Group V Secretory Phospholipase A2 Promotes Atherosclerosis
Evidence From Genetically Altered Mice

Meredith A. Bostrom, Boris B. Boyanovsky, Craig T. Jordan, Marilyn P. Wadsworth, Douglas J. Taatjes, Frederick C. de Beer, Nancy R. Webb

Objective—Group V secretory phospholipase A2 (GV sPLA2) has been detected in both human and mouse atherosclerotic lesions. This enzyme has potent hydrolytic activity towards phosphatidylcholine-containing substrates, including lipoprotein particles. Numerous studies in vitro indicate that hydrolysis of high density lipoproteins (HDL) and low density lipoproteins (LDL) by GV sPLA2 leads to the formation of atherogenic particles and potentially proinflammatory lipid mediators. However, there is no direct evidence that this enzyme promotes atherogenic processes in vivo.

Methods and Results—We performed gain-of-function and loss-of-function studies to investigate the role of GV sPLA2 in atherogenesis in LDL receptor–deficient mice. Compared with control mice, animals overexpressing GV sPLA2 by retrovirus-mediated gene transfer had a 2.7 fold increase in lesion area in the ascending region of the aortic root. Increased atherosclerosis was associated with an increase in lesional collagen deposition in the same region. Mice deficient in bone marrow–derived GV sPLA2 had a 36% reduction in atherosclerosis in the aortic arch/thoracic aorta.

Conclusions—Our data in mouse models provide the first in vivo evidence that GV sPLA2 contributes to atherosclerotic processes, and draw attention to this enzyme as an attractive target for the treatment of atherosclerotic disease.

Key Words: Group V secretory phospholipase A2 ■ atherosclerosis ■ retrovirus-mediated gene transfer ■ bone marrow transplantation

The secretory phospholipase A2 (sPLA2) family of enzymes hydrolyze the fatty acid esterified at the s n-2 position of glycerophospholipids.1 Of the 10 sPLA2s described in mammals, Group IIA (GIIA), Group V (GV), and Group X (GX) sPLA2 have been detected in human and/or mouse atherosclerotic lesions.2–4 These enzymes have been proposed to exert multiple proatherogenic effects in the arterial wall. Phospholipid hydrolysis by sPLA2 generates potentially bioactive lipids, namely free fatty acids and lysophospholipids, which may promote various proinflammatory processes. Hydrolysis by either GV or GX sPLA2 markedly reduces the capacity of HDL to promote cellular cholesterol efflux from lipid-loaded macrophages.5 Hydrolysis of LDL by sPLA2 in vitro results in an increased affinity for extracellular matrix proteoglycans and promotes LDL aggregation.5,6 When incubated with mouse peritoneal macrophages, LDL hydrolyzed by either GV or GX sPLA2 induces foam cell formation.2,3 Thus, in vitro studies suggest that sPLA2s could promote atherogenesis by increasing the retention of LDL particles in the subendothelium and by generating potent inducers of macrophage foam cells.

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In this study, we directly tested the hypothesis that GV sPLA2 promotes atherosclerosis in vivo. Using both gain-of-function and loss-of-function approaches, we demonstrate for the first time that bone marrow–derived GV sPLA2 contributes to atherogenesis in LDL receptor–deficient mice.

Methods

Generation of Retroviral Vectors
Retroviral vectors expressing GV sPLA2 and GFP or GFP only were produced in Phoenix ecotropic packaging cells (Dr G.P. Nolan, Stanford University Medical Center, Palo Alto, Calif).

Mice
Female C57BL/6 and LDL receptor–deficient (LDLR−/−) mice in C57BL/6 background were obtained from Jackson Labs (Bar Harbor, Me). Female GV sPLA2-deficient (GV sPLA2−/−) mice that had been backcrossed 11 times with the C57BL/6 strain were provided by Dr. Jonathan Arm ( Brigham and Women’s Hospital, Boston, Mass).7 For atherosclerosis studies, mice were maintained on a high-fat diet (Harlan Teklad #TD94059) for 12 or 14 weeks, as indicated. All
Gene Transduction Rates, Plasma Total Cholesterol Concentrations, and Phospholipase Activity in GFP → LDLR−/−, GV sPLA2 + GFP → LDLR−/−, GV sPLA2+/+ → LDLR−/−, and GV sPLA2−/− → LDLR−/− mice

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<tr>
<td></td>
<td>GFP</td>
<td>GF+GFP</td>
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<tr>
<td>Transduction rates*</td>
<td>10±2%</td>
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<td>Total Cholesterol, mg/dl</td>
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<td>Phospholipase activity, μmol/ml (mean±SEM)</td>
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<table>
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<td>Total Cholesterol, mg/dl</td>
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<td>182±42</td>
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<td>Phospholipase activity, μmol/ml (mean±SEM)</td>
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<td>5.5±0.2</td>
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*As determined by flow cytometry of peripheral white blood cells.

