Cloning of a Unique Lipase from Endothelial Cells Extends the Lipase Gene Family

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A new lipoprotein lipase-like gene has been cloned from endothelial cells through a subtraction methodology aimed at characterizing genes that are expressed with in vitro differentiation of this cell type. The conceptual endothelial cell-derived lipase protein contains 500 amino acids, including an 18-amino acid hydrophobic signal sequence, and is 44% identical to lipoprotein lipase and 41% identical to hepatic lipase. Comparison of primary sequence to that of lipoprotein and hepatic lipase reveals conservation of the serine, aspartic acid, and histidine catalytic residues as well as the 10 cysteine residues involved in disulfide bond formation. Expression was identified in cultured human umbilical vein endothelial cells, human coronary artery endothelial cells, and murine endothelial-like yolk sac cells by Northern blot. In addition, Northern blot and in situ hybridization analysis revealed expression of the endothelial-derived lipase in placenta, liver, lung, ovary, thyroid gland, and testis. A c-Myc-tagged protein secreted from transfected COS7 cells had phospholipase A1 activity but no triglyceride lipase activity. Its tissue-restricted pattern of expression and its ability to be expressed by endothelial cells, suggests that endothelial cell-derived lipase may have unique functions in lipoprotein metabolism and in vascular disease.

The process of angiogenesis, the formation of new blood vessels, is central to physiological and pathophysiological conditions including placental development, wound healing, diabetic retinopathy, and tumor growth (1–3). Because of the great potential for treatment of human disease through manipulation of angiogenesis, fundamental information regarding the molecular pathways that regulate endothelial cell differentiation is of central importance. To study the cellular and molecular basis of angiogenesis, a number of in vitro models of vascular formation have been derived, whereby cultured endothelial cells produce a capillary-like network on various extracellular matrices. In particular, a tumor-derived basement membrane preparation composed primarily of laminin, collagen, and growth factors has been shown to reproducibly induce human umbilical vein endothelial cells (HUVEC) to form a 3-dimensional capillary network (4, 5).

Through their functional activities, the proteins encoded by the lipase gene family are intimately linked to the endothelium. Lipoprotein lipase (LPL) is synthesized by several different types of parenchymal cells, including adipocytes, muscle cells, and macrophages. However, LPL protein released by these cells translocates to functional binding sites on the surface of vascular endothelial cells, where it participates in lipoprotein metabolism and uptake (6). Hepatic lipase (HL) is synthesized and secreted by hepatocytes where some protein is bound to the cell surface. In addition, HL translocates from the hepatocyte surface to the endothelial surfaces of liver sinusoids. Despite the association of both LPL and HL with vascular endothelium, extensive previous studies have failed to detect production of either lipase in endothelial cells (7). The critical association between lipids and vascular disease coupled with the central roles of LPL and HL as modulators of lipid levels suggests an intimate relationship between these enzymes and the vasculature (8–12). One of these lipases, LPL, has been implicated in the genesis and progression of atherosclerotic disease in the blood vessel wall through local expression (13). Further information regarding the role of the endothelial cell in lipid metabolism would thus be of great significance.

We have used the in vitro model of endothelial cell tube formation on the basement membrane material Matrigel coupled with a polymerase chain reaction-based suppression subtractive hybridization cloning strategy to identify genes that may be activated during vascular formation (14). One of these genes was found to encode a new member of the lipase gene family, endothelial cell-derived lipase (EDL). Despite a high sequence similarity to LPL, EDL was found to be expressed in a distinct and complementary tissue-restricted fashion, with high level expression in the liver, placenta, lung, and steroid hormone-producing organs. The cloning and characterization of EDL provides the first evidence for lipase production directly by the endothelial cell. Production of a lipase in the vessel wall would provide a mechanism for local regulation of lipolytic activity, allowing the vessel to participate in lipid metabolic processes that are related to atherosclerosis and other vascular diseases, as well as those that are related to angiogenesis.

* This work was supported in part by Grant KO8 HL 03865 01 (to H. L. D.) and a sponsored research agreement with Progenitor, Inc., Menlo Park, CA (to T. Q.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF118767 and AF118768.

