Pancreatic Bile Salt-Dependent Lipase Induces Smooth Muscle Cells Proliferation

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Background—Because bile salt-dependent lipase (BSDL), an enzyme secreted by the pancreatic acinar cells and associated with LDL in circulating blood, also locates with smooth muscle cells (SMCs) in atherosclerotic lesions, we aimed to investigate its effects on SMCs.

Methods and Results—Immunohistochemical experiments allowed us to detect an expression of BSDL in atherosclerotic lesions from hypercholesterolemic monkeys and from human arteries. BSDL was found to be associated with SMCs but not with macrophages. BSDL was significantly mitogenic for cultured SMCs. This effect was inhibited by heparin and anti-BSDL antibodies, whereas heat-denaturated and diisopropylfluorophosphate-treated BSDL were inefficient. The mitogenic effect of BSDL was associated with an activation of the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase pathway, which was inhibited by heparin, and involved several mechanisms, among them diacylglycerol and oleic acid production as well as a rapid basic fibroblast growth factor release.

Conclusions—Circulating BSDL is associated with SMCs within the intimal arteria and may trigger SMC proliferation, which could contribute to the development of atherosclerotic lesions. (Circulation. 2003;108:86-91.)

Key Words: atherosclerosis ■ arteries ■ muscle, smooth

M any observations suggest that the role of pancreatic bile salt-dependent lipase (BSDL) may not be restricted to the hydrolysis of dietary lipids (cholesteryl esters, triacylglycerides, and [lyso]phospholipids1) because the enzyme is found in the blood of normolipidemic patients associated with the apolipoprotein (Apo) B100 of LDL.2 The presence of the enzyme in the circulation could be the result of an orchestrated transepithelial movement through the enterocyte.3 BSDL was also detected in the vascular endothelium,4 where it could originate from macrophages,5 be produced by endothelial cells,6 or be derived from LDL-associated BSDL.2

The role of endothelial BSDL in atherosclerosis4–6 is speculative. BSDL in the endothelial wall could be protective in hydrolyzing lyso phosphatidylcholine (lysoPC) formed in the intima during the oxidative modification of LDL.4 LysoPC attracted monocytes/macrophages further, leading to foam cells7 and altered various intracellular signaling pathways.8 As a consequence, lysoPC has growth-promoting effects on smooth muscle cells (SMCs) that play a central feature in the development of atherosclerotic lesions. Therefore, endothelial BSDL may hydrolyze lysoPC to decrease the attraction of monocytes and the formation of foam cells or could lower SMC proliferation by decreasing the level of lipid second messenger (lysoPC8 or ceramide9–11) in the intima. However, BSDL could evoke SMC proliferation by generating lysoPC or free oleic acid12 from phosphatidylcholine (PC).13

In this context, endothelial BSDL, independently of its origin,2,4–6 may either generate a lipid second messenger that regulates the proliferation of SMC or hydrolyze this messenger to annihilate its proliferative effect. In this first approach, we attempted to delineate the effect of exogenous BSDL on SMC proliferation.

Methods

Materials

Methyl-[3H] choline chloride (86 Ci/mmol), [3H]-thymidine (5 Ci/mmol), [3H]-palmitic (53 Ci/mmol), [3H]-oleic acid (15 Ci/mmol), and cholesteryl [14C]-oleate (40 to 60 Ci/mmol) came from NEN. Heparin (3000 Da) was from Fluka, and PD98059 was from Calbiochem. Human atheromatous carotid and normal mammary arteries were gift from Drs J.B. Michel and O. Meilhac (INSERM U-460, Paris, France).

