

# Macrophage-Expressed Group IIA Secretory Phospholipase A<sub>2</sub> Increases Atherosclerotic Lesion Formation in LDL Receptor–Deficient Mice

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**Objective**—Transgenic mice expressing human group IIA secretory phospholipase A<sub>2</sub> (group IIA sPLA<sub>2</sub>) spontaneously develop atherosclerotic lesions. The mechanism for this proatherogenic effect is likely multifactorial, because HDL-cholesterol is significantly lower and LDL/VLDL cholesterol is slightly higher in transgenic mice compared with nontransgenic littermates. In the present study, we show for the first time that elicited peritoneal macrophages from transgenic mice express human group IIA sPLA<sub>2</sub>. This study tested whether macrophage-expressed sPLA<sub>2</sub> contributes to atherogenesis.

**Methods and Results**—Bone marrow cells from either sPLA<sub>2</sub> transgenic mice or control C57BL/6 mice were transplanted into LDL receptor–deficient mice. After hematopoietic engraftment, animals were fed a diet enriched with saturated fat and cholesterol for 12 weeks. Despite a lack of effect on serum lipoprotein concentrations, the presence of bone marrow–derived cells expressing human group IIA sPLA<sub>2</sub> resulted in a significant increase in the extent of atherosclerosis in the aortic arch (12.8±1.4% versus 7.4±0.9%;  $P<0.005$ ) and aortic sinus (0.3±0.03 mm<sup>2</sup> versus 0.2±0.04 mm<sup>2</sup>;  $P<0.05$ ).

**Conclusions**—Group IIA sPLA<sub>2</sub> can contribute to atherosclerotic lesion development through a mechanism that is independent of systemic lipoprotein metabolism. (*Arterioscler Thromb Vasc Biol.* 2003;23:263-268.)

**Key Words:** atherosclerosis ■ bone marrow transplant ■ transgenic mice  
■ group IIA secretory phospholipase A<sub>2</sub> ■ macrophages

Group IIA secretory phospholipase A<sub>2</sub> (group IIA sPLA<sub>2</sub>) is a member of a family of secreted phospholipases that hydrolyzes the *sn*-2 fatty acyl ester bond of glycerophospholipids to generate free fatty acids (FFAs) and lysophospholipids.<sup>1</sup> Group IIA sPLA<sub>2</sub> (traditionally referred to as nonpancreatic or synovial sPLA<sub>2</sub>) is thought to be responsible for amplifying the inflammatory component of many disease processes, including atherosclerosis. During acute or chronic inflammation, the concentration of group IIA sPLA<sub>2</sub> can increase more than 100-fold in inflammatory fluids and plasma.<sup>2</sup> In humans, serum concentration of sPLA<sub>2</sub> is an independent risk factor for coronary artery disease and a predictor of cardiovascular events.<sup>3</sup> Group IIA sPLA<sub>2</sub> has been detected in human atherosclerotic lesions by immunocytochemistry. In two studies, group IIA distribution corresponded mainly to that of  $\alpha$ -actin, suggesting that the primary cellular source of the enzyme in the vessel wall is smooth muscle cells.<sup>4,5</sup> However, some sPLA<sub>2</sub> staining associated with lipid-laden CD68-positive macrophages was also detected. These findings contrast with two other reports, where

sPLA<sub>2</sub> was present primarily in areas with macrophage-derived foam cells.<sup>6,7</sup> These discrepant results could be explained by differences in tissue preservation or the extent of atherosclerosis. It is also possible that the antibodies used for immunostaining may exhibit varying amount of cross-reactivity with a related sPLA<sub>2</sub>, group V.<sup>8,9</sup>

C57BL/6 transgenic mice that express human group IIA sPLA<sub>2</sub> provide a useful model to investigate the role of this enzyme in atherosclerotic lesion development.<sup>10</sup> The C57BL/6 strain lacks endogenous group IIA sPLA<sub>2</sub> protein because of a frame-shift mutation in exon 3.<sup>11</sup> Human group IIA sPLA<sub>2</sub> is present in a variety of tissues in the transgenic mice, including liver, kidney, lung, skin, and intima/media of the aorta.<sup>12</sup> Although serum enzymatic activity is elevated  $\approx$ 8-fold compared with nontransgenic littermates,<sup>10</sup> there is no evidence of systemic inflammation in the transgenic mice.<sup>13</sup> Notably, human group IIA sPLA<sub>2</sub> transgenic mice maintained for 12 weeks on a diet containing 1.25% cholesterol, 15.75% fat, and 0.5% sodium cholate have significantly increased vascular lipid deposition compared with nontrans-

Received November 14, 2002; revision accepted November 22, 2002.

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*Arterioscler Thromb Vasc Biol.* is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000051701.90972.E5

genic littermates.<sup>14</sup> The transgenic mice also develop spontaneous lesions when fed a low-fat diet.<sup>14</sup> Thus, the relationship in humans between sPLA<sub>2</sub> and atherosclerosis may be a causal one.

Group IIA sPLA<sub>2</sub> may promote atherosclerosis, at least in part, by altering systemic lipoprotein metabolism. The sPLA<sub>2</sub> transgenic mice have significantly lower plasma concentrations of HDL-cholesterol and phospholipids and slightly higher VLDL/LDL compared with nontransgenic littermates.<sup>13–15</sup> Differences in plasma lipoprotein concentrations do not seem to account entirely for the proatherogenic effect of sPLA<sub>2</sub>, however, because transgenic mice maintained on a normal diet spontaneously developed atherosclerotic lesions despite having relatively low LDL and VLDL.<sup>14</sup> Immunocytochemical analysis showed the presence of human group IIA sPLA<sub>2</sub> in aortic lesions of transgenic mice,<sup>14</sup> providing the possibility that sPLA<sub>2</sub> may provide a local proatherogenic effect in the microenvironment of the developing lesion.

