In Vitro Angiogenic Effects of Pancreatic Bile Salt-Dependent Lipase

Ouafa Rebaï, Josette Le Petit-Thevenin, Nadine Bruneau, Dominique Lombardo, Alain Vérine

Objective—Bile salt-dependent lipase (BSDL), a lipolytic enzyme secreted in the duodenum by pancreatic acinar cells, has been detected in the serum of all patients and in atheromatous plaque, suggesting its potential implication in vascular pathophysiology.

Methods and Results—In vitro pancreatic BSDL evokes human umbilical vein endothelial cell (HUVEC) proliferation and chemotactic migration. BSDL at mitogen concentration is capable to heal wounded HUVEC monolayer and to promote capillary network formation. HUVEC proliferation depends on the displacement of basic fibroblast growth factor and vascular endothelial growth factor from the extracellular matrix and the activation of extracellular signal-regulated kinases (ERK1/2), p38 mitogen-activated protein kinase, and focal adhesion kinase signaling pathways.

Conclusion—For the first time to our knowledge, it is suggested that circulating BSDL could be involved in pathophysiological angiogenesis. (Arterioscler Thromb Vasc Biol. 2005;25:359-364.)

Key Words: angiogenesis ■ human umbilical vein endothelial cells ■ bile salt-dependent lipase ■ growth factors

The bile salt-dependent lipase (BSDL) (EC.3.1.1.13) is a lipolytic enzyme, synthesized and secreted by the exocrine pancreas. Once in the duodenum, BSDL completes the digestion of dietary lipids.1 A fraction of duodenal BSDL is internalized by enterocytes via the Lox-1 scavenger receptor2 and transported to the blood compartment,3 where it partly associates with apolipoprotein B-containing lipoproteins in the plasma of normolipidemic patients.4 BSDL was detected in the human aortic endothelium and aorta BSDL could originate from macrophages, endothelial cells, eosinophils, or from low-density lipoprotein (LDL)-associated BSDL.5 However, endothelial BSDL may also originate from the pancreas via the circulation.3,6 BSDL has been detected in atherosclerotic lesions from hypercholesterolemic monkeys and from human arteries.6 The role of BSDL in atherosclerosis is still speculative; thus, this enzyme that has a wide substrate specificity and diffuse tissue distribution could have multiple functions in lipid and lipoprotein metabolism.5 BSDL in endothelial wall could be protective in hydrolyzing lysophosphatidylcholine (lysoPC) formed in the intima during the oxidative modification of LDL.7 BSDL could also modify the size and structure of weakly atherogenic LDL, making them more atherogenic.8 In this study, we have delineated the effects of BSDL on endothelial cells and we showed that BSDL promotes in vitro proliferation, migration, capillary network formation, and wound-healing of human umbilical vein endothelial cells (HUVECs) likely via the displacement of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) from the extracellular matrix (ECM) and the activation of multiple intracellular signaling pathways, suggesting that BSDL is involved in angiogenesis.

Materials and Methods

Materials

[3H]-thymidine (5 Ci/mmol), [125I]-bFGF (2958 Ci/mmol), and [125I]VEGF (4484 Ci/mmol) came from Perkin Elmer Life Sciences (Courtaboeuf, France). Fluorescein isothiocyanate-BSDL was prepared as described.3 Polyclonal antibodies (pAbL64) against human pancreatic BSDL were already described.6 bFGF and VEGF were from Sigma and AbCys (Paris, France), respectively. Secondary antibodies were from Sigma. Antibodies against extracellular signal-regulated kinases (ERK1/2), phosphorylated ERK1/2, p38 mitogen-activated protein kinase (p38-MAPK), phosphorylated p38-MAPK, focal adhesion kinase (FAK), phosphorylated FAK, bFGF, and VEGF were from Promega, Cell Signaling Technology, and R&D Systems. Way-121,898, a specific inhibitor of BSDL,9 was kindly released by Dr S. Adelman (Wyeth Research, Collegeville, Pa).

