Clinical Research

Molecular Basis for the Association of Group IIA Phospholipase A₂ and Decorin in Human Atherosclerotic Lesions

Peter Sartipy, Berit Johansen, Kathrine Gåsvik, Eva Hurt-Camejo

Abstract—Group IIA secretory nonpancreatic phospholipase A₂ (snpPLA₂) is associated with collagen fibers in the extracellular matrix of human atherosclerotic plaques. Decorin, a small proteoglycan (PG) carrying chondroitin/dermatan sulfate glycosaminoglycans (GAGs), forms part of the collagen network in human arteries. To explore whether snpPLA₂ may be associated with collagen fibers via interaction with decorin, we performed (1) immunohistochemistry to compare the relative in vivo localization of snpPLA₂ and decorin in human atherosclerotic tissue and (2) in vitro experiments to study the interaction between snpPLA₂ and decorin. In atherosclerotic lesions, decorin was detected within the snpPLA₂-positive part of the intima close to the media. Electrophoretic mobility shift assay showed that snpPLA₂ binds to decorin synthesized by human fibroblasts. Native and GAG-depleted decorin enhanced the association of snpPLA₂ to collagen types I and VI in a solid-phase binding assay. Furthermore, snpPLA₂ bound efficiently to a recombinant decorin core protein fragment B/E (Asp45-Lys359). This binding was competed with soluble decorin and inhibited at NaCl concentrations >150 mmol/L. The decorin core protein fragment B/E competed better than dermatan sulfate for binding of snpPLA₂ to decorin-coated microtiter wells. The enzymatic activity of snpPLA₂ increased 2- to 3-fold in the presence of decorin or GAG-depleted decorin. The results show that snpPLA₂ binds preferentially to the decorin protein core rather than to the GAG chain and that this interaction enhances snpPLA₂ activity. As a consequence, this active extracellular enzyme may contribute to the pathogenesis of atherosclerosis by modifying lipoproteins and releasing inflammatory lipid mediators at places of lipoprotein retention in the arterial wall. (Circ Res. 2000;86:707-714.)

Key Words: atherosclerosis • decorin • group IIA phospholipase A₂

Group IIA secretory nonpancreatic phospholipase A₂ (snpPLA₂) is a 14-kDa calcium-dependent enzyme that hydrolyzes the sn-2 fatty acid acyl ester bond of phosphoglycerides to free fatty acids (FFAs) and lysophospholipids. These products can act as intracellular second messengers or be further metabolized into proinflammatory lipid mediators. Immunohistochemical studies demonstrated the presence of snpPLA₂ in human arteries, and ultrastructural localization showed that some of the snpPLA₂ was located along collagen fibers in the extracellular matrix of human atherosclerotic lesions. Most of the collagen in arterial intima is collagen types I and III. Interspersed between these is collagen type VI, which appears in all vascular layers. Several studies have shown that collagen interacts with decorin, a chondroitin/dermatan sulfate (CS/DS) proteoglycan (PG) of the SLRP family class I. Decorin is present in the arterial wall, and its distribution is different in healthy arteries from that of atherosclerotic plaques. When decorin is associated with collagen, both the core protein and the glycosaminoglycan (GAG) chain are still available for binding to other ligands, eg, transforming growth factor (TGF)-β and LDL. These observations suggest that some of the extracellular snpPLA₂ detected in the arterial intima of lesions may be associated with collagen fibers via its interaction with decorin.

The hypothesis of the potential involvement of snpPLA₂ in the pathogenesis of atherosclerosis was recently reinforced with in vivo data. The proatherogenic mechanisms of snpPLA₂ may be potentiated by the capacity of both snpPLA₂ and apolipoprotein (apo) B lipoproteins to interact with sulfated PG/GAG. This may be a mechanism that contributes to colocalization of the enzyme and lipoproteins at cell membranes and extracellular space. However, the molecular basis for the interactions of snpPLA₂ with extracellular matrix components and how this may locate and control enzyme activity at sites of lesion development needs to be clarified. In the present work, we performed immunohistochemistry to explore the relative distribution of decorin and snpPLA₂ in human nonatherosclerotic and atherosclerotic tissue. We also

Received December 22, 1999; accepted February 3, 2000.
From Wallenberg Laboratory for Cardiovascular Research, Sahlgrenska University Hospital, Göteborg, Sweden (P.S., E.H.-C.), and the Department of Botany/UNIGEN Center for Molecular Biology, Norwegian University of Science and Technology, Trondheim, Norway (B.J., K.G.). Correspondence to Peter Sartipy, Wallenberg Laboratory for Cardiovascular Research, Sahlgrenska University Hospital, Göteborg 413 45, Sweden. E-mail peter.sartipy@wlab.wall.gu.se © 2000 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org

707
studied, in vitro, the binding of snpPLA₂ to decorin, the role of decorin in mediating the binding of snpPLA₂ to collagen types I and VI, and how decorin modulates snpPLA₂ activity.

