Glycosylphosphatidylinositol-Specific Phospholipase D Is Expressed by Macrophages in Human Atherosclerosis and Colocalizes With Oxidation Epitopes

Kevin D. O’Brien, MD; Christine Pineda; Winnie S. Chiu, BS; Rosario Bowen, BS; Mark A. Deeg, MD, PhD

Background—Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) may play an important role in inflammation, because it can hydrolyze the GPI anchors of several inflammatory membrane proteins (eg, CD106, CD55, and CD59) and its hydrolytic products upregulate macrophage cytokine expression (eg, interleukin-1 and tumor necrosis factor-α). Because of its potential regulatory role in inflammatory reactions, we hypothesized that GPI-PLD might be expressed in atherosclerosis.

Methods and Results—Immunohistochemistry using human GPI-PLD–specific rabbit polyclonal antiserum was performed on a total of 83 nonatherosclerotic and atherosclerotic human coronary arteries from 23 patients. Macrophages, smooth muscle cells, apoA-I, and oxidation epitopes also were identified immunohistochemically. Cell-associated GPI-PLD was detected in 95% of atherosclerotic segments, primarily on a subset of macrophages. Extracellular GPI-PLD was present in only 30% of atherosclerotic segments and localized to regions with extracellular apoA-I. In contrast, GPI-PLD was not detected in nonatherosclerotic segments. Expression of GPI-PLD mRNA by human macrophages was confirmed in vitro by reverse transcription/polymerase chain reaction. Further studies demonstrated that GPI-PLD–positive plaque macrophages contained oxidation epitopes, suggesting a link between oxidant stress and GPI-PLD expression. This possibility was supported by studies in which exposure of a macrophage cell line to H₂O₂ led to a 50±3% increase in steady-state GPI-PLD mRNA levels.

Conclusions—Collectively, these results suggest that oxidative processes may regulate GPI-PLD expression and suggest a role for GPI-PLD in inflammation and in the pathogenesis of atherosclerosis. (Circulation. 1999;99:2876-2882.)

Key Words: enzymes ■ proteins ■ cells ■ atherosclerosis

Regulation of biological events by enzymatic activity is a common paradigm in both normal and pathological states. In atherosclerosis, for example, diverse enzymatic systems mediate plaque stability,1,2 cell proliferation,3 and apoptosis.4 We noted that several groups have identified that important atherosclerotic plaque inflammatory proteins, including a splice variant of vascular cell adhesion molecule-1 (VCAM-1, or CD106), decay-accelerating factor (CD55), and CD595,6 may contain glycosylphosphatidylinositol (GPI) membrane anchors.7–9 This raised the possibility that an enzyme that specifically cleaves GPs might serve a regulatory function in the inflammation characteristic of atherosclerosis.

The only known mammalian GPI-specific enzyme is GPI-specific phospholipase D (GPI-PLD), an 814-amino-acid, N-glycosylated protein. GPI-PLD specifically cleaves GPs, including those of GPI-anchored proteins.10–12 Several lines of evidence suggest that GPI-PLD might participate in regulating inflammation in atherosclerosis. First, both murine and human macrophage cell lines express GPI-PLD activity,13 and macrophage infiltration is a hallmark of the chronic inflammation of atherosclerosis.14 Second, both intact GPs and GPI-PLD–generated cleavage products upregulate macrophage expression of both interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α).15,16 2 inflammatory cytokines believed to participate in human atherogenesis.17,18 Finally, GPI-PLD also is carried in plasma on a minor HDL particle,19,20 and HDL is present in atherosclerotic plaques.21,22 Another feature of the inflammation of atherosclerosis is oxidation, as demonstrated by the presence of oxidation epitopes in atherosclerotic plaques.23–25 The source of these oxidation epitopes is not known, but atherosclerotic plaque macrophages express a variety of oxidant-generating enzymes, including myeloperoxidase26 and 15-lipoxygenase.24

Received September 21, 1998; revision received March 17, 1999; accepted March 29, 1999.
From the Division of Cardiology, Department of Medicine, University of Washington, Seattle (K.D.O., C.P., W.S.C.), and the Division of Endocrinology and Metabolism, Department of Medicine, Indiana University, and the Roudebush Veterans’ Affairs Medical Center, Indianapolis, Ind (R.B., M.A.D.).
Correspondence to Kevin D. O’Brien, MD, Division of Cardiology, Box 356422, University of Washington, Seattle, WA 98195-6422. E-mail cardiac@u.washington.edu
© 1999 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org
Because of the link between inflammation and oxidation in atherosclerosis, we hypothesized that GPI-PLD may either promote or be regulated by oxidation.