BSDL Purification, Enzyme Activity, Protein Determination, and Inhibition

Methods used for BSDL purification, enzyme activity, and protein determination have been already described.6 Inhibition of BSDL activity was performed using Way-121,898 (20 μmol/L) added directly in the culture medium.9 At this final concentration, Way-121,898 totally inhibits BSDL within minutes and had no cytotoxic effect on HUVEC.

Cell Proliferation Assay and Cytotoxicity Measurement

HUVEC were cultured in EBM-2 medium (2% fetal calf serum [FCS]) according to manufacturer’s instructions (Cambrex Bio-
Science, Walkersville, Md). Cells were made quiescent by incubation for 48 hours in medium containing 0.3% FCS. Cell proliferation and cytotoxicity were evaluated as described.\(^6\)

**Cell Migration Assay**

HUVEC migration was determined using a 24-well chemotaxis chamber (Corning, NY) with polycarbonate filter (8-μm pore size). HUVEC (2×10\(^4\) cells) in serum-free medium (100 μL) were applied to the lower compartment of the chamber and incubated (37°C, 3 hours). After removing BSDL and a further incubation (37°C, 24 hours), the noninvading cells on the upper surface of the filter were completely wiped off with a cotton swab and filters were fixed (70% ethanol in phosphate-buffered saline [PBS]) and stained (hematoxylin). The number of migrated cells was counted in 10 random fields at 200× magnification under the microscope.

**Capillary Network Formation**

Matrigel (250 μL; BD Biosciences, Bedford, Mass) was poured into a 24-well culture plate and allowed to solidify (37°C, 1 hour). HUVEC (4×10\(^4\) cells/well) were seeded on the matrigel and cultured in media without or with BSDL (100 mU/mL, 37°C, 24 hours). After incubation, the formation of the capillary network was observed with in situ hybridization\(^6\) on HUVEC and VS2 BSDL-specific primers to amplify a 0.9-kb transcript.

**Preparation of ECM, Growth Factor Loading, and Displacement**

ECM were prepared according to Benezra et al.\(^1\) After confluence, HUVECs were removed from plastic plate using 20 mmol/L NH\(\text{OH}\) in PBS and 0.5% Triton X-100. The ECM remaining attached to plastic plate was washed (4 times) with PBS buffer, and we checked that no cells or cell debris was associated with this ECM preparation.\(^1\) Then, a solution (300 μL) of serum-free medium (0.2% gelatin) and [\(^{125}\)I]-radiolabeled growth factor to obtain ∼150 pg/well (30 000 cpm/well) was added in each well and plates were incubated (3 hours, room temperature). The remaining free radiolabeled growth factors were washed by removing (4 times) with 600 μL PBS and 0.02% gelatin. For the displacement of ECM-bound [\(^{125}\)I]-radiolabeled growth factors,\(^1\) BSDL was added to wells in 300 μL of serum-free medium (0.02% gelatin) and incubated (3 hours, 37°C); then, the medium was removed, centrifuged, and the supernatant (200 μL) used for radioactivity counting. Each well was washed (4 times) with 500 μL PBS and ECM was solubilized by overnight incubation (37°C, 300 μL, 1 mol/L NaOH). After sonication, the remaining radioactive link to ECM was determined on aliquots. The radioactivity released in the supernatant by BSDL was related to the sum (100%) of the radioactivity remaining in the ECM and that released on ECM incubation with BSDL.

**Polyacrylamide Gel Electrophoresis and Immunodetection**

Gel electrophoreses (SDS-PAGE) and immunodetections were performed using appropriate primary and secondary antibodies and revealed as described.\(^6\) Quantitations were performed by scanning fluororadiogram (National Institutes of Health Image software).