We now demonstrate the new finding that group IIA sPLA<sub>2</sub>, driven by its natural promoter, is expressed in macrophages in human group IIA transgenic mice. We hypothesized that increased sPLA<sub>2</sub> expression in macrophages within the microenvironment of the developing lesion may contribute to atherogenesis. To test this possibility, we transplanted bone marrow cells derived from human sPLA<sub>2</sub> transgenic mice into LDL receptor-deficient (LDLR<sup>-/-</sup>) mice that lack group IIA sPLA<sub>2</sub>. We reasoned that this approach would allow for a better mechanistic definition of the proatherogenic effects of sPLA<sub>2</sub>, because systemic influences on lipoprotein metabolism should be minimized. Our results show that expression of human group IIA sPLA<sub>2</sub> in bone marrow-derived cells promotes atherosclerotic lesion formation in the absence of any measurable changes in plasma lipoprotein concentrations.

## Methods

### Animals

Animals were maintained in a pathogen-free facility with equal light/dark cycle and free access to food and water. Male LDLR<sup>-/-</sup> and C57BL/6 mice were obtained from the Jackson Laboratory. LDLR<sup>-/-</sup> mice were backcrossed 10 times on a C57BL/6 background. Human sPLA<sub>2</sub> transgenic mice (backcrossed 12 times into C57BL/6) were obtained from Taconic. Recipient LDLR<sup>-/-</sup> mice were maintained on drinking water containing Sulfatrim (275 µg/mL) 1 week before and 2 weeks after bone marrow transplantation. Six weeks after transplantation, animals were maintained on a modified diet containing 20% fat and 0.15% cholesterol (wt/wt) (Harlan Teklad No. 88137) for 12 weeks. All animal procedures were approved by the Veteran's Administration Institutional Animal Care and Use Committee.

### Bone Marrow Transplantation

Ten-week-old LDLR<sup>-/-</sup> recipient mice were lethally irradiated with 9 Gy using a cesium  $\gamma$  source. Irradiated recipient mice were transplanted by tail vein injection of  $1 \times 10^7$  bone marrow cells harvested from tibia and femurs of age-matched C57BL/6 or sPLA<sub>2</sub> transgenic donor mice. To assess engraftment of donor hematopoietic cells, DNA was isolated from bone marrow cells of recipient mice using the DNeasy Tissue Kit (Qiagen). Polymerase chain reaction analysis was performed using DNA isolated from bone marrow of transplanted mice. Donor (LDLR<sup>+/+</sup>) DNA and recipient (LDLR<sup>-/-</sup>) DNA were amplified in a single reaction using a mixture of 3 oligonucleotide primers that distinguish the 2 alleles, as

described by the Jackson Laboratory. In all mice analyzed, the only amplification product detected corresponded to the LDLR<sup>+/+</sup> allele, indicating successful engraftment.

### Removal of Tissue and Blood Samples

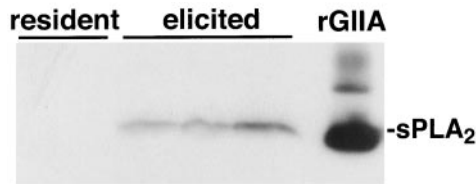
Recipient animals were bled via the retroorbital sinus 6 weeks after bone marrow transplantation, before initiation of modified diet. Animals were anesthetized using xylazine (4 mg/kg) and chloral hydrate (350 mg/kg). At the end of the study, bone marrow cells were flushed from the femur of each recipient mouse into 1 mL PBS. Terminal blood samples were collected, and samples were spun for 5 minutes at 10 000 rpm to obtain plasma. Animals were perfused with PBS by puncture of the left ventricle. The heart was separated from the aorta and frozen in OCT. The aortas were removed and immersed in 4% paraformaldehyde for 24 hours. After removal of adventitial tissue, the intimal aortic surface was exposed by a longitudinal cut and the tissue was pinned en face.

### Atherosclerotic Lesion Analysis

Lesion size was quantified in both the aortic arch and aortic root, as described previously.<sup>16</sup> The aortic arch was defined as the region from the ascending arch to 3 mm distal to the subclavian artery.<sup>16</sup> Percent lesion area was calculated using Image Pro software (Media Cybernetics). For analysis of the aortic root, tissue was frozen in OCT. Ventricular tissue was sectioned from the apical aspect until the aortic valves were visible. At this point, tissues were cut at 8-µm intervals and placed sequentially on 8 slides. Tissues were cut until  $\approx 10$  sections had been acquired per slide. The aortic sinus was defined as a region in which leaflet valves or cusps were present, while the ascending aorta was defined as the distal region of the root. Lesion areas were quantified by Image Pro Software using the luminal edge and internal elastic lamina as boundaries. Oil red O staining was used to assist in the visualization of lesions but not in the quantification of lesion size. Immunocytochemistry was performed on acetone-fixed, frozen sections as described previously.<sup>17,18</sup> A macrophage-specific antisera was obtained from Accurate (AI-AD31240). Anti-human group IIA sPLA<sub>2</sub> was provided by Dr T. Nevalainen<sup>19</sup> and used at a dilution of 1:1000. This rabbit antiserum was raised against recombinant human group IIA sPLA<sub>2</sub> and has been used extensively to stain group IIA sPLA<sub>2</sub> in human<sup>19</sup> and transgenic mouse tissues.<sup>12</sup> Previously, this antiserum was shown to exhibit no immunoreactivity with any tissue from nontransgenic C57BL/6 mice that lack endogenous group IIA sPLA<sub>2</sub>,<sup>12</sup> suggesting that this reagent does not cross-react with any other mouse sPLA<sub>2</sub>, including group V. Tissues were incubated with a species-specific biotinylated secondary antibody followed by an avidin-biotin peroxidase complex (Elite kits, Vector Laboratories). Immunoreactivity was visualized using the red chromogen amino-ethyl carbazole (Biomed), and sections were counterstained with hematoxylin.