**Materials and Methods**

**Immunohistochemistry**

Human carotid artery segments were obtained from autopsy or surgery and quickly frozen and stored at −20°C until sectioning. Uterine arteries (used as nonatherosclerotic control tissue) were obtained from women undergoing hysterectomy. Atherosclerotic regions were defined by the presence of intima thickness accompanied by extracellular lipid accumulations, cholesterol crystals, and foam-cell and non–foam-cell macrophages.

Immunofluorescence staining was performed on atherosclerotic (n=9) and nonatherosclerotic (n=4) arteries. A Mouse monoclonal antibody (mAb) BF1 was used to detect snpPLA₂. An antibody generated against smooth muscle cell actin, HHF-35, recognized vascular smooth muscle cells. Decorin was detected by an mAb generated against the core protein moiety. In addition, some sections were incubated with or without chondroitinase ABC (ChABC). Standard hematoxylin/eosin staining for routine histological evaluation of the sections was also performed.

**Isolation and Characterization of PG**

Confluent human skin fibroblasts maintained in Eagle’s minimum essential medium in bottles coated with collagen type I were incubated for 3 days with medium containing L-[4,5-³H]leucine and [³⁵S]sulfur at final specific activities of 17 µCi/mL and 33 µCi/mL, respectively. Conditioned medium was collected and supplemented with protease inhibitors.

PGs, isolated from conditioned medium, were digested with chondroitinase AC (ChAC), chondroitinase B (ChB), or heparitinase I (HS I) and analyzed by SDS-PAGE. PGs were also digested with ChABC, and Western blot was performed essentially as described.

**In Vitro Interactions of snpPLA₂ With Decorin and Collagen**

snpPLA₂ was purified from conditioned medium from a transfected CHO cell line as described. Binding of snpPLA₂ to fibroblast-derived PG was analyzed by electrophoretic mobility shift assay (EMSA).

Expression and purification of a recombinant decorin core protein fragment representing Asp45-Lys359 (B/E) was done essentially as described.

Microtiter wells were coated with collagen type I or VI or FFA-free BSA. Native decorin (from bovine articular cartilage) or
decorin digested with ChABC was added to some wells. Nonbound decorin was determined in the supernatants after incubation.\textsuperscript{24} snpPLA\textsubscript{2} was diluted with D-PBS and added to the wells in increasing concentrations. After incubation, the activity of bound snpPLA\textsubscript{2} was determined by measuring generation of FFA from PC mixed micelles (prepared as described below) with a commercial kit (NEFA-C).

Microtiter wells were coated with the B/E fragment. In some experiments, snpPLA\textsubscript{2} was added directly to the B/E-coated wells. In a second set of experiments, a constant concentration of snpPLA\textsubscript{2} (290 nmol/L) was mixed with increasing concentrations of decorin before it was added to the B/E-coated wells. In a third set of experiments, snpPLA\textsubscript{2} (290 nmol/L) was added to B/E-coated wells in D-PBS supplemented with NaCl. In all experiments, the activity of snpPLA\textsubscript{2} retained in the B/E-coated wells was determined as described above.

snpPLA\textsubscript{2} Activity

PC mixed micelles were prepared as described.\textsuperscript{21} Human LDL was isolated from healthy fasted male volunteers.\textsuperscript{25} snpPLA\textsubscript{2} and decorin or GAG-depleted decorin were preincubated (30 minutes, room temperature) before substrate was added. snpPLA\textsubscript{2} activity was determined by measuring release of FFA during the incubations. An expanded Materials and Methods section is available online at http://www.circresaha.org.

Figure 2. Immunofluorescence detection of decorin, $\alpha$-smooth muscle actin, and snpPLA\textsubscript{2} in a human atherosclerotic lesion from a carotid artery. Neighboring sections of frozen human atherosclerotic lesion: decorin-positive staining (B); $\alpha$-smooth muscle actin–positive staining (D); snpPLA\textsubscript{2}-positive staining (F). A, C, and E, Histology sections stained with hematoxylin. I indicates intima; M, media. Magnification $\times$37.
Results