**Reverse-Transcription Polymerase Chain Reaction**

Conditions for mRNA extraction, reverse-transcription polymerase chain reaction (RT-PCR) have been already described.\(^12\) Two pairs of primers were used, the first one (BSDL5; 5′-TTCGTAGGCTTGCGGCGTGTTACGACA-3′ and BSDL3; 5′-TTTCTGAGATTCCAGCTAAACCTAAATGCTGAACC-ATCTG-3′) was used to amplify the whole mRNA encoding human BSDL. The second one (VS5; 5′-CCTCGTGGTGTC-CTGCAGCTGGGAGA-3′ and VS2; 5′-CCCCATGCTGTC- GGCCGACCATAGCA-3′) should amplify a 0.9-kb transcript. β-Actin primers were from Proligo (Paris, France).

**Statistical Analyses**

All experiments were performed in triplicate and each experiment was reproduced at least 3 times. Error bars reflect the standard deviation and probability values were assessed using the Mann−Whitney nonparametric test. \(P<0.05\) was considered significant.

**Results**

**BSDL Is Not Expressed by HUVEC**

BSDL has been detected in human aorta,\(^7\) and a recent study showed that mRNA encoding the enzyme cannot be detected by in situ hybridization\(^6\) on human aorta, whereas a cDNA transcript was isolated by RT-PCR using human endothelial cells.\(^13\) However, this transcript was not sequenced and its specificity not ascertained. Therefore, a RT-PCR experiment using mRNA extracted from HUVEC and VS5 and VS2 BSDL-specific primers to amplify a 0.9-kb transcript was performed and results were negative, although a transcript for β-actin was amplified. We next used BSDL5 and BSDL3 primers to amplify the whole mRNA of BSDL,\(^12\) and no transcript of the expected size (ie, 2.2 kb\(^13\)) for human pancreas BSDL mRNA was obtained. Instead, a unique 1.7-kb transcript was obtained, the sequence of which had only 25% homology with that of BSDL.\(^14\) We then performed a Western blot on HUVEC homogenate using pAbL64 polyclonal antibodies to human BSDL and were unable to detect a 100-kDa material corresponding to the human enzyme. Also, no esterase activity depending on bile salts was recorded in HUVEC homogenates (data not shown). These results suggested that BSDL may not be expressed by HUVEC.

**BSDL Promotes HUVEC Proliferation and Migration**

Because BSDL promotes smooth muscular cell\(^6\) proliferation and migration, we have recorded the effect of pancreatic BSDL used as representative of the circulating enzyme\(^3,7\) on HUVEC. For this purpose, HUVECs were cultured to obtain a robust ECM\(^15\) and made quiescent; then, HUVECs were incubated with BSDL and [\(^3\)H]-thymidine incorporation was determined. As shown in Figure 1A, increasing BSDL concentration in incubation (1 hour) from 5 mU/mL up to 200 mU/mL enhanced the rate of [\(^3\)H]-thymidine incorporation. Compared with control without BSDL, this increase (46±4%) becomes significant at 100 mU/mL, a BSDL activity corresponding to that found in the human plasma.\(^4\) At higher concentrations, the enzyme inhibits the [\(^3\)H]-thymidine incorporation. This may be because of the capture by the excess of BSDL of heparan sulfate proteoglycans that are required to facilitate growth factor signaling.\(^15\) Incubation of HUVEC with BSDL (100 mU/mL) for various time periods indicated that the increase in [\(^3\)H]-thymidine incorporation appeared after 15 minutes of incubation and that a significant incorporation, compared with time 0, was recorded between 1 hour to 3 hours of incubation (Figure 1B). The decrease in [\(^3\)H]-thymidine incorporation observed after 24 hours of incubation could result from a denaturation of BSDL or because of its degradation. Under identical conditions, bFGF (25 ng/mL) or VEGF (10 ng/mL) increases the [\(^3\)H]-thymidine incorporation by 25% and 74%, respectively. The
proliferative effect of BSDL necessitates an active form of the protein as heat-denatured BSDL (1 μg/mL, amount corresponding to 100 mU/mL active enzyme) or cell incubation with BSDL in the presence of Way-121,898 (20 μmol/L) was unable to increase the [3H]-thymidine incorporation compared with native BSDL (1 hour, 100 mU/mL) (Figure 1C). [3H]-thymidine incorporation promoted by BSDL (100 mU/mL) in HUVECs was not modified by LDL (400 μg/mL in terms of protein) isolated from human plasma by sequential ultracentrifugations. LDL alone also induced a significant increase in [3H]-thymidine incorporation (30 ± 7%; P < 0.01; n = 4) that was annihilated by Way-121,898. These results suggested that BSDL, which is associated with these lipoproteins,4 is likely involved in the proliferative effects of LDL on HUVECs as observed here (data not shown).