### Lipid, Lipoprotein, and Phospholipase Analysis

Six weeks after transplant and before initiation of high-fat diet, plasma from 4 mice was pooled (3 pools per group) for quantifying lipids and phospholipase activity. For terminal samples, individual mouse plasmas were analyzed separately (C57BL/6  $\rightarrow$  LDLR<sup>-/-</sup>, n=11; sPLA<sub>2</sub> tg  $\rightarrow$  LDLR<sup>-/-</sup>, n=12). Total and HDL cholesterol concentrations were determined using an enzymatic assay (Wako Chemicals Inc). Aliquots of plasma pooled from 2 to 4 mice (200 µL) were clarified by centrifugation and resolved by size exclusion chromatography using a Superose 6 column (Pharmacia LKB Biotechnology Inc). The column was eluted at a flow rate of 0.5 mL/min in buffer containing 150 mmol/L NaCl and 10 mmol/L Tris/HCl, pH 7.4, 0.01% sodium azide. The cholesterol content of fractions (0.5 mL) was determined enzymatically (Wako Chemicals). Phospholipase activity in plasma was determined using a colorimetric assay (Wako Chemicals) with mixed micelles comprising 1-palmitoyl, 2-oleoyl phosphatidylglycerol, deoxycholate, and Nonidet-40 as substrate.<sup>20</sup> Values were expressed as the amount of FFA released in the assay per microliter of plasma.



**Figure 1.** Western blot analysis of peritoneal macrophages from human group IIA sPLA<sub>2</sub> transgenic mice. Macrophages were collected from duplicate untreated mice (resident) or from triplicate mice 5 days after intraperitoneal injection of 1% biogel (elicited) and cultured as described in the Experimental Procedures section. Aliquots corresponding to 10  $\mu$ g protein were separated by nonreducing SDS-PAGE, immunoblotted with rabbit anti-human group IIA sPLA<sub>2</sub>, and visualized by chemiluminescence detection. Purified recombinant human group IIA (rGIIA) sPLA<sub>2</sub> (100 ng) was analyzed for comparison. Similar results were obtained in 2 additional experiments.

### Immunoblot Analysis

Peritoneal macrophages were collected from untreated human group IIA sPLA<sub>2</sub> transgenic mice or mice 5 days after intraperitoneal injection of 1% biogel (1 mL). Adherent cells were cultured for 16 hours in DMEM containing 10% FBS. Postnuclear supernatants of cell lysates were separated by nonreducing SDS-PAGE, electroblotted onto 0.2  $\mu$ mol/L pore-size PVDF membrane (Schleicher and Schuell), and immunoblotted using rabbit anti-human synovial sPLA<sub>2</sub>.<sup>8</sup> Antibody binding was visualized by chemiluminescence detection (ECL, Amersham Corp).

### Statistical Analysis

All data are presented as mean  $\pm$  SEM. Student's *t* test was performed using Sigma Stat 2.03 (SPSS, Inc). All data met the constraints of normality and equivalence of variance to permit parametric analysis.

## Results

### Immunoblot Analysis of Human sPLA<sub>2</sub> Transgenic Mouse Peritoneal Macrophages

In humans, group IIA sPLA<sub>2</sub> mRNA is not detected in monocytes or terminally differentiated, unstimulated macrophages.<sup>7</sup> However, group IIA sPLA<sub>2</sub> mRNA transcription is induced in human monocyte-derived macrophages incubated with minimally modified or mildly oxidized LDL.<sup>7</sup> To assess whether macrophages from transgenic mice harboring the entire human group IIA sPLA<sub>2</sub> gene express human sPLA<sub>2</sub>, immunoblot analysis of peritoneal macrophages was performed (Figure 1). Human sPLA<sub>2</sub> protein was not detected in resident macrophages from the transgenic mice. In contrast, an  $\approx$ 14-kDa immunore-

active band, which comigrated with recombinant human group IIA sPLA<sub>2</sub>, was detected in elicited macrophages that were collected 5 days after peritoneal injection of a 1% solution of Biogel. As expected, group IIA sPLA<sub>2</sub> was not detected in either resident or biogel-elicited macrophages from nontransgenic littermates (data not shown). These results suggest that macrophage-derived sPLA<sub>2</sub> may contribute to the intense sPLA<sub>2</sub> staining in aortic lesions of human sPLA<sub>2</sub> transgenic mice.<sup>14</sup>