Immunohistochemistry

The selectivity of the mAb generated against decorin was initially tested and confirmed on sections of normal human skin segments (data not shown). In human nonatherosclerotic arteries (Figure 1), prominent staining of decorin was detected by immunofluorescence only in the adventitia. In sections of atherosclerotic lesions (Figure 2) showing a thickened intima and part of the media, decorin was detected mainly in the layer of intima close to the media and less in the subendothelial part of the intima, as shown in Figure 2A and 2B. Immunostaining of neighboring sections with mAb HHF-35 (Figure 2C and 2D) shows 2 regions within atherosclerotic lesions that are enriched in smooth muscle cells. One region is the subendothelial intima, which contains a belt of mostly spindle-shaped smooth muscle cells. The other region is the media and the medial intima. Immunostaining with mAb BF1 (Figure 2E and 2F) shows that most snpPLA2-positive cells have a distribution similar to that of the actin HHF-35-positive cells. Other snpPLA2-positive cells that are not smooth muscle cells (HHF-35-negative) were also detected in the arterial intima (Figure 2E and 2F). On the basis of their morphology and findings from earlier studies, these additional cells may be macrophages. In advanced atherosclerotic lesions (Figure 3), decorin was detected in the plaque core close to the media overlapping with snpPLA2 staining. Some atherosclerotic sections were also preincubated with ChABC before immunostaining was performed (see online supplementary information; http://www.circresaha.org). In brief, the immunohistological detection of snpPLA2 increased after ChABC treatment in regions corresponding to areas that also stain positive for decorin. This suggests that the removal of the GAG moiety unmasks but does not remove snpPLA2-antigen associated with decorin. A summary of the immunohistological detection of decorin and snpPLA2 is presented in the Table. The extent of decorin distribution in intima corresponds with severity of the lesion and coincides with snpPLA2 staining, except that more areas are positive for snpPLA2 than decorin.

Isolation and Characterization of PG

Decorin is a quiescence-inducible gene in fibroblasts, and reverse transcription–polymerase chain reaction evaluation of total RNA isolated from the cells indicated a high expression of decorin 1 to 7 days postconfluence (data not shown). The PG preparation obtained from conditioned medium contained mainly decorin (Figure 4A and 4B). The band migrating at \( \sim 100 \text{ kDa} \) was totally degraded by ChB, indicating the presence of DS. A protein band with a molecular weight of 45 to 50 kDa, corresponding to the size of the core protein of decorin, was left after digestion with ChB, and it was identified as the decorin core protein by Western blot analysis. The PG preparation also contained a small proportion of a material retained at the top of the gel that was susceptible to HS I digestion (perlecan-like).

In Vitro Interactions of snpPLA2 With Decorin and Collagen

We studied the interaction of snpPLA2 with decorin and with decorin bound to collagen type I or VI in 2 different in vitro models: (1) EMSA with decorin isolated from human fibroblasts and (2) a solid-phase binding assay using collagen and collagen-decorin–coated microtiter wells.

In the EMSA assay (Figure 4C), the intensity of the band corresponding to free decorin decreases in the presence of 0.25 \( \mu \text{mol/L} \) of snpPLA2 and also with 0.5 \( \mu \text{mol/L} \) of snpPLA2. Furthermore, the band corresponding to decorin migrates differently and becomes wider in the presence of 0.5 \( \mu \text{mol/L} \) of snpPLA2, compared with the control (without snpPLA2). At concentrations \( >1 \mu \text{mol/L} \) of snpPLA2, there is no free decorin, and all decorin molecules have formed...
Immunofluorescence Detection of Decorin and snpPLA$_2$ in Atherosclerotic Lesions and Control Nonatherosclerotic Human Arteries

<table>
<thead>
<tr>
<th>Arterial Tissue</th>
<th>Subendothelial Intima</th>
<th>Medial Intima</th>
<th>Media</th>
<th>Adventitia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherosclerotic lesions (n=5)</td>
<td>Decorin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Advanced lesions (n=4)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control arteries (n=4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Presence of moderate staining is indicated with +, very intense staining is indicated with ++, and the absence of staining is indicated with −.

complexes with snpPLA$_2$ and remain at the origin of the gel. These results indicate that snpPLA$_2$ binds to decorin in vitro at physiological ionic conditions.

The possibility that decorin may serve as a link between collagen fibers and snpPLA$_2$ was investigated with a microtiter-well binding assay. The wells were first coated with collagen type I or VI, and then decorin was added. The wells coated with collagen type VI bound more decorin (370±50 ng, n=9) than the wells coated with collagen type I (160±60 ng, n=16). Control wells contained either collagen type I or VI or BSA. Measuring snpPLA$_2$ activity monitored binding of snpPLA$_2$. snpPLA$_2$ did not bind to the wells coated only with collagen type I or with BSA (Figure 5A). However, snpPLA$_2$ was able to bind to collagen type VI (Figure 5B). The binding to collagen type VI was higher than that of control wells coated with BSA. This suggests that snpPLA$_2$ may associate with collagen type VI even in the absence of decorin. Interestingly, the addition of decorin to collagen-coated wells significantly enhanced the binding of active snpPLA$_2$ to both collagen types I and VI (Figure 5A and 5B), indicating that decorin may mediate binding of snpPLA$_2$ to collagen fibers.

snpPLA$_2$ may bind to decorin through the GAG and/or the protein moiety of the PG. To discriminate between these possibilities, we performed experiments with decorin and decorin pretreated with ChABC. There was no difference in the amount of active snpPLA$_2$ binding to untreated decorin or GAG-depleted decorin in plates coated with collagen type VI (Figure 5C). Importantly, the GAG moiety in decorin was totally degraded, according to analysis of ChABC-treated decorin by SDS-PAGE (data not shown). These results suggest that snpPLA$_2$ binds to GAG-depleted decorin, possibly through a direct interaction with the core protein of decorin. Notably, in similar experiments performed with versican-coated plates, the retention of active snpPLA$_2$ decreased significantly after GAG digestion (see online supplementary information; http://www.circresaha.org).