Immunocytochemistry and Microscopy

Segments of aorta were obtained from male adult cynomolgus monkeys (Macacus fascicularis) fed with cholesterol-rich diet,14 fixed in Bouin’s solution, and paraffin-embedded. Serial sections of 5 μm were obtained with a Leica microtome. For histologic observation, deparaffinized sections were stained with Masson’s...
trichrome blue. Immunolabelings were carried out using monoclonal antibodies to CD68 specific of macrophages (Dako, Trappes, France), α-actin labeling SMCs (Sigma, St Louis, Mo), polyclonal antibodies to C-reactive protein (Rockland, Tebu, France), C3-complement fraction (Dako, France), monoclonal antibody to 4-hydroxynonenal–modified ApoB (a gift from Dr G. Jürgens, Graz University, Graz, Austria), or pAbL64 antibodies specific for human pancreatic BSDL. These primary antibodies were revealed using an immunoperoxidase kit (Dako, StrepABC/Complex/HRP Duet). For each detection, a control was done by omitting the primary antibody to determine the nonspecific binding. Nuclei were counterstained with hematoxylin.

BSDL Purification, Enzyme Activity, and Protein Determination

BSDL was purified from human pancreatic juice and, when required, inhibited by diisopropyl fluorophosphate (DFP)18 or heat denatured (95°C, 5 minutes.). The esterolytic activity of BSDL was recorded in the presence or absence of sodium taurocholate (4 mmol/L) using 4-nitrophenyl hexanoate (4-NPC).16 The cholesterol esterase activity of BSDL was determined on cholesteryl [14C]oleate.17 Protein concentration was determined with the microBCA kit (Pierce). Basic fibroblast growth factor (bFGF) was quantified by an ELISA kit (R&D System).

Cell Proliferation and Cytotoxicity Measurements

Human SMCs (hSMCs) and rabbit femoral SMCs (rSMCs) were from ATCC and cultured as described.9,10 Cells were seeded at a density of 25,000 cells/mL in 24-multwell Nunc culture plates, in RPMI 1640 (Life Technology) with 10% FCS. At confluence, the medium was removed and replaced for 24 hours by RPMI containing 0.5% FCS. Quiescent cells were then incubated with the different agonists. Cell proliferation was evaluated by [3H]-thymidine (0.5 μCi/mL) incorporation.9 The cytotoxicity was evaluated using the MTT test.19

Metabolic Labeling, Lipid Extraction, and Analyses

For the estimation of cell lipid variations, cells were preincubated with radiolabeled precursors for 24 hours ([3H]-oleic acid, 4.5 μCi/mL) or 48 h ([3H]-palmitic acid, 1 μCi/mL and [3H]-choline, 0.5 μCi/mL). Cells were washed with PBS and treated with BSDL in the absence or presence of effectors. At the end, medium was withdrawn and replaced for 24 hours by RPMI containing 0.5% FCS. Quiescent cells were then incubated with the different agonists. Cell proliferation was evaluated by [3H]-thymidine (0.5 μCi/mL) incorporation.9,10 The cytotoxicity was evaluated using the MTT test.19

SDS-PAGE and Immunoblotting

Proteins were separated on SDS-PAGE (12.5% polyacrylamide and 0.1% SDS) and electrotransferred onto nitrocellulose membranes. Immunodetections were carried out using pAbL64, antibodies to extracellular signal regulated kinase (ERK) 1 and ERK2 (Santa Cruz), or antibodies to phosphorylated mitogen-activated protein kinase (MAPK) (Promega, Madison, Wis) as primary antibodies17 and chemoluminescence kit (Roche).

Northern Blotting and In Situ Hybridization

RNA was extracted from SMCs using Trizol (Life Technologies), separated on 1% agarose gel, and then transferred onto a nitrocellu-

Pancreatic BSDL Locates in Atheromatous Aortas

In some sections of aortas from hypercholesterolemic monkeys, no abnormalities were seen by gross examination (Figures 1A through 1D). However, Masson’s trichrome blue (Figure 1A) revealed a thickness of the intima that presents fibrous areas with distinctive proliferative SMC, the media being normal. The labeling of SMCs of the media and proliferative SMCs of the intima with anti–α-actin (Figure 1B) can be superimposed on that obtained with pAbL64 (Figure 1C), whereas the labeling with antibodies to CD68 is negative (Figure 1D).