### Plasma Cholesterol, Phospholipase Activity, and Lipoprotein Distributions in Mice After Bone Marrow Transplantation

To test the hypothesis that macrophage-expressed sPLA<sub>2</sub> contributes to atherosclerotic lesion development, 10-week-old LDLR<sup>-/-</sup> mice were transplanted with bone marrow cells derived from either human group IIA sPLA<sub>2</sub> transgenic mice or wild-type mice. Six weeks after bone marrow transplantation, mice were maintained for 12 weeks on a modified diet containing 20% fat and 0.15% cholesterol (wt/wt). Plasma concentrations of total cholesterol, HDL-cholesterol, and phospholipase activity were quantified before and after feeding a high-fat diet. In both sPLA<sub>2</sub> tg $\rightarrow$ LDLR<sup>-/-</sup> and C57BL/6 $\rightarrow$ LDLR<sup>-/-</sup> mice, total cholesterol concentrations were highly elevated after modified diet feeding (Table). None of the parameters measured were significantly different between the two groups of mice, either before or after the atherogenic diet. Separation of plasma lipoproteins by size exclusion chromatography showed that most plasma cholesterol in mice fed the modified diet was in the VLDL/LDL fractions (Figure 2). There was no significant difference in lipoprotein cholesterol distribution in C57BL/6 $\rightarrow$ LDLR<sup>-/-</sup> and sPLA<sub>2</sub> tg $\rightarrow$ LDLR<sup>-/-</sup> mice.

### Quantification of Atherosclerotic Lesions

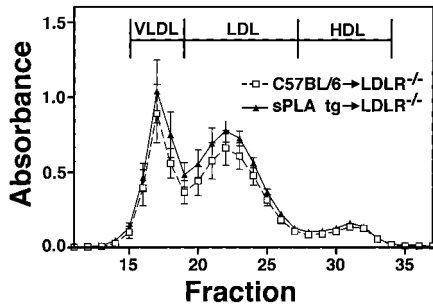
The extent of atherosclerosis was measured on the intimal surface of the aortic arch and in the aortic root. No discernible lesions were detected in either the abdominal aorta or thoracic aorta in any of the recipient mice. En face analysis of the aortic arch showed a highly significant 73% increase in lesion area in mice transplanted with bone marrow cells derived from human group IIA sPLA<sub>2</sub> transgenic mice compared with control bone marrow (percent lesion area, 12.8 $\pm$ 1.4% versus 7.4 $\pm$ 0.9% for transgenic and nontransgenic, respectively; *P*<0.005; Figure 3A). In addition, there was a significant increase in mean atherosclerotic lesion area within the aortic sinus of the aortic

**Total Cholesterol (TC), HDL Cholesterol (HDL-C), and Phospholipase (PLase) Concentrations in C57BL/6 $\rightarrow$ LDLR<sup>-/-</sup> and sPLA<sub>2</sub> tg $\rightarrow$ LDLR<sup>-/-</sup> Mice**

	6 Weeks After BMT, Normal Diet		19 Weeks After BMT, 12 Weeks High-Fat Diet	
	C57 $\rightarrow$ LDLR <sup>-/-</sup>	sPLA <sub>2</sub> $\rightarrow$ LDLR <sup>-/-</sup>	C57 $\rightarrow$ LDLR <sup>-/-</sup>	sPLA <sub>2</sub> $\rightarrow$ LDLR <sup>-/-</sup>
TC	234 $\pm$ 17	237 $\pm$ 5	1204 $\pm$ 103	1292 $\pm$ 116
HDL-C	91 $\pm$ 2	91 $\pm$ 2	67 $\pm$ 6	76 $\pm$ 3
PLase	2.7 $\pm$ 0.2	2.9 $\pm$ 0.1	3.3 $\pm$ 0.4	4.0 $\pm$ 0.2

Cholesterol values are in mg/dL, mean  $\pm$  SEM. Phospholipase values are nmol FFA released from mixed micelles containing 1-palmitoyl, 2-oleoyl phosphatidylglycerol per  $\mu$ L sera. Corresponding values for control C57BL/6 and human Group IIA sPLA<sub>2</sub> transgenic mouse sera were 2.4 $\pm$ 0.26 and 37.3 $\pm$ 1.8, respectively.

BMT indicates bone marrow transplantation.



**Figure 2.** Lipoprotein cholesterol distributions of C57BL/6 →LDLR<sup>-/-</sup> and sPLA<sub>2</sub> tg→LDLR<sup>-/-</sup> mice fed a modified diet for 12 weeks. Values represent the mean cholesterol content of each fraction (±SEM) for 4 pools per group, with 2 to 3 mice in each pool.

root (mean lesion area per section, 0.2±0.04 versus 0.3±0.03 mm<sup>2</sup> for nontransgenic and transgenic, respectively; *P*<0.05; Figure 3B).

**Immunocytochemical Analysis of Lesions**

Immunocytochemical analysis of aortic root sections confirmed the presence of human group IIA sPLA<sub>2</sub> in lesions of sPLA<sub>2</sub> tg→LDLR<sup>-/-</sup> mice that was associated with lipid-laden macrophages (Figure 4). As expected, human sPLA<sub>2</sub> was not detected in C57BL/6→LDLR<sup>-/-</sup> mice (data not shown). Inspection of all sections from aortic roots of each animal did not reveal overt differences in the cellular characteristics of lesions from C57BL/6→LDLR<sup>-/-</sup> and sPLA<sub>2</sub> tg→LDLR<sup>-/-</sup> mice.