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‡‡The abbreviations used are: HUVEC, human umbilical vein endothelial cells; apo, apolipoprotein; EDL, endothelial cell-derived lipase; HL, hepatic lipase; LPL, lipoprotein lipase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Cell Culture—HUVEC and human coronary artery endothelial cells were obtained from Clonetics, Inc. (San Diego, CA). Namalwa (human B-cell malignancy), MOLT4 (human T-cell malignancy), JEG3 (human choriocarcinoma cells), HeLa (human epithelial cell tumor), 143B (human osteosarcoma cells), A549 (human lung epithelial cells), MEL (mouse erythroleukemia cell line), NIH3T3 (mouse embryonic fibroblast cell line), C2C12 (mouse myoblast cell line), RAW 264.7 (mouse monocyte/macrophage cell line), HepG2 (human hepatoma cell line), Hepa 1–6 (mouse hepatoma cell line), and COS7 were obtained from American Type Culture Collection (Manassa, VA). These cells were cultured under recommended conditions. Yolk sac cells were derived and cultured as described previously (15, 16). To facilitate in vitro tube formation, HUVECs (3.9 × 10⁴ cells/cm²) were plated onto Matrigel (Becton Dickinson, Bedford, MA) at 480 µg/ml dish and incubated at 37 °C for 3 h. The medium was removed, the cells were rinsed three times with cold phosphate-buffered saline, 2 ml of MatriSperse solution (Becton Dickinson) was added to each 35 mm dish, and the cell/gel layer was scraped into 50-ml conical tubes. An additional 2 ml of MatriSperse solution was added to the dishes, which were then transferred to the tubes, and the Matrigel dissolved at 4 °C for 1 h. Released HUVEC were washed with phosphate-buffered saline three times and used for isolation of mRNA with a MicroFast Track kit (Invitrogen, San Diego, CA).

Cloning by Suppression Subtractive Hybridization—A polymerase chain reaction-based cDNA subtraction methodology employing agents supplied in the polymerase chain reaction Select cDNA Subtraction kit (CLONTECH, Palo Alto, CA), was employed to identify genes preferentially expressed in tube-forming endothelial cells (14). Tester DNAs were derived from 2 μg of total RNA from tube-forming HUVEC poly(A)⁺ RNA and driver DNA was derived from 2 μg of poly(A)⁺ RNA from growth-arrested HUVEC. Subtraction hybridization was performed according to the manufacturer's instructions, and the products of secondary polymerase chain reaction were examined by dieoxy sequencing. One cloned cDNA fragment encoded a novel sequence and was used to screen a HUVEC CAGTGGGACT-3' peptide tag (EQKLISEED), was added at the 3'-end by polymerase chain reaction with the human LPL and HL amino acid sequences revealed conservation of the catalytic residues serine (Ser-169), histidine, respectively, and that presumably are important for the catalytic pocket and serves to confer substrate specificity for the monoclonal antibodies (Roche Molecular Biochemicals). Those clones that synthesized c-Myc-tagged EDL protein sequences were for further study. Negative control clones were randomly selected from a transfection with the empty phbAPr-3-neo vector. The COST cells stably expressed EDL-c-Myc fusion protein (EDL-c-Myc/phiAPr-3-neo construct) did not express an exogenous protein (phiAPr-3-neo vector). To obtain the c-Myc-tagged EDL protein, the COST cells were cultured in serum-free DMEM with 2 units/ml heparin. After 36 h, the cells and conditioned media were harvested. The medium was concentrated from 10 ml to 1 ml by Centricon 10 (Millipore, Bedford, MA), brought to 30% final concentration glycero1, and stored at −80 °C. Cells were harvested by scraping into lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% CHAPS, 10 mM EDTA, 10% glycero1, 10 µg/ml aprotinin, 1 µM phenylmethylsulfonyl fluoride).