In atheromatous segment, the Masson’s trichrome blue (Figure 1E) detects, under spumous cells, a fibrosis area indicating secreting SMCs. The intima displays classic foam-thickened lesions that are positive to anti–α-actin (Figure 1F), pAbL64 (Figure 1G), and anti-CD68 (Figure 1H). SMCs of the media and those migrating within the intima react with anti–α-actin antibodies (Figure 1F) and with pAbL64 (Figure 1G). SMCs of the intima seem more responsive to pAbL64 than contractile cells of the media. Anti-CD68 labeling is never superimposed to that of pAbL64 (Figures 1G and 1H). Same BSDL expression was observed in human artery atheromatous lesions. Preincubation of slides with free BSDL (250 μg/mL, 30 minutes) and detection with pAbL64 (40 μg/mL) plus BSDL (250 μg/mL) abolished the antibodies’ reactivity (Figure 1J), meaning that pAbL64 recognized BSDL in atheroma plaques. Immunohistochemical detection of 4-hydroxynonenal–modified ApoB indicated (Figure 1K) the presence of oxidized-LDL in the lesions, whereas plasmatic proteins such as C reactive protein or the fraction C3 of complement were not detected (data not shown).

Smooth Muscle Cells Do Not Express Pancreatic-Like BSDL

We additionally investigated BSDL activity and expression in hSMCs. The esterolytic activity of hSMC lysate on 4-NPC is weak and does not differ in the absence (31±0.2 mU/mg cell proteins) or in the presence of sodium taurocholate (28±0.3 mU/mg cell proteins). The cholesterol esterase activity in SMC lysate is low (100 pmol/h per mg cell proteins) and inhibited by sodium taurocholate. Obviously, this activity cannot be attributed to BSDL, because sodium taurocholate represents a potent activator of the enzyme. Furthermore, no activity on these substrates can be detected in the 10-times concentrated culture medium of hSMCs.
Western blotting was performed after separation of SMC lysate proteins. Although human pancreas lysate and pancreatic BSDL are responsive to pAbL64 (Figure 2A), SMC lysate does not react. Northern blotting (Figure 2B) showed that in the pancreas, the nucleotide probe of BSDL hybridizes with mRNA species of 2.2 kb, corresponding with that encoding this enzyme.23 No trace of this latter mRNA can be detected in hSMCs, whereas pancreas and hSMCs are positive to β-actin probe (Figure 2B). Reverse transcriptase–polymerase chain reaction using BSDL probes17,19,23 failed to detect any specific transcript (not shown). To ascertain the absence of mRNA encoding BSDL in atheromatous arterial wall, we performed in situ hybridization. As shown in Figure 3, antisense RNA sequence strongly hybridized with human pancreatic acinar cells but not with islets (Figure 3B, IL). However, this probe hybridized neither with endothelial cells nor with SMCs of human atheromatous coronary section (Figure 3D); the same negative results were obtained with normal mammary artery (not shown). No unspecific labeling of these tissues was detected using the sense probes (Figures 3A and 3C).

Figure 1. Microscopic study of atherosclerotic lesions in aortas. Male cynomolgus monkeys were killed after 12 months of hypercholesterolemic diet, and thoracic aorta were examined. A through D, Grossly normal aorta (×25), Masson’s trichrome blue coloration (A). Immunodetection using anti-α-actin (B), pAbL64 (C), and anti-CD68 (D). E through H, Atheromatous section of thoracic aorta (×40), Masson’s trichrome blue coloration (E). Immunodetection using anti-α-actin (F), pAbL64 (G), and anti-CD68 (H). The arrows indicate the colocalization of anti-α-actin and pAbL64 immunoreactivity in fibrous areas. I through K, Atheromatous section of human carotid artery (×40). Immunodetection using pAbL64 (I) and pAbL64+BSDL (J) and anti-4-hydroxynonenal–modified ApoB (K).