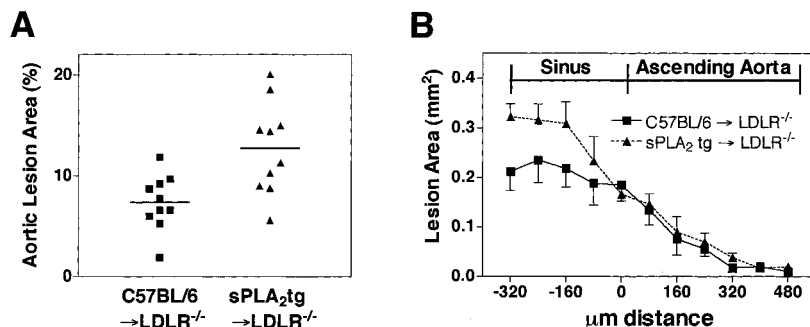
**Discussion**

C57BL/6 transgenic mice expressing human group IIA sPLA<sub>2</sub> have increased vascular lipid deposition compared with nontransgenic littermates.<sup>14</sup> The present study demonstrates for the first time that macrophage cells in these transgenic mice express human group IIA sPLA<sub>2</sub>. This finding suggested the interesting possibility that macrophage-expressed sPLA<sub>2</sub> contributes to atherosclerotic lesion formation in the transgenic mice. To test this possibility, lethally irradiated LDLR<sup>-/-</sup> mice were reconstituted with bone marrow cells derived from either human sPLA<sub>2</sub> transgenic mice or wild-type mice. Both donor and recipient mice were C57BL/6 background and thus lack the expression of mouse group IIA sPLA<sub>2</sub> because of a natural mutation in the endogenous gene.<sup>11</sup> We selected male mice for our analysis because studies in sPLA<sub>2</sub> transgenic mice have shown that the

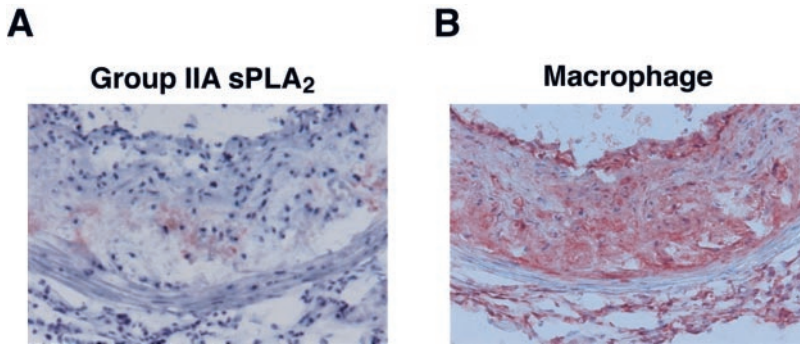
magnitude of the sPLA<sub>2</sub> effect is more pronounced in this sex compared with females.<sup>14</sup> Our results demonstrate that mice reconstituted with bone marrow cells from human group IIA sPLA<sub>2</sub> transgenic mice develop significantly larger lesions in both the aortic sinus and aortic arch compared with mice repopulated with wild-type bone marrow cells.

Immunocytochemical analysis of lesions from sPLA<sub>2</sub> tg→LDLR<sup>-/-</sup> mice verified the presence of sPLA<sub>2</sub> associated with macrophage foam cells. Group IIA sPLA<sub>2</sub> has similarly been detected in human lesions.<sup>4-7</sup> It is possible that such lesional sPLA<sub>2</sub> immunostaining represents enzyme that has infiltrated into the intima from another source rather than macrophage expression. However, it seems likely that group IIA detected in our transplantation experiment is primarily macrophage derived, because (1) group IIA was not detected in any other region in the vessel wall that appeared to be devoid of macrophage cells; (2) we would expect that most donor-derived cells present in lesions would be macrophages; and (3) we have demonstrated that macrophage cells in the transgenic mice do indeed express human group IIA sPLA<sub>2</sub>. It is notable that in situ hybridization analyses of sPLA<sub>2</sub> transgenic mouse tissues showed no hybridization signal in spleen or lymph nodes, suggesting that the human group IIA promoter is not constitutively active in transgenic mouse macrophages.<sup>12</sup> In this study, we show that human sPLA<sub>2</sub> protein is also not present in resident peritoneal macrophages from the transgenic mice but can be detected in biogel-elicited peritoneal macrophages. There is evidence that the endogenous group IIA sPLA<sub>2</sub> promoter is under transcriptional regulation in human monocyte macrophages.<sup>7</sup> Taken together, the evidence suggests that macrophage cells can be a source of group IIA sPLA<sub>2</sub>. This conclusion is consistent with the finding that macrophage expression of group IIA sPLA<sub>2</sub> has been reported in association with other human inflammatory diseases in addition to atherosclerosis, including Crohn’s disease<sup>21</sup> and acute pancreatitis.<sup>22</sup>

Our data showed regional-specific differences in the proatherogenic response to group IIA sPLA<sub>2</sub> expression. Whereas lesion formation was significantly enhanced in the aortic sinus and aortic arch, there was no difference in the extent of lesions in the ascending aorta. A similar region-specific difference in response to macrophage expression of lipoprotein lipase has also been reported.<sup>23</sup> In fact, in the relatively few studies where atherosclerosis has been quantified in the aortic root as well as throughout the aorta, many have reported nonuniform alterations in atherogenesis in response to an intervention.<sup>24-27</sup> Whether such discrepancies are attributable to



**Figure 3.** A, En face lesion area in aortic arch of C57BL/6→LDLR<sup>-/-</sup> and sPLA<sub>2</sub> tg→LDLR<sup>-/-</sup> mice as percent of total intimal area. Horizontal bars indicate the mean for each group (*P*<0.005). B, Lesion size in the aortic root of C57BL/6→LDLR<sup>-/-</sup> and sPLA<sub>2</sub> tg→LDLR<sup>-/-</sup> mice (n=8). Lesion area was determined in 8-μm-thick sections at 80-μm intervals after Oil red O staining. Values represent mean lesion areas (±SEM) for each section. The transition zone between the aortic sinus and the ascending aorta, defined by disappearance of the valve cusps, is 0 on the x-axis.