Triglyceride lipase activity were quantitated in triplicate using the long chain triglyceride substrate [14C]triolein activity assays (18). Phospholipase activity was measured using diacylglycerylphosphatidylcholine liposomes, which were synthesized using a modification of the triolein emulsion, Diacylglycerol phosphatidylcholine (20 mg/ml, Sigma) was used in place of egg yolk extract (18). Labeled triolein was substituted with 1.2 dl-[14C]oleoyl-1,2-diacetyl-sn-glycerol (Amersham Life Science) at an activity of 0.06 µCi/ml of substrate. Substrate (200 µl) was added to 100 µl of medium from transfected cells in a final volume of 330 µl (150 mM NaCl, 100 mM Tris-HCl, pH 8.5, 2.5% bovine serum albumin, 2 units/ml heparin (Eli Lilly and Co., Indianapolis, IN)) in the presence or absence of 10 µl of human plasma as source of apoC-III (19). The samples were incubated at 37 °C for 2 h followed by oleic acid extraction and scintillation counting (20). The presence of functional catalytic residues was established in an assay employing the water soluble substrate p-nitrophenylbutyrate (21).

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of EDL—We performed subtractive suppression hybridization to isolate genes preferentially expressed in HUVEC undergoing tube formation on Matrigel, compared with growth-arrested HUVEC in monolayers (14). Two cDNA fragments isolated in this screen appeared to encode a novel lipoprotein lipase-like gene and represented a gene that was documented by Northern blot to be up-regulated in HUVEC in the early phase of tube formation on Matrigel (Figs. 1 and 2). This novel lipase is also expressed in cultured endothelial cells isolated from human coronary artery and in murine cell lines derived from the primitive yolk sac that have properties of endothelial progenitor cells (15, 16). Because there was no prior evidence for a lipase produced within endothelial cells, this new protein was named endothelial cell-derived lipase (EDL). Through subsequent screening of a HUVEC cDNA library and a mouse embryonic cDNA library, the entire open reading frame of human and mouse EDL was determined.

The open reading frames of both the human and mouse EDL sequences were 1500 nucleotides, encoding highly conserved proteins of 500 amino acids (Fig. 1). A hydrophobicity analysis of the predicted amino acid sequence revealed a hydrophobic leader peptide with a putative signal cleavage site located 18 amino acids downstream of the translation initiation site in both proteins (22). The mature human and mouse EDL proteins thus consist of 482 amino acids. EDL shares sequence similarity with the mammalian lipases, including 44% amino acid identity to LPL and 41% amino acid identity to HL. Alignment with the human LPL and HL amino acid sequences revealed conservation of the catalytic residues serine (Ser-169), aspartic acid (Asp-193), and histidine (His-274) as well as of the 10 cysteine residues involved in disulfide bridge formation (23). Similarly, two stretches of hydrophobic amino acids (163–172 and 271–279) that are adjacent to the catalytic serine and histidine, respectively, and that presumably are important for binding with lipid substrate are also conserved. In addition, like LPL and HL, EDL possesses a lid consisting of 19 amino acids. By analogy with the predicted three-dimensional structures of LPL and HL, the EDL lid probably covers a catalytic pocket and serves to confer substrate specificity for
Because the EDL lid region has minimal sequence homology with the LPL and HL lids, the EDL substrate may be different from those of other mammalian lipases. Interestingly, the regions bordering the lid are almost identical among EDL, LPL, and HL. In addition, alignment with the human LPL sequence indicates conservation of positively charged clusters involved in heparin binding (23). The corresponding clusters in EDL include: cluster 1, Arg-327—Lys-329—Arg-330—Lys-333; cluster 2, Arg-312—Lys-313—Arg-315; cluster 3, Arg-188; and cluster 4, Lys-352—Arg-450—Lys-452–Lys-459. Finally, five potential glycosylation sites are predicted by the presence of the universal acceptor sequence Asn-Xaa-(Thr-Ser) at positions 80, 136, 393, 469, and 491. These glycosylation sites may modulate the heparin binding properties of EDL.

**Expression Analysis**—The developmental-specific and tissue-specific pattern of EDL expression was investigated by Northern blotting and \(^{35}\)S-labeled cRNA in situ hybridization. The tissue-specific expression pattern of EDL in the adult is unique and complementary to that documented for LPL. LPL is expressed in muscle tissues, adipose tissue, mammary gland, brain, and macrophages, whereas EDL is expressed in placenta, liver, lung, testis, thyroid, and corpus luteum of the ovary (6). This difference in expression pattern suggests distinct and nonoverlapping functions for these two lipases with similar primary sequence.