Bone Marrow Transduction and Transplantation

Bone marrow cells were cultured for 48 hours in DMEM supplemented with 13% FBS, 5 μg/mL polybrene (Sigma H-9268), 10 ng/mL interleukin (IL)-3, 20 ng/mL IL-6, and 100 ng/mL mouse stem cell factor (mSCF). Cells were then transduced by two consecutive 24-hour incubations with retroviral supernatants. Cells (~1×106; 100 μL) were injected into lethally irradiated (9 Gy) female C57BL/6 mice. For atherosclerosis studies, bone marrow cells from 3 primary recipients that had >30% of peripheral white blood cells expressing GFP were injected into 15 lethally irradiated (9 Gy) female LDLR−/− mice.

Generation of GV sPLA2−/− → LDLR−/− and GV sPLA2+/+ → LDLR−/− Mice

Female LDLR−/− mice (6- to 8-week-old) were transplanted with 1×105 bone marrow cells harvested from age-matched female GV sPLA2−/− or GV sPLA2+/+ mice.

Lipid, Lipoprotein, and Phospholipase Analyses

Plasma total cholesterol and triglyceride concentrations were measured using colorimetric assays (Wako; Thermo Electron Corporation). Plasma lipoprotein cholesterol distributions and phospholipase activity were determined as described previously.

Real-Time RT-PCR

RNA was isolated from bone marrow cells and cardiac tissue using the TRIzol reagent (Molecular Research Center, Inc.). Semi-quantitative real-time RT-PCR was performed using the standard curve method and normalized with 18S.

Quantitation of Atherosclerosis

Atherosclerosis was quantified in the aortic arch/thoracic aorta and the aortic root as described previously. Aortic root sections were also stained for collagen using picrosirius red and photographed under polarized light.

Further detailed materials and methods are provided in supplemental materials, available online at http://atvb.ahajournals.org.

Results

Generation of Chimeric Mice Expressing GV sPLA2 and GFP

Chimeric LDL receptor–deficient (LDLR−/−) mice overexpressing GV sPLA2 and GFP or GFP only were generated by transducing bone marrow cells with retrovirus ex vivo, followed by two rounds of transplantation (supplemental Figure I). In a control experiment, atherosclerosis was assessed in LDLR−/− mice transplanted with nontransduced bone marrow cells, or cells transduced with the retroviral vector expressing only GFP. Retrovirus transduction of GFP had no effect on plasma total cholesterol in LDLR−/− mice fed normal diet (supplemental Table I). After high-fat diet, mice transduced with GFP had a slight reduction in plasma triglycerides compared with nontransduced mice. Importantly, there was no significant effect of retrovirus transduction on high-fat diet–induced hypercholesterolemia or atherosclerosis, despite persistent GFP expression in transplanted mice throughout the course of the 22-week experiment (supplemental Table I, supplemental Figure II).

Overexpression of GV sPLA2 in Bone Marrow–Derived Cells of LDLR−/− Mice

The expression of retroviral vector-encoded genes in transplanted mice was assessed by several methods. First, transduction rates in GFP → LDLR−/− and GV sPLA2−/− + GFP → LDLR−/− mice were quantified by determining the number of peripheral white blood cells that express GFP (Table). Flow cytometric analysis of mice 6 weeks and 18 weeks after transplantation (ie, before initiation of atherogenic diet and at the termination of the experiment) indicated that transduction rates were similar among mice within each group, and persisted throughout the course of the experiment. Mean transduction rates in GFP → LDLR−/− mice (~10%) were considerably lower compared with GV sPLA2−/− + GFP → LDLR−/− mice (~52%). However, as noted above, we established that GFP expression in bone marrow–derived cells does not influence the extent of atherosclerosis in mice.

GFP could also be detected by indirect immunofluorescent staining in atherosclerotic lesions of GFP → LDLR−/− and GV sPLA2−/− + GFP → LDLR−/− mice (green fluorescence, supplemental Figure IIIA). Staining of the same aortic root sections with a GV sPLA2−/− specific antibody provided strong evidence that GV sPLA2 expression was induced in GV sPLA2−/− + GFP → LDLR−/− mice above the endogenous levels expressed in GFP → LDLR−/− mice (red fluorescence, supplemental Figure IIIA). Consistent with the immunostaining data, we determined that GV sPLA2 mRNA was significant...
cantly increased both in bone marrow cells and in cardiac tissue encompassing the aortic root region of GV sPLA2 + GFP → LDLR−/− mice compared with GFP → LDLR−/− mice (supplemental Figure IIIB). Taken together, our data clearly show that retroviral vector-encoded genes were persistently expressed in the transduced mice, and GV sPLA2 + GFP → LDLR−/− mice had higher levels of GV sPLA2 expression in bone marrow–derived cells compared with GFP → LDLR−/− mice.

**Plasma Phospholipase Activity and Lipids/Lipoproteins**

To determine whether overexpression of GV sPLA2 in bone marrow–derived cells alters plasma phospholipase activity or lipid/lipoprotein concentrations, we assessed these parameters in marrow–derived cells alters plasma phospholipase activity or modestly reduced amount of LDL-associated cholesterol in GV mice (Figure 1B). This difference in lipoprotein profiles did not appear to be associated with a difference in LDL particle size.