To further analyze the binding of snpPLA$_2$ to the core protein of decorin, a recombinant core protein fragment of human decorin, Asp45-Lys359 (fragment B/E), was expressed. Compared with the complete processed and secreted decorin core protein, this fragment lacks 14 amino acid residues at the N-terminal, it carries no GAG chain, and it is not glycosylated. snpPLA$_2$ was efficiently retained in B/E-coated wells (Figure 6A). The wells contained 910±430 ng (n=9) of the B/E fragment. The binding to the BSA-coated wells was low. It was possible to compete the binding of snpPLA$_2$ to immobilized B/E by soluble decorin (Figure 6B). At the highest concentration of competitor (0.2 μg/μL), only ~20% of the snpPLA$_2$ was bound, compared with 100% snpPLA$_2$ bound in the absence of competitor. The binding of snpPLA$_2$ to B/E-coated wells appears to be mediated mainly by electrostatic interactions, because the binding was almost completely inhibited by increasing the NaCl concentration up to 1 mol/L in the binding buffer (Figure 6C).

Experiments were also performed with decorin-coated microtiter wells using the B/E fragment or DS as competitors. The B/E fragment was more efficient in competing for binding of snpPLA$_2$ to decorin-coated wells compared with DS. Preincubation of 290 nmol/L snpPLA$_2$, together with 0.12 μg/μL of B/E (M$_w$ ~35 kDa) or DS (M$_w$ 11 to 25 kDa) reduced the binding of snpPLA$_2$ to the decorin-coated wells

Figure 4. A, PGs isolated from conditioned fibroblast culture media were digested with ChAC, ChB, HS I, or without enzyme (Ctrl) and analyzed by 4% to 12% SDS-PAGE. Radioactive bands were visualized by autoradiography (Std, molecular weight standards). Arrow indicates the remaining core protein of decorin. B, Western blot. An aliquot of the PG preparation was digested with ChABC and separated on a 4% to 12% SDS-PAGE. After transfer, the membrane was developed with an ECL+ Plus Western blotting detection system using monoclonal antibodies against human decorin. C, EMSA. A constant amount of PG was incubated with increasing concentrations of snpPLA$_2$ (as indicated). The PG-protein complexes were separated from free PG by agarose-gel electrophoresis. The radioactive bands were visualized by autoradiography. Results are representative for an experiment performed twice.
to 40% (B/E) and 90% (DS), compared with 100% binding in the absence of competitors. Ten times higher concentrations of the competitors further reduced the binding of snpPLA2 to the decorin-coated wells to 18% and 70%, respectively.

Effect of Decorin on the Activity of snpPLA2
To investigate whether the interaction with decorin could affect the enzyme activity, increasing concentrations of decorin or GAG-depleted decorin were incubated with snpPLA2. snpPLA2 activity was increased in the presence of decorin when PC micelles or LDL was used as substrate presenting structures (Figure 7). Interestingly, a similar enhancing effect on snpPLA2 activity was observed with untreated decorin and with GAG-depleted decorin. These results support the results from the microtiter binding assay indicating that snpPLA2 binds to the core protein of decorin and that this interaction enhances snpPLA2 activity.

Discussion
snpPLA2 displays 3 biochemical properties of a secretory enzyme. First, the cDNA encodes a 144-amino-acid protein including a 20-amino-acid signal peptide. Second, snpPLA2 has 7 disulfide bridges, which makes it a very stable protein against proteolysis and nonenzymatic hydrolysis. Third, it requires millimolar concentrations of calcium for its activity.
The immunofluorescence results in the present study show that staining for snpPLA2 coincides with regions that stain positively for the extracellular matrix PG decorin in human atherosclerotic arteries. The distribution of decorin and snpPLA2 in nonatherosclerotic and atherosclerotic arteries was similar to what has been reported.3,8,27 The immunofluorescence results on arterial wall sections indicate colocalization of snpPLA2 and decorin. However, the relative differences of the signal intensities suggest that other matrix components besides collagen-associated decorin may also bind snpPLA2. Hence, the presence of extracellular snpPLA2 is not dependent on decorin, because snpPLA2 shows a wider distribution pattern than decorin.

