vessel wall.

Keywords: Atherosclerosis; Endothelial factors; Enzyme; Lipid metabolism; Lipoproteins

1. Introduction

Lipoprotein metabolism has a central role in the etiology of atherosclerosis. The lipase gene family, including lipoprotein lipase (LPL) and hepatic lipase (HL), are key enzymes that hydrolyze triglycerides and phospholipids in circulating lipoproteins to provide free fatty acids for energy consumption and storage [1–3]. In addition to their enzymatic activity, lipases are thought to play a role in the progression of atherosclerosis through the accumulation of lipoproteins in the vascular wall [4,5]. Recently, we [6] and another group [7] have independently cloned endothelial cell-derived lipase (EDL), a new lipase that belongs to LPL gene family. Since overexpression of EDL in mice markedly reduced plasma concentrations of high-density lipoprotein (HDL) cholesterol, and targeted disruption of...
EDL increased HDL cholesterol level, EDL is considered to be a major regulator of HDL metabolism [7,8]. As EDL shares sequence homology with other lipases including LPL and HL, it is speculated that EDL may accumulate lipoproteins at the vascular wall like LPL.

The aim of this study was to investigate the localization of EDL in human coronary arteries to understand the functional role of EDL in the vascular disease such as atherosclerosis. In the present study, EDL expression was identified in endothelial and medial smooth muscle cells in normal and atherosclerotic coronary arteries. In addition, EDL was detected in infiltrating cells such as macrophages and smooth muscle cells in the atheromatous plaque. The local expression of EDL within human coronary arteries, taken together with its sequence homologies with LPL and HL, suggests that EDL may have unique functional roles in pathogenesis of coronary artery diseases as well as lipid metabolism in the vessel wall.

2. Methods

2.1. Human tissue

Human coronary artery tissues were obtained from 10 autopsy cases (age ranged between 47 and 83 years) within 5 h after death, after informed consents from their families. For immunohistochemical examination, tissues were embedded in O.C.T. compound, prepared by snap-freezing in liquid nitrogen, and stored at −80 °C until use. Serial cryostat sections (5-μm thick) were cut and fixed with 100% acetone at −20 °C for 10 min. All sections were examined by hematoxylin–eosin stain, and classified into non-atherosclerotic and atherosclerotic coronary arteries according to the reports of the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association [9–11]. A total of 12 non-atherosclerotic and 15 atherosclerotic samples were evaluated for histology. In non-atherosclerotic coronary arteries, only mild and diffuse adaptive intimal thickening was observed. On the other hand, various pathological changes including fatty streak, accumulation of lipid, intimal disorganization, and infiltration of inflammatory cells were observed in atherosclerotic segments.

2.2. Cell culture

Human coronary arterial smooth muscle cells (HCASMCs) and human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA, USA) and subcultured in our laboratory using media supplied by the manufacturer. HeLa cells were obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

2.3. Immunohistochemistry

Immunohistochemistry was performed as previously described [12]. The sections were incubated with 3% H₂O₂ in methanol to eliminate endogenous peroxidase for 20 min, washed in 50 mM Tris–HCl buffer (pH 7.6), blocked with carrier protein (Dako LSAB kit, Dako A/S, Glostrup, Denmark) for 60 min at room temperature, and then incubated with primary antibody overnight at 4 °C. Primary antibodies were two different rabbit polyclonal anti-human EDL antibodies developed against two different synthetic peptides (residues 422 to 445, SWYNLWKFRSYLSQPRNGRELN) and (residues 475 to 494, GRELWFRKCRDJWWRMKNETS). After washing with Tris–HCl buffer (pH 7.6), sections were incubated with biotinylated goat anti-rabbit immunoglobulins (Dako A/S) at a dilution of 1:300, washed in Tris–HCl buffer (pH 7.6), and then incubated with streptavidin–horseradish peroxidase conjugate (Dako A/S). After color development with 0.03% diaminobenzidine, the sections were counterstained with Mayer’s hematoxylin for 120 s. As negative control, the primary antibody was replaced with rabbit non-specific immunoglobulin.