**Quantification of Atherosclerosis**

Atherosclerotic lesion area was measured on the intimal surface of the aortic arch and thoracic aorta. There was no significant difference in atherosclerotic lesion area in the arch and thoracic regions of GV sPLA2 + GFP → LDLR−/− mice (mean=2.2±0.3%) compared with GFP → LDLR−/− mice (mean=2.7±0.5%). Atherosclerotic lesion area in aortic root sections was quantified after oil red O staining for neutral lipid (Figure 2A). GV sPLA2 + GFP → LDLR−/− mice had significantly more lesion area in the aortic root when compared with GFP → LDLR−/− mice (Figure 2B and 2C). In the ascending region of the aortic root (defined as the region anterior to the aortic valves), average lesion area was 2.7-fold greater in mice overexpressing GV sPLA2.

**Inflammatory Gene Expression in Aortic Tissue**

To assess inflammatory gene expression in lesions, real time RT-PCR was used to measure COX-2, tumor necrosis factor (TNF)-α, and IL-6 mRNAs in cardiac tissue containing the aortic root, where GV sPLA2 mRNA was shown to be significantly induced in GV sPLA2 + GFP → LDLR−/− mice (supplemental Figure IIIB). There were no significant differences in mRNA levels of any of these genes for the 2 groups of mice (supplemental Figure IVA through IVC), although there was a trend for increased TNF-α and IL-6 mRNA in mice overexpressing GV sPLA2.

**Quantification of Lesional Collagen**

Two previous studies have reported increased collagen deposition in atherosclerotic lesions of mice with macrophage-specific expression of human GIIA sPLA2. Thus, it was of interest to determine whether GV sPLA2 overexpression similarly promotes collagen deposition. Lesional collagen was visualized by staining aortic root sections with picrosirius red followed by polarized light microscopy (Figure 2D). Collagen area, calculated as a percentage of atherosclerotic lesion area, was significantly increased (2-fold) in the ascending region of the aortic root in mice that overexpressed GV sPLA2 (Figure 2E and 2F). This increase in collagen area was not associated with any detectable difference in matrix metalloproteinase (MMP)-9 or MMP-13 mRNA expression (supplemental Figure IVD and IVE).
Deficiency of GV sPLA2 in Bone Marrow–Derived Cells of LDLR−/− Mice

Given the significant proatherogenic effect of GV sPLA2 overexpression in transduced LDLR−/− mice, it was of interest to investigate whether endogenous GV sPLA2 in bone marrow–derived cells plays a significant role in atherogenesis. LDLR−/− mice were transplanted with bone marrow harvested from either GV sPLA2+/+ or GV sPLA2−/− mice.6 Six weeks after transplantation, plasma phospholipase activity and total cholesterol levels were not different between GV sPLA2+/+ → LDLR−/− and GV sPLA2−/− → LDLR−/− mice (Table). There was also no detectable difference in the lipoprotein-associated cholesterol distribution between the two groups (Figure 1C).

Mice were fed a high-fat diet for 14 weeks to accelerate atherosclerotic lipid deposition. We chose to maintain the mice on the atherogenic diet for a somewhat longer period than the overexpression study, because we anticipated that this would help to define a protective effect of GV sPLA2 depletion. After high fat diet feeding, plasma phospholipase activity, total cholesterol, and lipoprotein cholesterol distributions were similar in GV sPLA2+/+ → LDLR−/− and GV sPLA2−/− → LDLR−/− mice (Table; Figure 1D).

Figure 2. A, Oil Red O staining of representative aortic root sections from GFP → LDLR−/− and GV sPLA2−/− → LDLR−/− mice. Sections are located ~200 μm above the disappearance of the aortic valves. Images were taken at 50× under light microscopy. The box indicates the approximate region of a nearby section (within 64 μm) shown in D. B, Atherosclerotic lesion area in the aortic root. Values are mean lesion areas (±SEM) per section for sections ~64 μm apart; n = 6. The transition zone between the aortic sinus and the ascending aorta, defined by disappearance of the valve cusps, is 0 on the x axis. C, Mean lesion area (±SEM) in the ascending aorta (n = 6; *P < 0.05). D, Picrosirius red staining of aortic root sections from GFP → LDLR−/− and GV sPLA2−/− → LDLR−/− mice. Images were photographed under polarized light (magnification, 400×). Blue areas delineate regions stained with oil red O; pink areas delineate regions stained with picrosirius red. Regions outside the lesions have been cropped from the image. E, Mean (±SEM) collagen area, represented as the percent of atherosclerotic lesion area, for aortic root sections ~64 μm apart (n = 6). Numbers on the x axis correspond to values depicted in B. F, Mean collagen area (±SEM) as a percent of lesion area in the ascending region of the aortic root (n = 6; *P < 0.05).