Decorin is one of the several PGs known to accumulate in human atherosclerotic plaques, and it colocalizes with collagen.27 Its major functions appear to be assembly of collagen fibers, binding and regulation of growth factor activity, and control of cell growth.7 Decorin possesses 2 main collagen-binding sites23 that do not involve the CS/DS moiety. The single GAG chain located at Ser7 close to the N-terminal of the core protein protrudes away from decorin and is free to interact with other proteins. The results obtained in this work with 2 different in vitro binding assays show that snpPLA2 is able to interact with both the GAG moiety and the core protein of decorin at physiological salt and pH conditions. The mobility of decorin in the EMSA experiments, in which snpPLA2 was present in large excess, was inhibited, suggesting interaction with the GAG side chain. The mobility of the PG in the gel electrophoresis is dependent on its negatively charged GAG. Association of the snpPLA2 with the GAG appeared to be responsible for the inhibited mobility. However, the solid-phase binding assay, in which decorin and snpPLA2 were present in similar amounts, showed that snpPLA2 also binds to GAG-depleted decorin. These results were confirmed with experiments performed with a human recombinant decorin core protein fragment (Asp45-Lys359). Furthermore, this fragment competed more efficiently than the DS side chain. Taken together, these data suggest that PGs known to accumulate in atherosclerosis8 might bind snpPLA2 secreted by smooth muscle cells and thereby be partially responsible for the abundant extracellular snpPLA2 detected in arterial plaques,1,3,4 thus reinforcing the hypothesis that the presence of active extracellular snpPLA2 in the arterial wall may contribute to lipoprotein modification and generation of lipid mediators at places of atherosclerotic plaque formation and progression.

**Acknowledgments**

This work was supported by grants from the Medical Research Council (project No. 12129 to E.H.-C.), the Swedish Heart and Lung Foundation (project No. 61538 to E.H.-C.), and AstraZeneca R&D, Mölndal, Sweden. The authors are grateful to Prof Germán Camejo for valuable discussions during the course of this work and also for critically reading the manuscript.
References

Molecular Basis for the Association of Group IIA Phospholipase A₂ and Decorin in Human Atherosclerotic Lesions
Peter Sartipy, Berit Johansen, Kathrine Gåsvik and Eva Hurt-Camejo

Circ Res. 2000;86:707-714
doi: 10.1161/01.RES.86.6.707

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/86/6/707

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2000/04/11/86.6.707.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
METHODS

Materials.

Benzamidine-HCl, ε-amino n-caproic acid, HEPES, FFA-free BSA, cetyl pyridinium chloride, β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine, decorin (from bovine articular cartilage) were purchased from Sigma Chemical Co. (St. Louis, USA). Precast Tris-Glycine gels were bought from Novex (San Diego, CA, USA). Sulfur-35 (1.87 mCi/ml), L-(4,5-3H)-leucine (155 mCi/mmol), hyper films for autoradiography, and ECL+Plus Western blotting detection system were purchased from Amersham International (Buckinghamshire, England). Dermatan sulfate, Chondroitinase B (ChB), Chondroitinase AC (ChAC), Chondroitinase ABC (ChABC), and Heparitinase I (HS I) were bought from Seikagaku Co. (Tokyo, Japan). Collagen type VI (bovine) was purchased from Biodesign Int. (Kennebunk, ME, USA). Collagen type I (rat tail tendon) was from Collaborative Biomedical Products (Bedford, USA). Maxisorp ELISA-strips were from NUNC (Roskilde, Denmark). Dulbecco’s phosphate buffered saline (DPBS), culture media, antibiotics, and non-essential amino acids were purchased from BioWhittaker (Walkersville, MD, USA). Fetal bovine serum (FBS) was from Biochrom KG Seromed (Berlin, Germany). Spectra/Port® membranes 3 Mₘ cut-off 3,500 for dialysis were purchased from Spectrum Laboratory Products (Houston, Texas USA). NEFA-C kit was from Wako Chemicals GmbH (Neuss, Germany). Salts and other buffer substances or detergents were of analytical grade and purchased from Merck (Darmstadt, Germany). All the water used was filtered through a Milli-Pore® Milli-Q system and was of high purity (resistivity >18MΩcm⁻¹).

Human Tissues

Human carotid arterial segments were obtained from autopsy or surgery and quickly frozen and stored at -20°C until sectioning. Uterine arteries (used as non-atherosclerotic control tissue) were obtained from women undergoing hysterectomy. The
arteries were dried in filter paper, cut into 0.5 to 1.0-cm rings, and placed on pieces of cork covered with filter papers. The tissue was then embedded in Optimum Cutting Temperature compound (OCT, Tissue-Tek, Miles Laboratories, Elkhart, USA) and snap frozen in liquid nitrogen. The frozen tissue was stored at -80°C until sectioned. Freshly cut frozen sections (3 μm) were collected on poly-L-lysine-coated glass cover slips, air dried for about 1 hour and stored at -20°C. Immediately before staining, the sections were place at room temperature (RT) for 5-10 min and fixed in cold acetone for 5 min. Atherosclerotic regions were defined by the presence of intima thickness accompanied by extracellular lipid accumulations, cholesterol crystals, foam cell, and non-foam cell macrophages.