This report demonstrates that GPI-PLD is present in human atherosclerotic lesions and is expressed by human macrophages. Furthermore, it demonstrates an in vivo relationship between GPI-PLD and oxidation in atherosclerosis and in vitro regulation of GPI-PLD expression by oxidants in a macrophage cell line. Overall, these data suggest that GPI-PLD participates in the pathogenesis of human atherosclerosis.

Methods

Human Coronary Arterial Tissue
A total of 83 coronary arterial segments were obtained from 23 hearts removed at the time of cardiac transplantation for either ischemic (n=10) or nonischemic (n=13) cardiomyopathies. Patient ages at the time of organ removal ranged from 29 to 64 years (median, 52 years) for all patients, 46 to 64 years (median, 52.5 years) for those with ischemic cardiomyopathy, and 29 to 64 years (median, 46 years) for those with nonischemic cardiomyopathies. Arterial tissue was fixed in methanol–Carnoy’s fixative. The coronary artery segments were classified according to conventional histological criteria as atherosclerotic or nonatherosclerotic.27

Cell Culture

Human Monocyte–Derived Macrophages and HepG2 Cells
Human monocyte–derived macrophages were isolated from volunteer donors by the method of Boyum28 and cultured on 35-mm plastic dishes (Costar Corp) in RPMI-1640 medium (BioWhittaker) with 20% autologous serum for 2 days before use. Cells of the HepG2 human hepatoma cell line (American Type Culture Collection [ATCC]) were grown in DMEM (Gibco BRL) supplemented with 10% FBS (Hyclone). Total RNA was isolated from 7-day-old human monocyte–derived macrophages and HepG2 cells by the guanidinium–isothiocyanate method.29

J774A.1 Murine Macrophage Cell Line
J774A.1 cells (ATCC) were plated at 0.5×10^6 cells per 35-mm dish and grown to 90% confluence in DMEM (Gibco BRL) supplemented with 10% FBS (Hyclone) containing 25 mmol/L glucose. To examine the effect of oxidant stress on GPI-PLD mRNA steady-state levels, cells were incubated with medium with or without 500 μmol/L H₂O₂ for 60 minutes. At the end of the incubation, the cells were washed once with ice-cold PBS prepared with diethylpyrocarbonate-treated water. Total RNA was extracted with TriPure (Boehringer). Northern blotting was performed as described previously.22,25

Immunohistochemistry

Single-label immunohistochemistry was performed as described previously.22,23 Standard peroxidase enzyme substrate, 3,3'-diaminobenzidine with nickel chloride (Sigma), yielded a black reaction product. The slides were counterstained with methyl green.

Negative controls for each of the antibodies included substitution of primary antibody with either PBS or isotype-matched, irrelevant monoclonal antibodies (for monoclonal antibodies) or nonimmune serum (for polyclonal antibodies) to abolish specific immunohistochemical staining.

Reverse Transcription/Polymerase Chain Reaction

Reverse Transcription
Reverse transcription (RT) was performed by incubating 2 μg of total RNA with 50 pmol random hexamer at 70°C for 10 minutes, followed by addition of the following to the final concentrations indicated: 1× reverse transcriptase buffer (Gibco BRL), 0.01 mol/L DTT, 2 U/μL RNasin, 0.6 mmol/L each of dGTP, dATP, dTTP, and dGTP, and 500 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The mixture then was incubated at 42°C for 1 hour to generate cDNA.

Polymerase Chain Reaction and DNA Sequencing
Polymerase chain reaction (PCR) used 4 overlapping primer pairs (Table) designed to span 2.5 kb of the GPI-PLD cDNA.31 PCR was performed with 5 μL of cDNA. PCR buffer (Boehringer-Mannheim), 1 mmol/L each of dGTP, dATP, and dCTP, 0.5 pmol each of the 5'- and 3'-primers, and 10 U Taq polymerase (Boehringer-Mannheim). The tubes were heated on an MJ Research PTC 100-96 thermocycler according to the following protocol: 1 cycle of denaturation at 94°C for 1 minute and annealing/extension at 68°C for 1 minute 15 seconds; 25 cycles of denaturation at 94°C for 30 seconds and annealing/extension at 68°C for 1 minute; and a final extension at 72°C for 10 minutes. Presence of the appropriate PCR product then was confirmed both by molecular sizing with 1% Tris