The distribution of GV sPLA2 in atherosclerotic lesions of GV sPLA2+/+ → LDLR−/− and GV sPLA2−/− → LDLR−/− mice was assessed by indirect immunofluorescence and confocal microscopy. GV sPLA2 was detected in GV sPLA2+/+ → LDLR−/− mice, associated with lesional macrophages and to a lesser extent, vascular smooth muscle cells (Figure 3B and 3C, left panels). In contrast, there was a notable absence of GV sPLA2 colocalized with macrophages in lesions of GV sPLA2−/− → LDLR−/− mice (Figure 3B, right panel).

Atherosclerosis was quantified by en face analysis of the aortic root, and in serial sections throughout the aortic root. Compared with GV sPLA2+/+ → LDLR−/− mice, there was a 36% reduction in atherosclerotic lesion area in the aortic arch and thoracic aorta of GV sPLA2−/− → LDLR−/− mice (Figure 4A). However, there was no significant difference in atherosclerotic lesion area in the aortic root between the two groups (Figure 4B).

Discussion

An abundance of data implicates sPLA2s as mediators of atherosclerosis.14,15 Notably, expression of human GIIA sPLA2 in bone marrow–derived cells of LDLR−/− mice promotes atherosclerosis in the absence of alterations in plasma lipopro-
providing compelling evidence that increased sPLA2 activity within the vessel wall is proatherogenic. Recently, other members of the sPLA2 family in addition to GIIA have been speculated to play a role in atherosclerosis. Although GV sPLA2 has been detected in atherosclerotic lesions, there is no direct evidence that this enzyme contributes to atherogenesis in vivo. Results from this study provide the novel finding that GV sPLA2 promotes vascular lipid deposition in LDLR/H11002/H11002 mice.

Using retroviral vector mediated gene transfer, we investigated whether increased GV sPLA2 expression in bone marrow–derived cells modulates atherosclerosis. By coexpressing GFP in the transduced mice, we were able to specifically monitor transduction rates, and verify that expression of retroviral vector-encoded genes was maintained throughout the course of the ~20-week experiment. Although GFP has been used previously as a control in atherosclerosis studies using gene transfer, to our knowledge the effect of GFP on atherosclerosis has not been specifically addressed. Given the known effect of GFP to stimulate immune responses, it was important to confirm that GFP expression in bone marrow–derived cells does not alter the extent of atherosclerosis, because this could confound the interpretation of our results. Using this gene transfer approach, we unequivocally showed that mice expressing GV sPLA2 and GFP by retroviral vector had significantly increased lesion area compared with mice expressing only GFP.

We also showed that deficiency of GV sPLA2 in bone marrow–derived cells protects against atherosclerosis. We reported previously that GV sPLA2 is present in atherosclerotic

Figure 3. A, Aortic root sections from GV sPLA2+/→LDLR+/− (left) and GV sPLA2+/−→LDLR+/− mice (right) stained with oil red O (ORO) to visualize atherosclerotic lesions (50×). The boxes indicate the approximate regions of nearby sections (within 64 μm) depicted in B and C. B, Indirect immunofluorescent staining of GV sPLA2 (green) and CD68 (MΦ; red) (confocal image using a 20× objective). White arrows indicate colocalization of GV sPLA2 and macrophages. C, Indirect immunofluorescent staining of GV sPLA2 (green) and smooth muscle cell actin (SMC; red) (confocal image using a 20× objective). White arrows indicate colocalization of GV sPLA2 and smooth muscle cells.

Figure 4. A, Mean percent atherosclerotic lesion area (±SEM) in the aortic arch/thoracic aorta of GV sPLA2+/−→LDLR+/− and GV sPLA2+/−→LDLR+/− mice (n=12; *P<0.05). B, Atherosclerotic lesion area in the aortic root. Values shown are mean lesion areas (±SEM) for sections located ~64 μm apart (n=6). Numbers on the x axis correspond to those described in Figure 2B Legend.
lesions of both apoE−/− and LDLR−/− mice; however, the cellular source of the secreted enzyme was not established.3 By analyzing the lesional distribution of GV sPLA2 in mice transplanted with GV sPLA2−/− and GV sPLA2−/+ cells, we determined that macrophages are the major source of this enzyme in mouse lesions. Colocalization of GV sPLA2 with smooth muscle cells was also detected; however, this represented only a minor fraction of the total GV sPLA2 present in lesions.