2.4. Double labeling immunofluorescence

To identify cell types expressing EDL, double labeling immunofluorescence was performed [12]. The sections were blocked with carrier protein (Dako A/S) for 60 min at room temperature, and incubated with anti-EDL antibodies and antibodies against cell specific markers overnight at 4 °C. These antibodies were mouse monoclonal anti-human CD68 antibody (clone KP-1, Dako A/S) for macrophages at a dilution of 1:1,000, mouse monoclonal anti-human smooth muscle α-actin antibody (clone 1A4, Dako A/S) for smooth muscle cells at a dilution of 1:100, and mouse monoclonal anti-human von Willebrand factor antibody (clone F8/86, Dako A/S) for endothelial cells at a dilution of 1:50. After 15 min, slides were washed with 50 mM Tris–HCl buffer (pH 7.6), Texas Red-conjugated donkey anti-rabbit immunoglobulins (Amersham Biosciences, Tokyo, Japan) at a dilution of 1:50 and fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulins (Amersham) at a dilution of 1:50 were applied as secondary antibodies for 1 h at room temperature. These samples were examined by laser scanning confocal imaging system (MRC-1024, Nippon Bio-Rad Laboratories, Tokyo, Japan). The presence of EDL was demonstrated by red immunofluorescence labeling. The presence of cell markers was demonstrated by green
immunofluorescence labeling. Colocalization of red and green labeling was shown by yellow immunofluorescence labeling. Immunofluorescence of HCASMCs, HUVECs and HeLa cells was carried out with the same method using EDL antibodies as primary antibody and Texas Red-conjugated anti-rabbit immunoglobulins as second antibody.

2.5. Northern blotting

Total RNA was isolated from cultured cells by the acid guanidium thiocyanate–phenol–chloroform extraction method. For Northern blot analysis, 20 µg of total RNA was size fractionated on 1.3% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes. Membranes were then hybridized with a human EDL cDNA fragment radiolabeled with [32P]dCTP by random priming. Blots were hybridized at 42 °C for 16–24 h in the presence of 48% formamide and 10% dextran sulfate. After hybridization, the membranes were washed at high stringency conditions, 65 °C in the presence of 0.2×SSC buffer and 0.5% sodium dodecyl sulfate (SDS). Visualization was achieved by exposure to Hitachi Bas 2000 (Hitachi, Tokyo, Japan).

2.6. Western blotting

Culture cells were disrupted in ice-cold homogenate buffer [50 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, and 0.1 mM EGTA] containing protease inhibitors (0.5 mM leupeptin, and 0.7 mM pepstatin A) by a sonicator (VP-5s, Taitec, Japan). Samples were ultracentrifuged at 100,000 g for 60 min at 4 °C and separated into supernatants and pellets. Each supernatants and pellets were dissolved in the buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, 1% Triton-X, and 0.1% SDS] with proteinase inhibitors, and sonicated. Protein concentrations of cell extracts were determined by the method of Bradford [13]. After measurement of protein content, 100 µg of each protein sample was heat-denatured with Laemmli buffer containing 2% 2-mercaptoethanol, electrophoresed on 15% polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in TBS-T [10 mM Tris–HCl (pH 7.6), 100 mM NaCl, 0.1% Tween 20] containing 5% nonfat dry milk for 1 h. EDL protein expression was detected by incubating the membranes with polyclonal anti-human EDL antibody at a dilution of 1:1,000. The membranes were washed, hybridized with horseradish peroxidase-labeled donkey anti-rabbit immunoglobulins; (Amersham) at a dilution of 1:1,000, developed with the ECL chemiluminescence method (Amersham), and then exposed to X-ray film.

3. Results

We employed immunohistochemistry using polyclonal antibodies against two different peptides of human EDL to examine the expression of EDL in human coronary arteries. Both antibodies showed identical staining in human coronary arteries (Fig. 1). In non-atherosclerotic coronary arteries,...
arteries with mild diffuse intimal thickening, immunoreactivity of EDL was detected in endothelial cell layer and medial smooth muscle cells (Fig. 1A–C). EDL was also detected in nutrient arteries in adventitia. In atherosclerotic coronary arteries, various atherosclerotic changes were observed including hypercellular lesions and advanced atheromatous lesions such as fibrous and lipid-rich plaques. EDL was also detected in endothelium and medial smooth muscle cells in atherosclerotic arteries (Fig. 1E–G). In addition, the expression of EDL was detected in accumulating cells, macrophages (Fig. 1G and H) and smooth muscle cells (Fig. 1G and I), in the atherosclerotic lesion.

EDL immunoreactivity was detected in the neovascularure, which was found within atherosclerotic plaques in obstructive coronary arteries (Fig. 2A). By higher magnification, EDL immunoreactivity was observed in endothelial cells and medial smooth muscle cells in the neovascularure (Fig. 2B and C). The formation of neovascularure in the advanced atherosclerotic lesions was characterized by the immunostaining of von Willebrand factor, an endothelial marker, for vascular endothelial cells (Fig. 2D, E and F).

To identify EDL-expressing cells, double labeling immunofluorescence was performed (Fig. 3). Double staining demonstrated that von Willebrand factor was positive in EDL-expressing cells in endothelium in both non-atherosclerotic and atherosclerotic coronary arteries (Fig. 3A–C). EDL-expressing infiltrating cells seen in atherosclerotic lesion were also positive for CD68, a marker of macrophages (Fig. 3D–F). Moreover, EDL positive cells in the medial layer (Fig. 3G–I) and in atherosclerotic lesions (Fig. 3J–L) were positive for α-smooth muscle actin and thus identified as smooth muscle cells (Fig. 3G–I).