Immunohistochemistry

Immunofluorescence staining of tissue were performed on human atherosclerotic (n=9) and non-atherosclerotic (n=4) arteries as previously described (1). Mouse monoclonal antibody (mAb) BF1 (30μg/mL, subclass IgG2b, a generous gift from Dr. Jeff Browning, Biogen Inc., MA, USA) was used to detect snpPLA2. An antibody generated against smooth muscle cell actin, HHF-35 (Enzo Diagnostics Inc., dilution 1:35), recognized vascular smooth muscle cells. Decorin was detected by a specific mAb (subclass IgG1) generated against the core protein moiety of dermatan sulfate PG (dilution 1:2000, clone 6-B-6, Seikagaku, Tokyo, Japan) (16). Control mAb TWAR (IgG2a) generated against Chlamydia Pneumonia, kindly provided by Are Dalen, Faculty of Medicine, Norwegian University of Science and Technology, was used in final concentration 30μg/mL. Control sections where the primary antibody was omitted were also included. All primary antibodies were diluted in PBS with 1.2% BSA (Sigma, fraction V), and 2% human serum. The biotinylated rabbit antimouse IgG and FITC/streptavidine were diluted 1:40 and 1:50 respectively in PBS with 1.2% BSA. Additionally, some sections were incubated with or without ChABC (10 mU/mL) in PBS with 0.1 mg/mL protease free BSA (Promega Corp., Madison, WI, USA). After
incubation for 2h at 37°C the sections were washed 3 times in PBS for 10min. Immunohistologic detection of snpPLA₂ was then performed as described above. Standard hematoxylin/eosin staining for routine histologic evaluation of the sections were also performed.

**Cell culture of human skin fibroblasts.**

Human skin fibroblasts were cultured to confluency for seven days in EMEM-medium. Cells were seeded at 5000 cells/cm² in 80 cm² bottles coated with collagen type I (2) in medium containing 10% FBS. After 2 days the cells were washed 3 times with DPBS and synchronized for 2 days in fresh serum poor medium (0.5% FBS). To start the proliferation of the cells 10% FBS was added to fresh medium on day 4. After 7 days of culture the cells were confluent and the medium was changed to BME-diploid sulfate-free medium containing 10% FBS. The next day, L- (4,5-³H) leucine and ³⁵S-sulfur were added to the medium to a final specific activity of 17µCi/mL and 33µCi/mL respectively and the cells were incubated for 3 days. The cell culture medium containing ³⁵S- and ³H-biolabeled molecules was removed. Protease inhibitors were added to a final concentration of 10 mmol/L EDTA, 1mg/mL benzamidine-HCl, and 10mmol/L ε-amino n-caproic acid and the medium was stored at -20°C until used for isolation of PG.

**Isolation and characterization of fibroblast cell derived proteoglycans**

Thawed medium from human fibroblasts cultured as indicated above was dialyzed against binding-buffer containing 8 mol/L Urea, 2 mmol/L EDTA, 0.5 % Triton X-100, 20 mmol/L Tris-HCl, pH 7.5 using dialysis tubes with 3,500 Mₚ cut-off. The ³⁵S- and ³H-labeled PG were isolated by ion-exchange chromatography as described (3). PG were equilibrated in DPBS and divided in four equal parts. One part was left untreated (control) while the others received chondroitinase AC (ChAC) (200 mU/mL), chondroitinase B (ChB) (80 mU/mL), or heparitinase I (HS I) (80 mU/mL) respectively. After incubation over night at
37°C the material resistant to GAG digestion was analyzed by SDS-PAGE on a 4-12 % precast Tris-glycine gel (4). After electrophoresis the gel was fixed in 0.1 % cetyl pyridinium chloride in HAc:Isopropanol:H₂O (10:30:60), vacuum dried, and the radioactive bands were visualized by autoradiography. For Western blot analysis, PG were digested with ChABC (200 mU/mL) over night at 37°C. Proteins were separated on a 4-12% SDS-PAGE as described above. Western blotting was then carried out essentially as described (3) using polyclonal rabbit anti-human decorin antibodies diluted 1:10,000 (Cat. No AB1909, Chemicon International Inc., CA, USA). A swine anti-rabbit peroxidase conjugated antibody (Cat.No P0217, Dako, Denmark) was used as secondary antibody at dilution 1:25,000. The blot was finally developed using an ECL+Plus Western blotting detection system following the protocol indicated by the manufacturer.