EDL expression in placenta was detected in both mouse and human by Northern analysis and confirmed by in situ hybridization in the mouse (Figs. 3 and 4). The signal in the 7-day embryonic mouse sample likely represents expression in the
placenta, which was included in the RNA extraction. Intense hybridization of the $^{35}$S-labeled cRNA in situ probe was detected in a crescent shape in the placenta, consistent with labeling of trophoblast cells. This very high level of EDL expression suggests that EDL may play an important role in placental lipid metabolism. Fatty acids are required by the fetus, placenta, and fetal membranes for the synthesis of complex lipids such as phospholipids, triacylglycerols, and cholesterol esters. These lipids form cell membranes, are precursors for hormones, and may provide metabolic substrates. LPL hydrolyzes maternal very low density lipoprotein triglyceride to release free fatty acid and diacylglycerol. The majority of these released fatty acids are then transferred to the fetus by an unknown mechanism. There is, however, evidence for placental phospholipid transfer involving lipid breakdown and resynthesis (26). In addition, recent studies suggest that cholesterol plays a crucial role in specific processes during mammalian embryonic development, including modification of the Hedgehog protein (27). By analogy with the hydrolytic action of HL, which may facilitate transfer of lipoprotein-derived cholesterol to the liver, the hydrolytic action of EDL may facilitate transfer of lipoprotein-derived cholesterol to the feto-placental tissues (28). Thus, EDL may facilitate the uptake of lipoprotein-derived lipids from circulating maternal blood into the fetal membranes.

EDL mRNA was detected by Northern blot at high levels in mouse lung tissue and low levels in human lung tissue. The punctate hybridization pattern detected by in situ hybridization was not consistent with alveolar type I epithelial cell or endothelial cell EDL expression but was highly suggestive of EDL expression by macrophages or alveolar type II epithelial cells (Fig. 4). Recent experiments have documented EDL expression in differentiated RAW 264.7 cells, and EDL expression in the spleen could represent macrophage expression (Figs. 2 and 3). However, Northern blotting experiments with total RNA isolated from primary mouse peritoneal macrophages have failed to detect EDL transcripts, leaving unresolved the question of EDL expression in macrophages in vivo. A role for EDL in alveolar type II epithelial cells remains an intriguing possibility. These cells synthesize surfactant, which maintains alveolar patency (29). Disaturated phosphatidylcholine, which is synthesized from long chain fatty acids, is the major lipid component of surfactant (30). Surfactant synthesis is critically dependent on the availability of free fatty acids. One source of these lipids may be very low density lipoprotein-transported triglycerides, which are hydrolyzed locally by lipases to free fatty acids (31). We propose that EDL may hydrolyze lipoprotein-derived phospholipids and thereby play an integral role in providing fatty acid or lysophospholipid substrates for surfactant phospholipid synthesis by alveolar type II epithelial cells.

EDL expression was detected by Northern blot in testis and by in situ hybridization in the corpus luteum of the ovary (Figs. 3 and 4). Expression in these tissues, in conjunction with the placenta, raises the possibility that EDL might also play a role in the uptake of lipoprotein-derived cholesterol into steroidogenic tissues. Lipoprotein-derived cholesterol is synthesized from long chain fatty acids, is the major lipid component of surfactant (30). Surfactant synthesis is critically dependent on the availability of free fatty acids. One source of these lipids may be very low density lipoprotein-transported triglycerides, which are hydrolyzed locally by lipases to free fatty acids (31). We propose that EDL may hydrolyze lipoprotein-derived phospholipids and thereby play an integral role in providing fatty acid or lysophospholipid substrates for surfactant phospholipid synthesis by alveolar type II epithelial cells.

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ting, suggesting that EDL is expressed at least in the hepatocyte of the liver (Fig. 2). In situ studies in the adult mouse did not localize EDL expression to a single cell type in the liver and could represent widespread expression in this tissue (data not shown). Interestingly, a small transcript was identified in the human embryonic liver and must represent either an alternative spliced transcript from the EDL locus or a transcript from a different homologous gene (Fig. 3). The role of EDL in the liver, an organ which plays a central role in lipid metabolism, warrants detailed study and careful comparison of the activities of EDL and HL.

EDL message was detected in one of two mouse spleen samples, where it may be expressed by macrophages. Further insights into the functional role of EDL in this organ as well as in other tissues such as the thyroid, heart, brain, and kidney will require in situ hybridization experiments to localize expression to the cellular level.