We have also determined whether BSDL promoted HUVEC chemotactic migration through the filter of a migration chamber. For this, HUVECs (200 μL of 2 × 10⁵ cells/mL) in 0.3% FCS medium were applied in the upper compartment of chemotaxis cell, and medium with (+BSDL) or without (−BSDL) enzyme was added in the lower compartment. At the end of the incubation (3 hours, 37°C), migrated cells were fixed and stained, and 10 random fields were counted (P < 0.05). B, HUVECs were wounded and cultured without (control) or with BSDL. The same experiment was conducted with heat-denatured BSDL or with native BSDL in the presence of Way-121,898 or cycloheximide. After incubation (3 hours, 37°C), mediums were removed and cells were incubated in fresh medium for another 24 hours and photographed (10× magnification). The boundary of the wound is indicated by dashed lines.

Washed and allowed to migrate into the free space for 24 hours in the presence of fresh medium. In the presence of BSDL, HUVECs migrated actively and filled the wound within 24 hours (Figure 3B). The effects of BSDL were identical in the presence of cycloheximide (1 μg/mL, 3 hours), which inhibits the protein synthesis (Figure 2B). Once again, BSDL must be in an active form, because after an incubation of wounded cells with heat-denatured BSDL (1 μg/mL, 3 hours) or with BSDL in the presence of Way-121,898, no wound-healing can be detected (Figure 2B).

**BSDL Promotes Angiogenesis via bFGF and VEGF**

Our results suggest that BSDL may evoke angiogenesis. Therefore, HUVEC (4 × 10⁵ cells/well) were seeded on Matrigel in 12-well culture plate and cultured in EBM-2 containing 0.3% FCS (control medium), 10% FCS, or BSDL (100 mU/mL in control medium, 3 hours). After BSDL removal, cells were further incubated for 24 hours and the capillary network formation was analyzed under a phase-contrast microscope. As shown in Figure 3A, addition of BSDL (100 mU/mL, 3 hours) induced marked changes in HUVEC morphology, with structural rearrangements leading to the formation of capillary-like networks. Neither heat-denatured BSDL nor the enzyme in the presence of Way-121,898 was able to promote the formation of the network (Figure 3A). A significant promotion in tube length was
observed at 100 mU/mL BSDL (62% increase over control medium), whereas 10% FCS produced a weak effect (15%) (Figure 3B).

We have shown that BSDL provoked the rapid release of bFGF to induce smooth muscle cell proliferation. Therefore, the possible involvement of growth factors in HUVEC proliferation promoted by BSDL was determined by using antibodies to bFGF and VEGF. For this purpose, HUVECs were cultured to form a robust ECM and then made quiescent. Fresh medium depleted in FCS and growth factor was then added and incubated for 3 hours, and without (control) or with anti-bFGF (2 μg/mL), with anti-VEGF (2 μg/mL), or in the presence of a mixture of both antibodies (2 μg/mL each). Then, [3H]-thymidine incorporation was recorded. The increase in [3H]-thymidine incorporation was abolished when HUVECs were incubated with BSDL and antibodies against bFGF and/or VEGF (Figure 3C). Blocking either bFGF or VEGF by antibodies results in similar effects. This agrees with the well-known synergistic effect of bFGF and VEGF on HUVECs, in which bFGF potentiates VEGF by inducing the expression of VEGF, which in turn increases VEGF receptors expression.