An interesting aspect of our results is the finding that GV sPLA2 overexpression had region-specific effects on atherosclerosis that were different from GV sPLA2 deficiency. Whereas GV sPLA2 overexpression produced increased lipid deposition in the ascending aorta, GV sPLA2 deficiency resulted in decreased lesion area that was limited to the aortic arch/thoracic aorta. Although the reason for this discrepancy is unclear, studies investigating the effect of scavenger receptor A overexpression must also be considered. GX sPLA2 has been detected in mouse atherosclerotic lesions and has similar hydrolytic activity as GV sPLA2. Because we anticipated that GV sPLA2 deficiency in bone marrow–derived cells had no detectable effect on plasma lipoproteins or phospholipase activity, indicating that the protective effect was mediated within the vascular intima. That there was no detectable increase in plasma phospholipase activity in GV sPLA2+/GFP → LDLR−/− compared with GFP → LDLR−/− mice suggests that systemic effects of retroviral vector-mediated expression of GV sPLA2 were minimal. Nevertheless, we were able to detect a modest decrease in LDL-sized particles in mice overexpressing GV sPLA2 after atherogenic diet feeding when plasma lipoproteins were separated by size exclusion chromatography that was not observed in mice before high-fat diet feeding. We have shown previously that LDL particles hydrolyzed by GV sPLA2 are significantly smaller than native LDL. Small dense LDL particles are associated with increased atherogenicity.46 However, there was no evidence from fast protein liquid chromatography (FPLC) data that overexpression of GV sPLA2 resulted in the accumulation of smaller LDL particles. GV sPLA2 binds extracellular matrix proteoglycans and thus has the potential to hydrolyze LDL retained in the subendothelium. Rosengren et al recently showed that binding to proteoglycans significantly enhances GV sPLA2-mediated hydrolysis of LDL, and in turn, sPLA2 hydrolysis increases LDL–proteoglycan complex formation.70 We have shown that hydrolysis by GV sPLA2 alters the interaction of LDL particles with proteoglycans expressed on the surface of macrophages, leading to foam cell formation.7 The new finding that GV sPLA2 expression in bone marrow–derived cells is directly correlated with atherosclerotic lipid deposition in vivo is consistent with these in vitro results.

Transgenic expression of human GIIa sPLA2, promotes collagen deposition in atherosclerotic lesions of LDLR−/− mice.12,13 In the current study, mice overexpressing GV sPLA2 had significantly increased collagen area (normalized for lesion area) in the ascending region of the aortic root, the same region where GV sPLA2 overexpression increased lipid deposition. Although the molecular mechanisms are unknown, the possibility that sPLA2 may regulate the signaling pathway that leads to collagen deposition is intriguing. A recent study reported that pharmacological inhibition of GIIa sPLA2 prevents collagen deposition in the left ventricle that normally occurs during the development of hypertension in young spontaneously hypertensive rats.29 Because there is evidence that collagen content is in part regulated by matrix metalloproteinases (MMPs; reviewed in33), it is possible that increased GV sPLA2 activity leads to generation of specific arachidonate metabolites which have been shown to modulate MMP expression.34,35 However, we found no evidence that MMP-9 or MMP-13 transcripts are altered in mice overexpressing GV sPLA2.

In summary, using gain-of-function and loss-of-function approaches, we demonstrate for the first time that GV sPLA2 mediates atherosclerosis in vivo, consistent with abundant in vitro data. As with GIIa sPLA2, overexpression of GV sPLA2 in bone marrow...
cells leads to increased collagen deposition in atherosclerotic lesions. Future studies will clarify the mechanisms by which GV sPLA2 modulates atherosclerotic lesion development.

Acknowledgments

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Disclosures

None.

References

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METHODS

Generation of retroviral vectors  The cDNA encoding mouse GV secretory phospholipase A₂ (GV sPLA₂) was inserted into the retrovirus expression vector, MIEV (provided by Dr. R.G. Hawley). MIEV was derived from the murine Moloney leukemia virus and is a variant of the retroviral vector MINV in which the neomycin sequence is replaced with sequences encoding enhanced green fluorescent protein (GFP) (1). Retroviral vector expression plasmids encoding GV sPLA₂ and GFP or GFP only were transfected into Phoenix ecotropic packaging cells (provided by Dr. G.P. Nolan) using the Lipofectamine Reagent (Gibco 10964-013). Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 4 mM L-glutamine, 4500 mg/L glucose (Hyclone) supplemented with 10% heat inactivated fetal bovine serum (HI FBS), 1% penicillin-streptomycin, and 1% L-glutamine (Gibco) (“complete DMEM”). Transfected cells were cultured for 3 weeks, and then subjected to two rounds of fluorescence activated cell sorting on a Beckton-Dickinson Facsvantage cell analyzer to isolate clonal packaging lines that stably express GFP. Supernatants from these cells were titered by the limiting dilution method using NIH 3T3 cells (ATCC# CRL-1658). The rate of viral transduction in the limiting dilution assay was determined by flow cytometry, which quantified the number of NIH 3T3 cells expressing GFP. Clones that produced retroviral titers >1x10⁶ ifu/ml were expanded and used to produce high titer retrovirus stocks.
**Mice** Female C57BL/6 and LDL receptor deficient (LDLR\(^{-/-}\)) mice in C57BL/6 background were obtained from Jackson Labs. Female GV sPLA\(_2\) deficient (GV sPLA\(_2\)^{-/-}) mice that had been back-crossed 11 times into the C57BL/6 strain were generously provided by Dr. Jonathan Arm (2). Mice were maintained in a pathogen-free facility with equal light/dark cycle and free access to food (Teklad #TD02359) and water. For atherosclerosis studies, mice were maintained on a diet containing 7.5% cocoa butter and 1.25% cholesterol (Harlan Teklad #TD94059) for 12 or 14 weeks, as indicated. Two weeks prior to and six weeks following bone marrow transplantation, mice were provided drinking water containing Sulfatrim antibiotic (275 \(\mu\)g/ml). Throughout the post-transplantation period, mice were weighed twice per week to monitor general health. All procedures were done in compliance with the Lexington VA Medical Center Animal Care and Use Committee.