In vitro studies with cultured cells provide compelling evidence that endothelial cells can express EDL in a regulated fashion. Northern analysis of RNA isolated from HUVEC monolayers and HUVEC cultured on Matrigel verified that EDL mRNA was significantly increased in endothelial cells undergoing tube formation (Fig. 2). Northern blotting revealed EDL was highly expressed in human coronary artery endothelial cells (Fig. 2). In addition, an intense signal was detected in yolk sac cells, which express a number of endothelial cell markers and are felt to represent an in vitro model of primitive endothelial cells (33). Expression by these cells supports a potential role for EDL in embryonic vascular formation. EDL expression was detected at moderate levels in vascular organs in the mid-gestation human embryo and at low levels in the mouse embryo, consistent with a vascular expression pattern (Fig. 3). Embryonic endothelial cell expression of EDL was thus evaluated in a limited number of mouse embryo sections. Unfortunately, these preliminary in situ studies have not revealed embryonic endothelial cell expression (data not shown). Whether EDL is expressed during vascular formation in the embryo or in the adult in conditions of physiological or patho-

Fig. 4. In situ hybridization analysis of EDL expression. 35S-Labeled cRNA sense and antisense probes were hybridized to sections of murine embryos and adult tissues, with only the antisense probes showing a signal above background. Sections were photographed with brightfield (A, C, E, G) and darkfield (B, D, F, H) illumination. A and B, murine embryo at 7.5 days of development shows an intense signal over the placental tissue but no signal in the developing embryo. C and D, low power view of the lung reveals a punctate staining pattern over the alveoli. E and F, high power view of the lung, consistent with expression by type II alveolar cells or macrophages. G and H, mouse ovary shows an intense signal over corpus luteum. Photographs: A, B, G, and H at 4×; C and D at 10×, and E and F at 20×.
physiological angiogenesis will require further study.

Lipase Activity—To characterize the functional activities of EDL, and in particular, to determine its substrate specificity, we expressed recombinant protein in eukaryotic cells. An expression construct encoding a c-Myc-tagged human EDL fusion protein, EDL-c-Myc/phbAPr-3-neo, was transfected into COS7 cells. By Western blotting of cellular extracts and concentrated culture medium, it was determined that greater than 95% of the EDL-c-Myc-tagged protein was secreted into the supernatant (data not shown). The presence of functional catalytic residues in EDL-c-Myc was investigated using the water-soluble substrate p-nitrophenylbutyrate. The presence of lipid-hydrolyzing triglyceride lipase activity was investigated using the liposoluble substrate triolein. Because EDL demonstrates highest sequence similarity with LPL, and LPL activity is liposoluble, because both HL and LPL exhibit phospholipase A1 activity, the EDL-c-Myc protein did show phospholipase activity, which was approximately twice that of supernatant from vector alone-transfected cells (307 ± 25 versus 156 ± 34 nmol of free fatty acids/ml/h, p < 0.00005, Table I). Interestingly, this phospholipase A1 activity was partially inhibited by apo-CII. Thus, this novel protein demonstrates functional as well as structural characteristics of a lipase.

Summary and Implications—Data presented here showing EDL expression in a number of cultured endothelial cell lines and in a single macrophage cell line suggest that EDL can be expressed in the vessel wall. Such expression taken together with its structural and functional similarities with HL and LPL, molecules known to influence the atherosclerotic process, would suggest a role in vascular pathophysiology. Specifically, EDL may modulate the atherosclerotic process by facilitating cholesterol exchange between lipoproteins and the vessel wall. In addition, as a local regulator of lipid metabolism, EDL may support de novo angiogenesis by supplying fatty acids for energy required for endothelial cell proliferation and migration.

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| Construct              | Phospholipase activity | apo-CII (+) | apo-CII (-) |
|------------------------|------------------------|-------------|-------------|
| Vector alone           | 156 ± 34               | 174 ± 5     | p < 0.00005 |
| c-Myc-EDL              | 307 ± 25               | 249 ± 32    | p < 0.04    |
| Corrected c-Myc-EDL    | 151 ± 25               | 75 ± 32     |             |