GPI-PLD Primers Used in PCR-Based Amplification and Sequencing Reactions

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLD5'-1</td>
<td>5'-GAGGTTCACCCTGGTTGCCCTTTG-3'</td>
</tr>
<tr>
<td>PLD3'-1</td>
<td>5'-GCCATATCATACAGTGCTCAGAG-3'</td>
</tr>
<tr>
<td>PLD5'-2</td>
<td>5'-CCTGCCACAGCCGCTGTTATG-3'</td>
</tr>
<tr>
<td>PLD3'-2</td>
<td>5'-GATCCCTGTTGGCCTCTTGGTC-3'</td>
</tr>
<tr>
<td>PLD5'-3</td>
<td>5'-CATCCACATCCGCCCCTGAC-3'</td>
</tr>
<tr>
<td>PLD3'-3</td>
<td>5'-GCTTTGCGGTTTGTGGGAAAG-3'</td>
</tr>
<tr>
<td>PLD5'-4</td>
<td>5'-GGGAGCCCGACCTGGAAAGATG-3'</td>
</tr>
<tr>
<td>PLD3'-4</td>
<td>5'-GAGAAGGTCGGCAGATGTTGGG-3'</td>
</tr>
</tbody>
</table>
boric acid ethylenediamine tetra-acetic acid (TBE) gel electrophoresis and by direct DNA sequencing of gel-purified PCR products by a PCR-based dye termination method using the Taq-FS polymerase (ABI Prism Dye Terminator Cycle Sequencing Kit, Perkin-Elmer).33

Results

GPI-PLD Is Specifically Localized to Atherosclerotic Arteries

Immunohistochemistry was performed on 26 nonatherosclerotic and 57 atherosclerotic coronary artery segments. GPI-PLD often was detected in the intima of atherosclerotic coronary artery segments, where both extracellular and cell-associated GPI-PLD could be found (Figure 1). Extracellular GPI-PLD (Figure 1A) localized to regions that also contained apoA-I (Figure 1B), the predominant apolipoprotein of HDL. Cell-associated GPI-PLD (Figure 1C) was the most prominent pattern of GPI-PLD immunostaining in atherosclerotic segments. Immunohistochemistry performed on adjacent sections with cell-specific markers for macrophages, SMCs, and endothelial cells demonstrated that cell-associated GPI-PLD (C) colocalizes with macrophages (D) (magnification \( \times 200 \), methyl green counterstain).

In contrast, GPI-PLD immunostaining was absent from virtually all nonatherosclerotic segments (Figure 2). In particular, GPI-PLD was not detected in adventitial macrophages, indicating that this population of tissue macrophages does not express GPI-PLD at levels detectable by immunohistochemistry.

Both extracellular and cell-associated GPI-PLD were highly specific for atherosclerosis. However, whereas extracellular apoA-I was detected in all atherosclerotic plaques, extracellular GPI-PLD immunostaining was detected in only 30% of plaques (Figure 3). Cell-associated GPI-PLD was the most prevalent pattern of GPI-PLD staining in atherosclerosis; it was detected in 95% of atherosclerotic coronary artery segments (Figure 3).

Human Monocyte–Derived Macrophages Express GPI-PLD

Although previous studies had reported GPI-PLD enzymatic activity in a variety of macrophage-like cell lines, none had confirmed GPI-PLD mRNA expression, studied human macrophages, or determined the sequence of macrophage-expressed GPI-PLD. To better characterize GPI-PLD expression in human macrophages, total RNA was isolated from 7-day-old human monocyte–derived macrophages and, as a control, from the HepG2 (human hepatoma) cell line. The latter RNA source was chosen because GPI-PLD is expressed in the liver. RT-PCR then was performed with 4 overlapping primer pairs designed to span 2.5 kb of the hepatic and pancreatic mRNAs, a region that includes the entire mature peptide sequence. All 4 PCR products were gel-purified and sequenced to confirm their identity as GPI-PLD products.
Products of the predicted sizes of 706, 790, 673, and 771 bp were amplified with each of the 4 GPI-PLD primer pairs from both macrophage and HepG2 cell RNAs (Figure 4). Sequencing of the 4 macrophage PCR products demonstrated 100% nucleotide sequence identity with the reported sequence for GPI-PLD isolated from pancreatic islets and 96% nucleotide sequence identity with the reported sequence for GPI-PLD isolated from a liver cDNA library.31

Cell-Associated GPI-PLD and Cell-Associated Oxidation-Specific Epitopes Colocalize in Atherosclerotic Lesions

Because we hypothesized that GPI-PLD expression might be related to oxidation, immunohistochemistry was performed on adjacent sections with monoclonal antibody Ox5. Ox5 recognizes epitopes formed on proteins by products of polyunsaturated fatty acid oxidation.25 Co-localization of cell-associated GPI-PLD and Ox5 was found primarily on a subset of foam cell macrophages (Figure 5) but was also present on a subset of non–foam cell macrophages. Correlation of cell-associated staining for GPI-PLD and oxidation epitopes was high in both nonatherosclerotic and atherosclerotic segments (Figure 6). The immunohistochemical localization of GPI-PLD and oxidation epitopes raised the possibility that oxidants might regulate GPI-PLD expression.