The ECM is critical for normal vessel growth and maintenance by acting in part as a reservoir for growth factors. Therefore, we have investigated whether bFGF and VEGF actually originate from the ECM. Consequently, experiments were first conducted before a significant ECM deposition was reached. For this purpose, HUVECs were seeded in tissue culture plates at low density (4000 cells/cm²), maintained for 3 days to induce the minimal ECM deposition (−ECM), and then incubated (3 hours) with heparinase III (25 ng/mL), and without (control) or with anti-bFGF (2 μg/mL), with anti-VEGF (2 μg/mL), or in the presence of a mixture of both antibodies (2 μg/mL each). Then, [3H]-thymidine incorporation was recorded. The increase in [3H]-thymidine incorporation was abolished when HUVECs were incubated with BSDL and antibodies against bFGF and/or VEGF (Figure 3C). Blocking either bFGF or VEGF by antibodies results in similar effects. This agrees with the well-known synergistic effect of bFGF and VEGF on HUVECs, in which bFGF potentiates VEGF by inducing the expression of VEGF, which in turn increases VEGF receptors expression.

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tion, amounts of radiolabeled growth factors released in the supernatant were determined. BSDL induces a displacement of [\(^{3}H\)]-bFGF and of [\(^{3}H\)]-VEGF, which increases with increasing amounts of enzyme and significantly overcomes the spontaneous release of each growth factor (≈15%).

**BSDL Activates MAPK**

The release of growth factors from the ECM should be associated with the activation of multiple intracellular signal transduction pathways. Thus, to investigate how BSDL evoked HUVEC proliferation and migration, quiescent cells were incubated with BSDL (100 mU/mL) for the time periods indicated (Figure I, available online at http://atvb.ahajournals.org). At the end of the incubation time, cells were lysed and cell proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies to (phosphorylated) ERK1/2 (p42/p44) MAPK or antibodies to (phosphorylated) p38-MAPK. As shown on Western blots, BSDL provoked a net phosphorylation of ERK1/2 MAPK that culminates after 10 to 15 minutes of incubation, and then decreases progressively to reach basal value (Figure IA). BSDL also induces the rapid phosphorylation of p38-MAPK and of FAK within 5 minutes of incubation of HUVECs (Figure IB and IC). These results are consistent with the activation of intracellular pathways consecutive to the release of bFGF and VEGF from the ECM by BSDL.

**Discussion**

In this study, we showed that in vitro BSDL promoted HUVEC proliferation and migration; these effects are significant at BSDL concentrations found in the circulation (i.e., 100 mU/mL). We further showed that wound-healing promoted by BSDL is independent of protein synthesis, suggesting that the enzyme increased HUVEC motility. Furthermore, in the presence of blood concentration of BSDL, HUVECs presented changes in morphology and structural rearrangements leading to the formation of a capillary network. All these effects are characteristic of angiogenesis.

We next showed that BSDL is susceptible to displace bFGF and VEGF from the ECM, which supports that antibodies to these growth factors inhibited the \([^{3}H]\)-thymidine incorporation in HUVECs promoted by BSDL. Because BSDL has no heparinase-like activity,\(^1\) the destruction of the ECM\(^15\) by the enzyme cannot be argued to explain the observed effects. However, the heparin-binding site of BSDL\(^17\) has to be functional to displace sequestrated growth factors from the ECM,\(^18,19\) as suggested by the lack of effect of heat-denatured BSDL on angiogenesis events.