**Figure 4.** Immunocytochemical analysis of sections from the same aortic sinus region of a sPLA<sub>2</sub> tg→LDLR<sup>-/-</sup> mouse stained with rabbit anti-human group IIA sPLA<sub>2</sub> (A) or anti-mouse macrophage antisera (B). Immunoreactivity was visualized using the red chromogen, aminoethyl carbazole. Sections were counterstained with hematoxylin. Magnification, ×200.

regional differences in hemodynamics or other factors requires additional investigation.

Certain mouse strains, in particular the C57BL/6 mouse, lack the expression of functional group IIA protein because of a frame-shift mutation in exon 3.<sup>11</sup> The fact that C57BL/6 mice are relatively susceptible to the development of atherosclerosis suggests that group IIA sPLA<sub>2</sub> is not required for atherosclerotic lesion formation in these mice. In a recent study, it was reported that apoE<sup>-/-</sup>/sPLA<sub>2</sub><sup>+/+</sup> and apoE<sup>-/-</sup>/sPLA<sub>2</sub><sup>-/-</sup> mice fed a high-fat diet for 22 weeks had no significant difference in aortic cholesterol content.<sup>28</sup> The authors concluded that endogenous mouse sPLA<sub>2</sub> does not significantly affect atherogenesis in apoE<sup>-/-</sup> mice. This result contrasts with our finding that human sPLA<sub>2</sub> expression significantly enhances atherogenesis in LDLR<sup>-/-</sup> mice. Several factors could account for the discrepancy between our study and the published report, including possible species differences between mouse and human sPLA<sub>2</sub>. It is also possible that the lack of effect of the sPLA<sub>2</sub> genotype in the previous study was attributable to deficiencies in immune function in apoE<sup>-/-</sup> mice<sup>29</sup> that have not been documented in LDLR<sup>-/-</sup> mice.

Accumulating data from studies in vitro show that phospholipids on lipoprotein particles are substrates for group IIA sPLA<sub>2</sub> hydrolysis.<sup>30–32</sup> In the human group IIA sPLA<sub>2</sub> transgenic mice, increased sPLA<sub>2</sub> activity is accompanied by a 30% to 40% decrease in HDL-cholesterol concentrations.<sup>13,14</sup> In the present study, we assessed whether mice repopulated with macrophages expressing human group IIA sPLA<sub>2</sub> have altered plasma phospholipase activity or lipoprotein cholesterol concentrations, because such systemic effects could influence the extent of vascular lipid deposition. Our results show that mice repopulated with bone marrow cells expressing human group IIA sPLA<sub>2</sub> had no detectable alterations in plasma cholesterol concentrations, lipoprotein cholesterol distribution, or sPLA<sub>2</sub> activity compared with mice repopulated with wild-type bone marrow. We conclude from these findings that sPLA<sub>2</sub> expressed by macrophages within the microenvironment of developing lesions can promote the atherogenic process.

Local expression of group IIA sPLA<sub>2</sub> in the vessel wall could have multiple proatherogenic effects. One of the potential products of sPLA<sub>2</sub> hydrolysis is lysophosphatidyl choline, which has chemoattractant, chemostatic, and mitogenic effects on monocytes,<sup>33</sup> macrophages,<sup>34</sup> smooth muscle cells,<sup>35,36</sup> and T-lymphocytes.<sup>37</sup> Lysophospholipids can also serve as the substrate for the generation of potent proinflammatory lipid mediators, including platelet activating factor and lysophosphatidic

acid. FFAs released by sPLA<sub>2</sub> hydrolysis may be metabolized into proinflammatory agents such as eicosanoids or undergo oxidative modification. In addition to generating lipid mediators of inflammation, several lines of evidence suggest that sPLA<sub>2</sub> modification of LDL can result in structural alterations of the particle that promotes lipid accumulation in the vessel wall. Studies in vitro indicate that lipolysis of LDL with sPLA<sub>2</sub> results in partial lipoprotein aggregation and increased affinity for proteoglycans.<sup>38,39</sup> In addition, sPLA<sub>2</sub> modification increases the susceptibility of LDL to hydrolysis by secretory sphingomyelinase.<sup>40</sup> This leads to the accumulation of ceramide within the particles, which can also promote particle aggregation and fusion. Aggregated/fused LDL, which is prominent in atherosclerotic lesions,<sup>41–43</sup> is one of the most potent inducers of macrophage foam-cell formation in vitro.<sup>44–46</sup> Thus, although not directly shown, sPLA<sub>2</sub> hydrolysis of LDL could promote atherogenesis by increasing the retention of LDL particles in the subendothelium and by generating potent inducers of macrophage foam cells. Alternatively, sPLA<sub>2</sub> may promote atherosclerosis by modifying HDL in the vessel wall to reduce its protective activity.<sup>47</sup> Given the multitude of potential mechanisms whereby local expression of sPLA<sub>2</sub> could promote vascular lipid deposition, additional studies are necessary to delineate its proatherogenic effect.

### Acknowledgments

This work was supported by National Institutes of Health Grants HL-65730 (to D.R. van der Westhuyzen) and HL-69463 (to F.C. de Beer). The authors thank Dr T. Nevalainen for generously providing rabbit anti-human group IIA sPLA<sub>2</sub>. We also thank Jin Yu, Hong Xhu, and Wei Shi for assistance with mouse dissections and Deborah Howatt and John Burckle for tissue sectioning, image analysis, and immunocytochemistry.