Oxidant Stress Increases Steady-State GPI-PLD mRNA Levels in a Macrophage Cell Line

To determine whether oxidant stress regulates GPI-PLD expression, the effect of H2O2 on GPI-PLD expression was examined. J774A.1 is a murine macrophage–like cell line shown previously to contain functionally active GPI-PLD protein.13 J774A.1 cells were incubated in the presence or absence of 500 μmol/L H2O2 for 60 minutes. As shown in Figure 7, steady-state levels of GPI-PLD mRNA were 50±3% (mean±SD) higher (n=3, P<0.01) in H2O2-treated than in control J774A.1 cells. This result demonstrates that an oxidant can regulate GPI-PLD expression. It also raises the possibility that oxidant stress may mediate the upregulation of GPI-PLD seen in oxidation epitope–positive plaque macrophages.

Discussion

This study documents the presence of GPI-PLD in human atherosclerosis. The results show that (1) GPI-PLD is highly specific for atherosclerotic compared with nonatherosclerotic...
tissue and (2) GPI-PLD colocalizes with oxidation epitopes in a subset of plaque macrophages. Furthermore, this study documents regulation of GPI-PLD expression by exposure to H2O2. That GPI-PLD was detected almost exclusively in atherosclerotic lesions and not in nonatherosclerotic areas is consistent with a role for GPI-PLD inflammation. The in vivo colocalization of GPI-PLD protein with oxidation epitopes on plaque macrophages and the in vitro upregulation of macrophage GPI-PLD expression by exposure to H2O2 suggest that oxidants may be important regulators of GPI-PLD expression in inflammation.

Extracellular GPI-PLD in Atherosclerosis

Extracellular GPI-PLD staining was found in 30% of atherosclerotic plaques, where it colocalized with some but not all of the apoAI staining. This suggests that extracellular GPI-PLD may be derived from the influx of the GPI-PLD:apoA-I complex from serum. We recently showed that apoA-I localizes to proteoglycan-enriched regions of atherosclerotic plaques. Thus, the selective retention of extracellular GPI-PLD in atherosclerotic plaques could be due to increases in the number and/or affinity of HDL-retaining proteoglycans in atherosclerotic compared with nonatherosclerotic tissue. However, because macrophage cell lines secrete GPI-PLD activity in vitro, it is possible that secretion of GPI-PLD by plaque macrophages might account for some of the extracellular GPI-PLD seen in atherosclerotic lesions.

Cell-Associated GPI-PLD in Atherosclerosis

Cell-associated GPI-PLD, confined to macrophages, was found in nearly all atherosclerotic segments. Macrophage staining for GPI-PLD was seen in both the foam cell and non–foam cell phenotypes. However, only a subset of macrophages expressed GPI-PLD protein at a level detectable by immunohistochemistry. Macrophage expression of GPI-PLD mRNA was confirmed both in human macrophages and in J774 murine macrophage cell line, consistent with a previous report that murine and human macrophage cell lines contain GPI-PLD activity. In addition, our results suggest that some of the macrophage GPI-PLD is derived from endogenous synthesis of GPI-PLD rather than from uptake of exogenous GPI-PLD.

The sequence of human macrophage GPI-PLD mRNA had 100% nucleotide sequence identity to the reported human pancreatic coding sequence. A similar, although not identical, GPI-PLD cDNA has been identified from a human liver cDNA library. The relationship between these 2 forms is unclear. It is possible that they represent products of 2 different genes in humans. In mice, however, only 1 GPI-PLD gene has been identified, raising the possibility that polymorphisms may explain the difference between the 2 reported human GPI-PLD sequences.

These results do not rule out the possibility that all macrophages may express GPI-PLD below the level of detection of immunohistochemistry. However, the observation that only subsets of macrophages have detectable GPI-PLD in human atherosclerotic plaques, where it colocalized with some but not all of the apoAI staining. This suggests that extracellular GPI-PLD may be derived from the influx of the GPI-PLD:apoA-I complex from serum. We recently showed that apoA-I localizes to proteoglycan-enriched regions of atherosclerotic plaques. Thus, the selective retention of extracellular GPI-PLD in atherosclerotic plaques could be due to increases in the number and/or affinity of HDL-retaining proteoglycans in atherosclerotic compared with nonatherosclerotic tissue. However, because macrophage cell lines secrete GPI-PLD activity in vitro, it is possible that secretion of GPI-PLD by plaque macrophages might account for some of the extracellular GPI-PLD seen in atherosclerotic lesions.