**Bone marrow transduction and transplantation** Bone marrow cells were collected from 6 to 8-week old female C57BL/6 mice intraperitoneally injected 4 days previously with 150 mg/kg 5-fluorouracil (5-FU). Cells from 15 mice were pooled and resuspended in 5 ml sterile PBS supplemented with 2% heat inactivated fetal bovine serum (PBS/2%HIFBS). To lyse red blood cells, 10 ml of red blood cell lysis buffer (RCRB: 8.24 g/l \(\text{NH}_4\text{Cl}\), 37 mg/l EDTA, 1 g/l \(\text{NaHCO}_3\) in \(\text{dH}_2\text{O}\)) was added. After 5 minutes incubation at room temperature, cells were washed twice in PBS/2%HIFBS, and then passed over a 40 \(\mu\)m cell strainer (BD Falcon). Cells were cultured for 48 hours at 37\(^{\circ}\)C in complete DMEM supplemented with 13% HI FBS, 5 \(\mu\)g/ml polybrene (hexadimethrine bromide,
Sigma H-9268), 10 ng/ml interleukin-3 (IL-3), 20 ng/ml IL-6, and 100 ng/ml mouse stem cell factor (mSCF). Recombinant IL-3, IL-6 and mSCF were generously provided by Kirin Brewery Co, LTD, Gunma, Japan. Cells were collected, and then transduced by two consecutive 24-hour incubations with ecotropic retroviral supernatants in complete DMEM. This was carried out by pre-incubating 2 ml of viral supernatant in 6 cm tissue culture dishes pre-coated with fibronectin (Becton Dickinson; 63 µg per dish) for 10 minutes, and then adding 2-5x10^6 bone marrow cells in 4 ml of complete DMEM with 15% HIFBS and supplemented with IL-3, IL-6 and mSCF in the final concentrations described above. For the second round of transduction, nonadherent cells were collected from the dish, resuspended in fresh complete DMEM supplemented as described above, and then added back to the same dish containing a fresh aliquot of viral supernatant. After a total of 48 hours incubation with retrovirus, cells were collected by gentle scraping, pooled, and resuspended in PBS (~1x10^7 cells/ml). Retroviral transduction rates after ex vivo incubations were 14-36%, as determined by flow cytometry to detect GFP expression. Cells (100 µl) were injected via the tail vein into each of 10 lethally irradiated (9 Gy) 6 to 8-week old female C57BL/6 mice.

To determine whether this procedure led to efficient gene transfer in vivo, mice transduced with the retrovirus expressing only GFP were bled 6 weeks after bone marrow transplantation and GFP expression in peripheral white blood cells was assessed by flow cytometry. Six weeks after bone marrow transplantation of primary recipients, 75 µl of blood was collected from the retro-orbital sinus. Red
blood cells were lysed by 2 consecutive 5-minute incubations in 4 ml of RCRB. The peripheral white blood cells were washed twice with 4 ml of PBS, resuspended in 300 µl PBS, and analyzed by flow cytometry. The percentage of GFP-positive peripheral white blood cells in transduced mice ranged from 2-53% (mean 38 ± 5%).

Secondary transplantation of retrovirus transduced bone marrow cells

Since wide variations in gene transduction rates could confound the interpretation of atherosclerosis results, we investigated whether a second transplantation, whereby bone marrow cells from three of the initially transplanted mice were pooled and transplanted into a second group of recipient mice, would yield less variable results. Bone marrow was collected from 3 primary recipients that had a high percentage (>30%) of peripheral white blood cells expressing GFP. Cells were pooled and injected via the tail vein into 15 lethally irradiated (9 Gy) 6 to 8-week old female LDLR−/− mice. Following a 6-week recovery period, the percentage of peripheral white blood cells in the transplanted mice that expressed GFP was quantified by flow cytometry as described above. Gene transduction rates were considerably more consistent in recipients of secondary transplants, ranging from 26-49% (mean 36 ± 2%) (Supplemental Table 1). For all of the studies reported here, atherosclerosis studies were performed following the double transplantation procedure (Supplemental Figure I).