### References

1. Six DA, Dennis EA. The expanding superfamily of phospholipase A2 enzymes: classification and characterization. *Biochem Biophys Acta*. 2000;1488:1–19.
2. Vadas P, Browning J, Edelson J, Pruzanski W. Extracellular phospholipase A2 expression and inflammation: the relationship with associated disease states. *J Lipid Mediat*. 1993;8:1–30.
3. Kugiyama K, Ota Y, Takazoe K, Moriyama Y, Kawano H, Miyao Y, Sakamoto T, Soejima H, Ogawa H, Doi H, Sugiyama S, Yasue H. Circulating levels of secretory type II phospholipase A(2) predict coronary events in patients with coronary artery disease. *Circulation*. 1999;100:1280–1284.
4. Hurt-Camejo E, Andersen S, Standal R, Rosengren B, Sartipy P, Stadberg E, Johansen B. Localization of nonpancreatic secretory phospholipase A2 in normal and atherosclerotic arteries: activity of the isolated enzyme on low-density lipoproteins. *Arterioscler Thromb Vasc Biol*. 1997;17:300–309.

5. Elinder LS, Dumitrescu A, Larsson P, Hedin U, Frostegard J, Claesson HE. Expression of phospholipase A2 isoforms in human normal and atherosclerotic arterial wall. *Arterioscler Thromb Vasc Biol.* 1997;17:2257–2263.
6. Menschikowski M, Kasper M, Latke P, Schiering A, Schiefer S, Stockinger H, Jaross W. Secretary group II phospholipase A2 in human atherosclerotic plaques. *Atherosclerosis.* 1995;118:173–181.
7. Anthonson MW, Stengel D, Hourton D, Ninio E, Johansen B. Mildly oxidized LDL induces expression of group IIA secretory phospholipase A(2) in human monocyte-derived macrophages. *Arterioscler Thromb Vasc Biol.* 2000;20:1276–1282.
8. Balboa MA, Balsinde J, Winstead MV, Tischfield JA, Dennis EA. Novel group V phospholipase A2 involved in arachidonic acid mobilization in murine P388D1 macrophages. *J Biol Chem.* 1996;271:32381–32384.
9. Tischfield JA. A reassessment of the low molecular weight phospholipase A2 gene family in mammals. *J Biol Chem.* 1997;272:17247–17250.
10. Grass DH, Felkner RH, Chiang M-Y, Wallace RE, Nevalainen TJ, Bennett CF, Swanson ME. Expression of human group II PLA<sub>2</sub> in transgenic mice results in epidermal hyperplasia in the absence of inflammatory infiltrate. *J Clin Invest.* 1996;97:2233–2241.
11. Kennedy BP, Payette P, Mudgett J, Vadas P, Pruzanski W, Kwan M, Tang C, Rancourt DE, Cromlish WA. A natural disruption of the secretory group II phospholipase A2 gene in inbred mouse strains. *J Biol Chem.* 1995;270:22378–22385.
12. Nevalainen TJ, Laine VJ, Grass DS. Expression of human group II phospholipase A2 in transgenic mice. *J Histochem Cytochem.* 1997;45:1109–1119.
13. De Beer FC, de Beer MC, van der Westhuyzen DR, Castellani LW, Lusis AJ, Swanson ME, Grass DS. Secretary non-pancreatic phospholipase A2: influence on lipoprotein metabolism. *J Lipid Res.* 1997;38:2232–2239.
14. Ivandic B, Castellani LW, Wang X-P, Qiao J-H, Mehrabian M, Navab M, Fogelman AM, Grass DS, Swanson ME, de Beer MC, de Beer F, Lusis AJ. Role of group II secretory phospholipase A<sub>2</sub> in atherosclerosis I: increased atherogenesis and altered lipoproteins in transgenic mice expressing group IIA phospholipase A<sub>2</sub>. *Arterioscler Thromb Vasc Biol.* 1999;19:1284–1290.
15. Tietge UJF, Maugeais C, Cain W, Grass D, Glick JM, de Beer FC, Rader DJ. Overexpression of secretory phospholipase A2 causes rapid catabolism and altered tissue uptake of high density lipoprotein cholesteryl ester and apolipoprotein A-I. *J Biol Chem.* 2000;275:10077–10084.
16. Daugherty A, Whitman SC. Quantification of atherosclerosis in mice. *Methods Mol Biol.* 2003;209:293–309.
17. Daugherty A, Rateri DL. Presence of LDL receptor-related protein/alpha 2-macroglobulin receptors in macrophages of atherosclerotic lesions from cholesterol-fed New Zealand and heterozygous Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb.* 1994;14:2017–2024.
18. Whitman SC, Ravisankar P, Elam H, Daugherty A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E<sup>-/-</sup> mice. *Am J Pathol.* 2000;157:1819–1824.
19. Nevalainen TJ, Marki F, Kortesus PT, Grutter MG, Di Marco S, Schmitz A. Synovial type (group II) phospholipase A2 in cartilage. *J Rheumatol.* 1993;20:325–330.
20. Hoffmann GE, Schmidt D, Bastian B, Guder WG. Photometric determination of phospholipase A. *J Clin Chem Clin Biochem.* 1986;24:871–875.
21. Lilja I, Gustafson-Svard C, Franzen L, Sjobahl R, Andersen S, Johansen B. Presence of group IIA secretory phospholipase A2 in mast cells and macrophages in normal human ileal submucosa and in Crohn's disease. *Clin Chem Lab Med.* 2000;38:1231–1236.
22. Talvinen KA, Kemppainen EA, Nevalainen TJ. Expression of group II phospholipase A2 in the liver in acute pancreatitis. *Scand J Gastroenterol.* 2001;36:1217–1221.
23. Babaev VR, Patel MB, Semenkovich CF, Fazio S, Linton MF. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. *J Biol Chem.* 2000;275:26293–26299.
24. Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF, Funk CD. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. *J Clin Invest.* 1999;103:1597–1604.
25. Tangirala RK, Tsukamoto K, Chun SH, Usher D, Pure E, Rader DJ. Regression of atherosclerosis induced by liver-directed gene transfer of apolipoprotein A-I in mice. *Circulation.* 1999;100:1816–1822.
26. Witting PK, Pettersson K, Letters J, Stocker R. Site-specific antiatherogenic effect of probucol in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* 2000;20:E26–E33.
27. King VL, Szilvassy SJ, Daugherty A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor<sup>-/-</sup> mice. *Arterioscler Thromb Vasc Biol.* 2002;22:456–461.
28. Burton CA, Patel S, Mundt S, Hassing H, Zhang D, Hermanowski-Vosatka A, Wright SD, Chao YS, Detmers PA, Sparrow CP. Deficiency in sPLA(2) does not affect HDL levels or atherosclerosis in mice. *Biochem Biophys Res Commun.* 2002;294:88–94.
29. Roselaar SE, Daugherty A. Apolipoprotein E-deficient mice have impaired innate immune responses to *Listeria monocytogenes* in vivo. *J Lipid Res.* 1998;39:1740–1743.
30. Pruzanski W, Stefanski E, de Beer FC, de Beer MC, Vadas P, Ravandi A, Kuksis A. Lipoproteins are substrates for human secretory group IIA phospholipase A2: preferential hydrolysis of acute phase HDL. *J Lipid Res.* 1998;39:2150–2160.
31. Hurt-Camejo E, Camejo G, Sartipy P. Phospholipase A2 and small, dense low-density lipoprotein. *Curr Opin Lipidol.* 2000;11:465–471.
32. Sartipy P, Hurt-Camejo E. Modification of plasma lipoproteins by group IIA phospholipase A(2): possible implications for atherogenesis. *Trends Cardiovasc Med.* 1999;9:232–238.
33. Quinn MT, Parthasarathy S, Steinberg D. Lysophosphatidylcholine. a chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc Natl Acad Sci U S A.* 1988;85:2805–2809.
34. Sakai M, Miyazaki A, Hakamata H, Sasaki T, Yui S, Yamazaki M, Shichiri M, Horiuchi S. Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages. *J Biol Chem.* 1994;269:31430–31435.
35. Stiko A, Regnstrom J, Shah PK, Cercek B, Nilsson J. Active oxygen species and lysophosphatidylcholine are involved in oxidized low density lipoprotein activation of smooth muscle cell DNA synthesis. *Arterioscler Thromb Vasc Biol.* 1996;16:194–200.
36. Chai YC, Howe PH, DiCorleto PE, Chisolm GM. Oxidized low density lipoprotein and lysophosphatidylcholine stimulate cell cycle entry in vascular smooth muscle cells: evidence for release of fibroblast growth factor-2. *J Biol Chem.* 1996;271:17791–17797.
37. McMurray HF, Parthasarathy S, Steinberg D. Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes. *J Clin Invest.* 1993;92:1004–1008.
38. Oorni K, Hakala JK, Annala A, Ala-Korpela M, Kovanen PT. Sphingomyelinase induces aggregation and fusion, but phospholipase A2 only aggregation, of low density lipoprotein (LDL) particles: two distinct mechanisms leading to increased binding strength of LDL to human aortic proteoglycans. *J Biol Chem.* 1998;273:29127–29134.
39. Hakala JK, Oorni K, Pentikainen MO, Hurt-Camejo E, Kovanen PT. Lipolysis of LDL by human secretory phospholipase A(2) induces particle fusion and enhances the retention of LDL to human aortic proteoglycans. *Arterioscler Thromb Vasc Biol.* 2001;21:1053–1058.
40. Schissel SL, Jiang X, Tweedie-Hardman J, Jeong T, Camejo EH, Najib J, Rapp JH, Williams KJ, Tabas I. Secretary sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH: implications for atherosclerotic lesion development. *J Biol Chem.* 1998;273:2738–2746.
41. Nievelstein PF, Fogelman AM, Mottino G, Frank JS. Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein: a deep-etch and immunolocalization study of ultrarapidly frozen tissue. *Arterioscler Thromb.* 1991;11:1795–1805.
42. Kruth HS. The fate of lipoprotein cholesterol entering the arterial wall. *Curr Opin Lipidol.* 1997;8:246–252.
43. Haberland ME, Mottino G, Le M, Frank JS. Sequestration of aggregated LDL by macrophages studied with freeze-etch electron microscopy. *J Lipid Res.* 2001;42:605–619.
44. Khoo JC, Miller E, McLoughlin P, Steinberg D. Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis.* 1988;8:348–358.
45. Suits AG, Chait A, Aviram M, Heinecke JW. Phagocytosis of aggregated lipoprotein by macrophages: low density lipoprotein receptor-dependent foam-cell formation. *Proc Natl Acad Sci U S A.* 1989;86:2713–2717.
46. Tertov VV, Sobenin IA, Gabbasov ZA, Popov EG, Orekhov AN. Lipoprotein aggregation as an essential condition of intracellular lipid accumulation caused by modified low density lipoproteins. *Biochem Biophys Res Commun.* 1989;163:489–494.
47. Leitinger N, Watson AD, Hama SY, Ivandic B, Qiao J-H, Huber J, Faull KF, Grass DS, Navab M, Fogelman AM, de Beer FC, Lusis AJ, Berliner JA. Role of group II secretory phospholipase A2 in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 1999;19:1291–1298.