Figure 4. Amplification of GPI-PLD products from human macrophages by RT/PCR. This photograph of an ethidium bromide–stained agarose gel demonstrates results of RT followed by 35 cycles of PCR with each 4 GPI-PLD–specific primer pairs using either positive control HepG2 cell RNA (H) or human monocyte–derived macrophage RNA (M). Left lane, 100-bp DNA ladder. Products of predicted sizes of 706, 790, 673, and 771 bp were obtained both with positive control liver RNA and with macrophage RNA. These results indicate that GPI-PLD is expressed by cultured human macrophages.

Figure 5. Colocalization of GPI-PLD with oxidation epitopes on a subset of atherosclerotic plaque macrophages. Immunohistochemical staining for macrophages (A), GPI-PLD (B), and oxidation epitopes (C). Most cells in this region are macrophages (A). A subset of macrophages (A, bottom) has positive immunostaining for GPI-PLD (compare bottoms of A and B). These GPI-PLD–positive macrophages also contain oxidation epitopes (compare B and C) (magnification ×200, methyl green counterstain).
PLD by immunohistochemistry suggests that GPI-PLD expression may be regulated.

Relationship Between Oxidation and GPI-PLD Expression

The immunohistochemical colocalization of macrophage GPI-PLD and oxidation epitopes in plaque macrophages raises the possibility of a link between oxidants and GPI-PLD expression. This possibility is supported by the observation that phorbol ester both stimulates production of oxidation epitopes in human monocyte-derived macrophages and increases GPI-PLD activity in murine and human macrophage cell lines. Moreover, the in vitro observation of this study that H2O2 increases GPI-PLD mRNA steady-state levels in J774A.1 cells implicates oxidant stress in regulation of GPI-PLD expression and is the first demonstration of regulation of GPI-PLD expression in any system. In future experiments, it will be important to clarify the exact relationship between oxidants and GPI-PLD expression in atherosclerosis.

Potential Role of GPI-PLD in Macrophage Function and Atherosclerosis

One proposed function of GPI-PLD is to cleave and release GPI-anchored proteins. Endogenously produced GPI-PLD releases GPI-anchored proteins from a number of cell types, including bone marrow stromal cells and COS cells cotransfected with GPI-PLD and the GPI-anchored protein alkaline phosphatase. In addition, a number of proteins, including decay-accelerating factor, CD59, and proteoglycans, accumulate in atherosclerotic lesions and may serve as substrates for GPI-PLD. Although GPI-anchored VCAM-1 has not yet been identified in humans, increased serum levels of soluble adhesion molecules, including VCAM-1, occur in patients with atherosclerosis. Furthermore, basic fibroblast growth factor, which binds to heparan sulfate proteoglycans, is released via GPI-PLD-mediated cleavage of a GPI anchor.

Finally, oxidative stress has been proposed to play a central role in the pathogenesis of atherosclerosis by mediating cytotoxicity/apoptosis, inducing cellular proliferation and regulating expression of a number of proatherogenic genes. We speculate that increased expression of GPI-PLD in macrophages may participate in these processes. Collectively, these observations raise the possibility that GPI-PLD-mediated release of GPI-anchored proteins and/or the generation of free glycosylphosphatidylinositolts involved in inflammation may represent a response to oxidative stress and play a role in atherogenesis.

Acknowledgments

This work was supported in part by grants HL-02788 and DK-02345 (Dr O’Brien) from the National Institutes of Health, Bethesda, Md, and by Grants-in-Aid 94-WA-518R and 96-WA-304 (Dr O’Brien) from the American Heart Association (AHA), Washington Affiliate, and 98-0625 from the AHA, Indiana Affiliate (Dr Deeg). C. Pineda was supported in part by an Introduction to Cardiovascular Scholarship Award from the AHA, Washington Affiliate. The authors wish to thank Renee LeBoeuf, Jay Heinecke, Alain Baron, and Alan Chait for helpful comments as well as Ginger Hays and Stephanie Kruzan for assistance in manuscript preparation.

References


Glycosylphosphatidylinositol-Specific Phospholipase D Is Expressed by Macrophages in Human Atherosclerosis and Colocalizes With Oxidation Epitopes
Kevin D. O'Brien, Christine Pineda, Winnie S. Chiu, Rosario Bowen and Mark A. Deeg

Circulation. 1999;99:2876-2882
doi: 10.1161/01.CIR.99.22.2876

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/99/22/2876