Generation of GV sPLA2−/−→LDLR−/− and GV sPLA2+/+→LDLR−/− mice

Six to 8-week old female LDLR−/− mice were lethally irradiated with 9 Gy, and then transplanted by tail vein injection of 1x10^7 bone marrow cells harvested from the
femurs of age-matched female GV sPLA$_2^{-/-}$ or GV sPLA$_2^{+/-}$ mice. To assess engraftment of donor hematopoietic cells, DNA was isolated from bone marrow cells of recipient mice using the DNeasy Tissue Kit (Qiagen). Donor (LDLR$^{+/-}$) DNA and recipient (LDLR$^{-/-}$) DNA were amplified by the polymerase chain reaction using a mixture of 3 oligonucleotide primers that distinguish the 2 alleles, as described by the Jackson Laboratory (jaxmice.jax.org). In all mice analyzed, the amplification product corresponding to the LDLR$^{+/-}$ allele was detected, indicating successful engraftment.

**Lipid, lipoprotein and phospholipase analyses** Plasma total cholesterol and triglyceride concentrations were measured using colorimetric assays (Wako; Thermo Electron Corporation). Plasma samples (50 µl) were separated on a Superose 6 column as described previously (3) and the total cholesterol content of each fraction was determined. Plasma phospholipase activity was estimated by measuring the amount of free fatty acids (FFA) generated when added to mixed micelles comprised of 1-palmitoyl, 2-oleoyl phosphatidylglycerol (POPG), sodium deoxycholate and NP-40 as previously described (4). Phospholipase activity is reported as the amount of free fatty acid released per ml plasma. The POPG substrate used in the overexpression and deficiency studies was from two separate lots supplied by the vendor. Therefore, the determinations of plasma phospholipase activity cannot be directly compared for the two studies.

**Tissue harvest** Animals were anesthetized using chloral hydrate (350 mg/kg) and xylazine (4 mg/kg). Terminal blood was taken from the animals by cardiac puncture. A 60 µl aliquot was removed for flow cytometry and plasma
was prepared from the remaining portion by centrifugation for 5 minutes at 10,000 rpm. Animals were perfused with 15 ml PBS via the left ventricle. The aorta and heart were removed and separated. Aortas were placed in 4% paraformaldehyde in PBS (pH 7.4) for 24 hours, and then transferred to PBS. The top one-third of the heart (containing the aortic root) was removed from the apex and for most animals, frozen in Optimal Cutting Temperature Compound (OCT, Tissue Tek, Sakura Finetek). For the remaining animals, the samples were snap frozen for RNA isolation. Bone marrow from each femur was harvested in PBS and cell pellets were frozen at -20°C.

**Real-time RT-PCR** RNA was isolated from bone marrow cells and cardiac tissue using the TRIzol reagent (Molecular Research Center, Inc). The reverse transcription reaction was performed using the Reverse Transcription System (Promega) according to manufacturer's instructions. Real-time RT-PCR was performed using SYBR® Green (Applied Biosystems) on a DNA Engine Opticon 2 System (MJ Research). The reaction mixture contained 1 X SYBR Green, 2.5 mM MgCl₂, 1 mM dNTP, 0.125 pmoles of primers, 0.25 U of Amperase UNG, and 0.625 U AmpliTaq Gold® DNA polymerase. The reaction proceeded as follows: 50°C for 2 minutes, 95°C for 15 minutes, 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Steps 3-5 were repeated 39 times. Quantitation was done using the standard curve method and normalized with 18S. PCR primers for mouse GV sPLA₂ were: ATG AAG GGT CTC CTC ACA CTG (forward) and TAG CAG CCG TAG AAG CCA TAG (reverse). PCR primers for mouse 18S were: CGG CTA CCA CAT CCA AGG AA (forward) and GCT
Quantitation of atherosclerosis The descending aorta and aortic arch were cleaned of adventitial tissue, the intimal aortic surface was exposed by a longitudinal cut, and the tissue was pinned en face. The aortic arch region was defined as the region from the ascending arch to 3 mm distal to the left subclavian artery. The thoracic region was defined as the region from the end of the aortic arch to 1 mm below the last intercostal branch. The abdominal region was defined as the region from the end of the thoracic region to the iliac bifurcation. Percent lesion surface area in each region was calculated using Image Pro Software (Media Cybernetics).

The aortic root was serially sectioned (8 µm thick sections) from the aortic sinus (where the aortic valve leaflets appear) to the distal region of the root, covering a length of approximately 600 µm. Sections ~64 µm apart were fixed with 4% paraformaldehyde and histochemically stained with oil red O. Oil red O (0.5% in isopropyl alcohol) was prepared by diluting with 2/3 volume dH₂O and filtering with 0.2 micron filter paper. Sections were stained with oil red O for ten minutes, washed with 60% isopropyl alcohol and stained with hematoxylin for 10 seconds to visualize nuclei. Aortic root sections were also stained for collagen using picrosirus red and photographed under polarized light as described previously (5,6). Atherosclerotic lesion area was delineated manually using oil red O staining and quantified using Image Pro software. For quantitation of
collagen, non-lesion areas (regions not stained by oil red O) were cropped from the image. Collagen area in the lesion was delineated manually, quantified using Image Pro software, and values were presented as a percentage of lesion area.