The need for an activity of BSDL is questionable, albeit the catalytic site-directed inhibitor Way-121,898 annihilates the enzyme effects on HUVEC. Effectively on inactivation by the inhibitor or by heating, BSDL seems no more able to interact with HUVEC membranes (or ECM, data not shown), and as a consequence might not displace growth factors from the ECM to evoke proliferative effects on HUVEC. Furthermore, in the presence of a minimal ECM that represents a poor reservoir for growth factors,\(^15\) BSDL is unable to evoke HUVEC proliferation. This eliminates the possible involvement of cell lipid hydrolysis and the production of lipid second messenger\(^6\) by the enzyme. Moreover, no bile salts susceptible to activate BSDL are present in incubation. All this agrees more with the requirement of a structurally active form of BSDL rather than with a need for BSDL hydrolytic activity. Therefore, BSDL effects seem mainly associated with the displacement of growth factors from the ECM, likely by means of a competition between BSDL and growth factors for ECM proteoglycans. This agrees with the blocking effect of antibodies to growth factors on HUVEC proliferation promoted by BSDL. Further studies are essential to clarify the need for an active form of BSDL in growth factor release.

Undoubtedly, BSDL (and likely LDL-associated BSDL) is susceptible to induce angiogenesis via the release of growth factors from the ECM. Angiogenesis is a multistep process involving the remodeling of the ECM and migration and proliferation of endothelial cells. These events are associated with activation of multiple intracellular signal transduction pathways, including ERK1/2 MAPK, p38-MAPK, and FAK. ERK1/2 is strongly induced by bFGF and by VEGF, and activation of this pathway presumably plays a central role in the stimulation of endothelial cell proliferation, migration, and survival.\(^20\) The p38-MAPK responds to proinflammatory cytokines and environmental stress.\(^21\) This MAPK, involved in cytoskeleton reorganization, cell migration, and vascular permeability, is activated by bFGF and also in HUVEC by VEGF.\(^22,23\) In HUVEC, VEGF\(^24\) also activates FAK that plays a key role in regulating the dynamic changes in actin cytoskeleton organization that is a prerequisite for cell migration.\(^25\) All these kinases are phosphorylated over the unstimulated culture conditions on incubation of HUVECs with mitogenic concentration of BSDL.

Independently of its origin,\(^3,5\) the role of the circulating BSDL could only be speculative. It is generally accepted that consecutive to a vascular lesion, the subendothelial retention of LDL constitutes the initiating event in atherogenesis.\(^26\) In the blood, BSDL is associated with LDL and linked to the LDL cholesterol level.\(^4\) Once in the intima, BSDL, which is associated with oxidized LDL,\(^6\) may have a protective effect in hydrolyzing lysoPC.\(^7\) This hydrolysis may annihilate negative effects of lysoPC on the attraction and growth of monocytes and macrophages,\(^27\) on the reduction of cytokines production,\(^28\) thus lowering the inflammatory response, and on the apoptosis of endothelial cells.\(^29\) Furthermore, BSDL, which evokes the proliferation and migration of smooth muscle cells and of endothelial cells, may balance the effects of lysoPC on these cells, may stabilize the plaque, and re-establish the arterial integrity.\(^30,31\) To these positive effects, one can add the involvement of BSDL in the high-density lipoprotein cholesterol clearance by the liver.\(^32\) However, BSDL may have negative effects on LDL structure as mentioned.\(^8\)

Therefore, on interaction with ECM, possibly at the level of the vascular lesion and the displacement of growth factors, circulating BSDL may activate multiple intracellular signaling pathways in smooth muscle cells and endothelial cells to participate in the fine balance regulating the angiogenesis.

**References**


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Figure I: Activation of kinases by BSDL

Quiescent HUVEC were incubated with BSDL (100mL/m) for indicated time periods. At the end of incubation time, cells were lysed and proteins (50 µg/ lane) separated on SDS-PAGE and electrotransferred onto nitrocellulose membrane. Membranes were then probed with anti-ERK1 (p44) ERK2 (p42) MAPK specific antibodies and with anti-phosphorylated -ERK 1/2 MAPK (A), with anti-p-38 MAPK antibodies and antiphosphorylated-p38-MAPK antibodies (B) or with anti-phosphorylated- FAK antibodies (C).