Figure 2. BSDL is not expressed in hSMCs. hSMCs were grown to subconfluence, scraped, and lysed. A, Proteins were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. A lysate of human pancreas and human pancreatic BSDL were analyzed on the same gel. The membrane was probed with pAbL64. Lane 1, human pancreas lysate (2 μg cell proteins); lane 2, hSMC lysate (2 μg cell proteins); lane 3, pure human pancreatic BSDL (100 ng). B, RNA extracted from pancreatic tissue and from hSMCs was separated on 1% agarose gel and transferred onto a nitrocellulose membrane. Membrane was probed with a radiolabeled oligonucleotide specific for BSDL and for β-actin mRNA. Lane 1, RNA from pancreas (20 μg); lane 2, RNA from hSMC (20 μg).

Figure 3. In situ hybridization. Slides of human pancreas (A and B) and of atheromatous carotid artery (C and D) were probed with digoxigenin-labeled sense (A and C) and antisense (B and D) cRNA of BSDL. The reaction was detected with alkaline-phosphatase–labeled anti-digoxigenin antibodies (for technical details, see Reference 21). VL indicates vessel lumen; EC, endothelial cells.
Human Pancreatic BSDL Induces SMC Proliferation

We next investigated the effect of pancreatic BSDL on SMC proliferation. For this purpose, rSMCs were incubated for various time periods or with increasing concentrations of BSDL (specific activity, 100 U/mg), and [\(^{3}H\)]-thymidine incorporation was determined. On rSMC incubation with BSDL (100 mU/mL), [\(^{3}H\)]-thymidine incorporation increased with time and BSDL concentration up to 100 mU/mL whereas BSDL cytotoxicity was observed at higher concentrations (Figures 4A and 4B). The proliferative effect of BSDL is blocked by heparin (0.5 mg/mL) and pAbL64 (10 \(\mu\)g/mL). It was also relevant to determine whether the enzyme has to be active to promote DNA synthesis. Therefore, BSDL was either heat-denatured or inhibited by DFP\(^{15}\) and, after dialysis, added to the conditioned medium (1 \(\mu\)g/mL). Neither DFP-treated nor heat-denatured BSDL (data not shown) was able to promote DNA synthesis.

BSDL Induces the Formation of Lipid Second Messengers

To additionally examine the connection between BSDL activity and SMC proliferation, cell lipids were labeled with radioactive precursors. Compared with control mock-treated rSMCs (100±2%), effective mitogenic dose of BSDL (100 mU/mL, 15 minutes) modified neither the cellular amount of sphingomyelin (103±9%) nor that of ceramide (104±2%). This means that BSDL did not induce the hydrolysis of cellular sphingomyelin into ceramide. However, the diacylglycerol cellular content of rSMC increased (130±14%, \(P<0.05\)) on cell treatment with BSDL, an increase that is inhibited by coincubation of rSMC with BSDL and heparin (108±4%). An increase in the cellular amount of DAG (115±4%, \(P<0.01\)) was also observed in hSMC on incubation with mitogenic dose of BSDL (100 mU/mL, 30 minutes). Under these conditions, BSDL is internalized by hSMC (Figure 5) and can be detected in punctated structures in the cytoplasm and perinuclear area. Furthermore, this mitogenic dose of BSDL lowered the hSMC cellular amount of phospholipids (90±2%, \(P<0.01\)). Under these conditions, BSDL also increases the amount of oleic acid (148±10%, \(P<0.001\)) and decreases that of lysoPC (85±2%, \(P<0.001\)) detected in the conditioned medium.

BSDL Activates bFGF Release and MAPK Cascade

We speculated that the mitogenic effect of BSDL could be partly mediated via the release of bFGF and the activation of MAPKs, which are classically associated with cell proliferation signaling.\(^{24}\) BSDL (like lysoPC) induces a rapid release of bFGF after short incubation time (<10 minutes), followed by a progressive return to the basal level (Figure 6A). However, challenging hSMCs with a mitogenic amount of bFGF (10 ng/mL, 24 hours) did not induce the expression of BSDL (not shown). BSDL also provoked a net phosphorylation of ERK1/ERK2 MAPK that culminated after 10 to 15 minutes of incubation and then decreased progressively to
reach basal value (Figure 6B). Heparin decreased the phosphorylation of MAPK (Figure 6C) promoted by BSDL. The MAPK inhibitor PD98059 inhibited efficiently the [3H]-thymidine incorporation triggered by BSDL (Figure 6D).