Indirect Immunofluorescence Aortic root sections were fixed in acetone for 5 minutes, washed two times with PBS, and blocked for one hour at room temperature with PBS containing 5% normal goat serum and 0.5% Triton X-100. Sections were then incubated for 1 hour at room temperature with a combination of primary antibodies, as indicated. The primary antibodies were chicken anti-mouse GV sPLA$_2$ IgG (10 µg/ml) (4), mouse anti-GFP IgG (1:200, Sigma), rabbit anti-mouse CD68 IgG (1:200; Serotec), and rabbit anti-smooth muscle actin IgG (1:100; Abcam). After 4 washes with 0.2% Tween in PBS, CD68 and smooth muscle actin were detected using Alexa Fluor 568-labeled goat anti-rabbit IgG (1:200; Molecular Probes). GV sPLA$_2$ was detected using Alexa Fluor 488-labeled goat anti-chicken IgG (1:200; Molecular Probes) or Alexa Fluor 568-labeled goat anti-chicken IgG (1:200; Molecular Probes). GFP was detected using biotinylated goat anti-mouse IgG (1:200, Jackson Immunoresearch Labs), followed by FITC-labeled Streptavidin (1:1000, Molecular Probes). Slides were mounted using fluorescence protecting medium containing DAPI (Vectashield, Vector Laboratories). Confocal microscopy was performed at the University of Kentucky Imaging Facility using a Leica laser scanning microscope with UV, argon (488 nm) and krypton (568 nm) lasers and a 20X or 60X oil immersion objective.
Statistical Analyses All data were analyzed using SigmaStat Software 2.03 (SPSS, Inc). Data was tested for normalcy and equal variance before analysis. A Student’s t-test or a Mann-Whitney test in lieu of a t-test was used where appropriate.
REFERENCES


Supplemental Figure I

1° Recipient

Chimera

2° Transplant

Transduced C57BL/6 bone marrow cells

1° recipients reconstituted with transduced bone marrow cells:
GFP
GFP + GV sPLA₂

Assess atherosclerosis

Initiate atherogenic diet

12 wks

6 wks

2° recipients reconstituted with pooled bone marrow derived from 1° transplants:
GFP
GFP + GV sPLA₂
Supplemental Figure II
A.

GV sPLA₂+GFP→LDLR⁻/⁻  GFP→LDLR⁻/⁻  GV sPLA₂+GFP→LDLR⁻/⁻

(no primary Ab)

B.

Bone Marrow

Aortic Root

Supplemental Figure III
Supplemental Figure IV
Supplemental Figure Legends

Supplemental Figure I

Overview of experimental design. Bone marrow cells transduced with retroviral vector expressing either GFP only or GFP and GV sPLA$_2$ were transplanted into lethally irradiated recipient mice. Bone marrow cells from these primary transplants were pooled, and then used to reconstitute LDLR$^{-/-}$ mice. After the 6-week engraftment period, mice were fed an atherogenic diet for 12 weeks, and then euthanized for analysis of atherosclerosis.

Supplemental Figure II

(A) En face lesion area (mean ± SEM) in the aortic arch and thoracic aorta of GFP-transduced (n = 12) and non-transduced (n = 9) mice as a percent of total intimal area. (B) Lesion size (mean ± SEM) in the aortic root of GFP-transduced (n = 5) and non-transduced (n = 5) mice. Lesion area was determined in 8-µm-thick sections ~64 µm apart after oil red O staining. Distance through the aorta is shown on the x-axis. Zero is defined as the transition zone, a region just proximal to the disappearance of the aortic valves. Negative distance is the aortic sinus (proximal to the transition zone) and the positive distance is distal to the transition zone, proceeding into the ascending aorta.
Supplemental Figure III

(A) Indirect immunofluorescence of aortic root sections from GFP → LDLR−/− and GV sPLA₂ + GFP → LDLR−/− mice. Confocal microscopy was performed using a 60X oil immersion objective in order to detect GFP (green) and GV sPLA₂ (red) secondary antibodies. Nuclei were visualized using DAPI. The section on the left is a negative control (no primary antibody). (B) Real-time RT-PCR (mean ± SEM) of GV sPLA₂ mRNA in bone marrow cells (left) and aortic root tissue (right) from GFP → LDLR−/− and GV sPLA₂ + GFP → LDLR−/− mice (n=3-4). GV sPLA₂ mRNA is normalized to 18S (* p<0.05).

Supplemental Figure IV

Real time RT-PCR quantification (mean ± SEM) of COX-2 (A), TNF-α (B), IL-6 (C), MMP-9 (D), and MMP-13 (E) mRNAs in aortic root tissue from GV sPLA₂ + GFP → LDLR−/− and GFP → LDLR−/− mice (n=4). All mRNAs were normalized for 18S.
Supplemental Table I. Gene transduction rates and plasma total cholesterol and triglyceride concentrations in GFP-transduced and non-transduced mice (*p < 0.05).

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<td>Transduction rates**</td>
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<tr>
<td>Plasma triglycerides (mg/dl)</td>
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** as determined by FACS analysis of peripheral white blood cells