Isolation of human recombinant secretory non-pancreatic phospholipase A₂

SnPPLA₂ was purified from conditioned medium from a transfected CHO-cell line (5) as described (6).

Electrophoretic mobility shift assay (EMSA)

Binding of fibroblast derived PG to snPPLA₂ was studied by EMSA essentially as described (7). Briefly, 9,000 cpm of 35S-3H-labeled PG was incubated at room temperature for 30 min in a final volume of 20 μL sample buffer (10 mmol/L HEPES, 5 mmol/L CaCl₂, 2 mmol/L MgCl₂, and 140 mmol/L NaCl pH 7.4) containing increasing concentrations of snPPLA₂ (0-16 μmol/L). The samples were then loaded on a 0.7 % agarose gel and electrophoresis was run at 60 V for 2 h in running buffer containing 10 mmol/L HEPES, 2 mmol/L CaCl₂, and 4 mmol/L MgCl₂ pH 7.2. The gel was fixed in 0.1 % cetyl pyridinium chloride in HAc:Isopropanol:H₂O (10:30:60), dried, and the radioactive bands were visualized by autoradiography.

Expression and purification of human recombinant decorin core protein fragment B/E
The expression vector pRSET A (Invitrogen, Carlsbad, CA, USA) containing the coding region representing Asp45-Lys359 (fragment B/E) of the human decorin core protein was kindly provided by Dr. Elke Schönherr, University of Munster, Munster, Germany (8). The plasmid was transformed into *E.coli* strain BL21(DE3)pLysS (Invitrogen, Carlsbad, CA, USA) following the protocol indicated by the manufacturer. Single positive colonies were selected and inoculated into 100mL-cultures. Expression of the B/E fragment was induced by addition of IPTG to a final concentration of 0.4mmol/L when the density of the cultures had reached an OD_{600nm} of 0.4-0.6. Purification of the B/E fragment was done by affinity chromatography on a Ni-NTA agarose gel (Qiagen, Hilden, Germany) as described previously (8).

**Solid phase binding assay I**

Microtiter plates were coated with collagen type I or collagen type VI as described (9). Briefly, collagen dissolved in diluted HAc was neutralized by addition of NaOH and diluted to 0.04 μg/μL with DPBS. Fifty μL was added to each well and the plates incubated at +4°C over night. As negative control, parallel wells were coated with 50 μL of DPBS containing 1% FFA-free BSA. Non-bound proteins were removed by rinsing the wells three times with DPBS. To block non-specific binding sites the wells were incubated at RT for 2 h with 200 μL of DPBS containing 1% FFA-free BSA. The wells were then rinsed three times with DPBS. Decorin (from bovine articular cartilage) was diluted to 0.02μg/μL with DPBS. Fifty μL was added to the wells and the plates incubated for 4 h at 37°C. In control wells, 50 μL of DPBS containing 1% FFA-free BSA was added. The amount of non-bound decorin was determined in the supernatants after incubation as described (10). The wells were then rinsed three times with DPBS. In some experiments decorin was pretreated with ChABC (100mU/mL) overnight at 37°C to remove the GAG from the core protein before added to the wells. The depletion of GAG by ChABC treatment was followed by SDS-PAGE analysis.
SnpPLA2 was diluted with DPBS and added to the wells in increasing concentrations (as indicated in the figure) in a final volume of 50 μL. The plate was incubated 1 h at 37°C. The wells were rinsed three times with DPBS to remove non-bound snpPLA2. The remaining snpPLA2 was determined by measuring the enzyme activity using PC-mixed micelles prepared as described below. Forty μL of PC-mixed micelles were added to the wells and the plates were incubated for 18 h at 37°C. The liberated FFA were determined using a commercial kit (NEFA-C).

**Solid phase binding assay II**

Microtiter wells were coated with purified B/E by adding 50 μL DPBS containing 6.5 μg B/E to each well following the same procedure as described above for collagen. In some experiments 50 μL of DPBS containing snpPLA2 in increasing concentrations (indicated in the figure) was added to the wells and the activity of bound snpPLA2 was determined as described above. In a second set of experiments, a constant concentration of snpPLA2 (290 nmol/L) was mixed with increasing concentrations of decorin (indicated in the figure), in a final volume of 50 μL of DPBS, before added to the B/E coated wells. The plates were incubated for 1 h at 37°C and bound snpPLA2 was determined as described above. Finally, to study the contribution of electrostatic forces to the binding of snpPLA2 to immobilized B/E, snpPLA2 (290 nmol/L) was added to B/E coated wells in 50 μL DPBS supplemented with NaCl in increasing concentrations (0.15 - 1 mol/L NaCl). After incubation for 1 h at 37°C the activity of bound snpPLA2 was determined as described above.