Discussion

In this study, we took into account that BSDL is a pancreatic cholesterol esterase also found in the blood circulation associated with LDL, that the fraction of insudated LDL may ferry BSDL into the vascular intima, and that cholesterol esterase-degraded LDL induces the SMC proliferation to delineate the role of BSDL once located in the vascular intima. Alternatively, BSDL detected in the vascular endothelium could originate from macrophages or from endothelial cells.

Our results showed that immunoreactive BSDL can be detected within atheromatous artery walls, where it is associated with SMCs, whereas activated macrophages were not reactive to BSDL antibodies. We were unable to detect BSDL (mRNA, protein, and activity) within cultured SMC or BSDL mRNA in human atheroma plaque, including SMC and endothelial cells. These data support an exogenous origin for BSDL (eg, pancreas) via intestinal transcytosis and blood LDL, which locate (like BSDL) in atheromatous lesions. Consequently, we have examined the effect of pancreatic BSDL on SMC proliferation. Results indicated that BSDL at circulating blood concentrations induced the proliferation of SMCs. This proliferation is linked to BSDL activity, because DFP-inhibited enzyme was ineffective. The simple binding of BSDL to SMC membrane cannot activate the cell proliferation, as shown with heat-denatured enzyme. However, BSDL needs to associate with the SMC plasma membrane, and heparin, which inhibits BSDL association with membranes, or pAbL64, which mainly recognized the O-linked sugars of the enzyme, decreased the proliferative effect of BSDL. These results suggested that BSDL may interact with SMC plasma membranes via either the heparin site or the O-linked sugar domain of the human BSDL or both. Consequently of its wide specificity, BSDL may generate many lipid second messengers susceptible to promote SMC proliferation. The amounts of cellular SPM and ceramide do not vary on SMC incubation with proliferating dose of BSDL, allowing us to rule out the SPM-ceramide activation pathway.

Cellular phospholipids and cell-free medium lysoPC significantly decrease, whereas free oleic acid appears in the medium, suggesting that this mitogenic fatty acid is generated by BSDL from PC and lysoPC. Consistently, BSDL, which may hydrolyze PC preferentially exposed to the outer leaflet of plasma membrane, could (through the generation of lipid second messenger) induce the release of the bFGF of the pericellular compartment. bFGF then associates with cognate receptor on SMC membrane to induce proliferation. The phosphorylation of bFGF receptor could additionally activate a phosphoinositide-specific phospholipase C that evokes the production of cellular DAG. However, DAG, which slightly increases in SMC on incubation with BSDL, may result from the hydrolysis of triglycerides by the enzyme once internalized by SMC.
Independently of their sequence production, these lipid second messengers transmit individually their mitogenic activity through the ubiquitous MAPK pathway.\textsuperscript{24} We showed that a marked activation of MAPK occurred within minutes of BSDL incubation, where rapid inactivation ensued. This activation time corroborates that of MAPK activation after SMC incubation with mitogenic lysoPC.\textsuperscript{30} Also, the ERK MAPK kinase inhibitor PD98059 induced a significant decrease in BSDL-evoked proliferation.

Briefly, our results show that pancreatic BSDL evokes SMC proliferation through intracellular and extracellular lipid messenger generation, bFGF release, and subsequent MAPK activation. From a pathophysiological point of view, SMC proliferation is a constant feature in atherosclerotic lesions.\textsuperscript{31} The presence of BSDL associated with SMCs in atheroma plaque, as well as its mitogenic effect of cultured SMCs, could suggest a role for this lipase in SMC proliferation and fibrous cap formation. However, its overall effect within the plaque is not elucidated, and additional investigations will be necessary to better determine the potentially proatherogenic function of BSDL.

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