To further evaluate the expression of EDL in smooth muscle cells, Northern blot, Western blot and immunostaining experiments were performed with cultured HUVECs and HCASMCs. Human cDNA fragments were employed as 33P-labeled probes for hybridization to blots containing total RNA (20 µg) isolated from HUVECs and HCASMCs. A dominant band was observed at the expected size, approximately 4.4 kilobases, in both RNA samples (Fig. 4A). When cell lysates were electrophoresed, transferred to nitrocellulose membrane and probed with the anti-human EDL antibody, a band was visualized that corresponded to the expected protein size of 62 kDa. EDL protein was found in the membrane and cytosolic fractions (Fig. 4B). Immunostaining experiments confirmed that EDL protein was expressed in HUVECs and HCASMCs, but not in HeLa cells (Fig. 4C).

4. Discussion

Because of implications for pathogenesis of atherosclerosis, localization of LPL in the vessel wall has been
Fig. 3. Immunofluorescence microscopy for EDL in human coronary arteries. Double labeling immunofluorescence was employed with confocal microscopy to identify vascular cells expressing EDL. Labeling with cell-specific markers is shown in the left panels; anti-von Willebrand factor (vWF) antibody for endothelial cells (A), anti-CD68 antibody for macrophages (D), and anti-α-smooth muscle actin (α-SA) antibody for smooth muscle cells (G and J). Middle panels (B, E, H and K) are immunofluorescence labeling of EDL protein. Right panels (C, F, I and L) are double immunofluorescence. Colocalization of cell specific marker (green) and EDL (red) was shown by yellow label immunofluorescence. EDL was expressed in endothelial cells (A–C) and medial smooth muscle cells (G–I). In addition, EDL was also expressed in infiltrating macrophages (D–F) and smooth muscle cells (J–L) in atherosclerotic lesions. (scale bar, 10 μm).
extensively studied. LPL is synthesized by several different types of parenchymal cells including adipocytes, muscle cells, and macrophages, and translocates to functional binding sites on the surface of vascular endothelial cells [14–16]. LPL protein has been identified within atherosclerotic vessels [4,15], and hypothesized to promote atherosclerosis by bridging between lipoprotein particle and matrix proteoglycans, resulting in accumulation of lipoproteins in the vessel wall [4,5,17]. EDL is a member of the lipase gene family and shares sequence homology with LPL and HL [6,7,18,19]. Given potential link between local vascular wall expression of a lipase and vascular disease such as atherosclerosis, we employed immunohistochemical analysis to investigate the expression of EDL in human coronary arteries. In the present study, EDL was expressed in the endothelial cells and medial smooth muscle cells in human coronary arteries. In addition, EDL was also expressed in the neovascularization in atherosclerotic lesion of coronary arteries. In contrast to LPL and HL, EDL is produced by endothelial cells and upregulated in the early phase of endothelial tube formation in an in vitro model of angiogenesis [6]. Moreover, we showed EDL is also produced by cultured human coronary arterial smooth muscle cells. Local expression of EDL in human coronary arteries taken together with its phospholipase A activity in vitro suggests that EDL may supply free fatty acids from phospholipid as an energy source for endothelial cells and smooth muscle cells in the blood vessels to maintain vascular function including cell proliferation and angiogenesis.

LPL gene expression has been identified in the vessel wall in macrophages [4,15,20]. LPL is postulated to facilitate the receptor-mediated uptake of lipoproteins by macrophages [21,22]. In contrast to many beneficial effects...
of LPL on lipoprotein metabolism in circulating blood [23]. LPL expressed in the vessel wall has been thought to be an atherogenic factor. A striking association between LPL activity in macrophages and susceptibility to develop atherosclerosis has been documented in inbred mouse lines, and high macrophage LPL is considered a potential risk factor for coronary artery disease [24]. In the present study, we demonstrated that EDL was expressed in infiltrating cells such as smooth muscle cells, and macrophages in atherosclerotic lesion of human coronary arteries. Further study is required to examine the effect of EDL on uptake of lipoproteins by macrophages.

Atherosclerosis is now widely considered to be an inflammatory process [25], and inflammatory cytokines such as IL-1β and TNF-α are thought to have an important role in initiating the expression of a variety of genes that in turn promote cell adhesion and other processes that are required for atherosclerotic progression. We have reported that EDL expression in endothelial cells is highly regulated in vitro by cytokines and physical forces considered to be involved in the pathogenesis of vascular disease including atherosclerosis and hypertension [26]. In addition, EDL was cloned independently by another group as a result of performing differential display in THP-1 cells after exposure to oxidized LDL [7]. From these observations, we speculate that EDL may be induced in vascular cells including macrophages by atherosclerotic stimuli, and may regulate the atherosclerotic process by facilitating accumulation of lipoproteins in the vessel wall.

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References

[25] Renier G, Skamene E, Desanctis JB, Radzioch D. High macrophage lipoprotein lipase expression and secretion are associated in inbred...