**SnpPLA2 activity**

Reaction mixtures containing 300 ng snpPLA2 and decorin or decorin pretreated with ChABC in increasing concentrations were incubated at room temperature for 30 min in a final volume of 20 μL DPBS. Mixed PC-micelles were prepared by dissolving 10 mg of L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl in 200 μL of 4 % Nonidet P40 and 2 %
deoxycholic acid. The micelles were diluted twenty times with DPBS. Twenty μL was added to each reaction and the samples were incubated for 4 h at 37°C. The enzyme activity was determined using a commercial kit (NEFA-C) that measures free fatty acids produced during the incubation. Human LDL (d. 1.019-1.063), isolated by sequential ultracentrifugation of plasma from healthy fasted male volunteers (11), was also used as substrate presenting structure. LDL was equilibrated in DPBS supplemented with 2 % FFA-free BSA. Reaction mixtures containing 700 ng snpPLA₂ and increasing concentrations of decorin were pre-incubated at room temperature for 30 min in a final volume of 20 μL. Twenty μL of LDL was added resulting in a final concentration of 0.63 mg apoB/mL. The samples were incubated at 37°C for 24 h and the enzyme activity was monitored by measuring FFA with the NEFA-C kit.

**Solid phase binding assay III**

Versican, isolated from pig aorta as described (12), was kindly provided by Dr. Germán Camejo, AstraZeneca R&D Mölndal, Sweden. GAG-digestion was performed by incubating 2μg/μL of versican in DPBS in the presence of ChABC (200mU/mL) and HS I (80mU/mL) over night at 37°C. The digested PG was equilibrated in DPBS on a NAP-5 column. The depletion of GAG was analyzed by agarose-gel electrophoresis followed by staining with toluidine-blue (7). Microtiter wells were coated with native- or GAG-depleted versican by adding 50 μL DPBS containing 2 μg versican to each well following the same procedure as described above for collagen. SnpPLA₂ was diluted with DPBS and added to the wells in increasing concentrations (as indicated in the figure) in a final volume of 50 μL. The plate was incubated 1 h at 37°C and the activity of bound snpPLA₂ was determined as described above.
REFERENCES


Online supplementary information

RESULTS

Immunodetection of snpPLA$_2$ in ChABC-treated sections

Some sections were also pre-incubated with ChABC to specifically degrade the GAG-chains before immunostaining was performed. As shown in Figure S1 the immunohistologic detection of snpPLA$_2$ increased in regions of the intima close to the media (indicated by the arrow) after ChABC-treatment. These regions correspond to areas that also stain positive for decorin in the atherosclerotic arteries suggesting that the removal of the GAG-moiety appears to unmask, but not remove, snpPLA$_2$-antigen associated with decorin.

*In vitro* interaction of snpPLA$_2$ with versican

Versican does not bind to collagen and was therefore coated directly onto the plastic surfaces in microtiter wells. Measuring the enzyme activity monitored snpPLA$_2$ binding to the versican-coated wells. As shown in Figure S2, snpPLA$_2$ bound efficiently to versican-coated wells, while binding of snpPLA$_2$ to the control wells coated with BSA was low. GAG-depleted versican displayed significantly decreased binding emphasizing the important role of the GAG moiety for interactions between versican and snpPLA$_2$. However, there was some residual binding of snpPLA$_2$ to GAG-depleted versican. Compared to the binding to native versican this residual binding was low and close to saturation ($B_{max}: 0.24\pm0.05\text{mM}$). On the other hand, a straight line, indicating a high capacity binding far from saturation, better represented the interaction of snpPLA$_2$ with native versican. The ChABC/HS I treatment of versican appeared to remove the GAG determined by agarose-gel electrophoresis followed by toluidine-blue staining (data not shown). However, it can not be
ruled out that there still are non-degradable short oligosaccharides bound to the versican core protein that may be responsible for the residual binding of snpPLA$_2$ to the ChABC/HS versican-coated wells.

**LEGENDS**

**Figure S1.** Immunofluorescence detection of snpPLA$_2$ in a ChABC treated atherosclerotic lesion from a carotid artery. **B)** SnpPLA$_2$-positive staining in media and intima in untreated section **D)** SnpPLA$_2$-positive staining in neighboring section to B, treated with 10 mU/mL ChABC in PBS containing 0.1 mg/mL protease free BSA for 2h at 37°C. **A, and C)** Histology sections stained with hematoxylin. **I = Intima, M = Media. Magnification X 74.**

**Figure S2.** Solid phase binding assay for evaluation of snpPLA$_2$ binding to versican coated microtiter-wells. The wells were coated with native versican, GAG-depleted versican (ChABC/HS versican), or FFA-free BSA as described in Methods. Increasing concentrations of snpPLA$_2$ (indicated on the x-axis) was added to the wells. The activity of bound snpPLA$_2$ was determined by measuring the release of FFA from PC-containing mixed micelles. The data presented are means ± S.D. of two separate experiments each performed in triplicate. Statistical differences between native and ChABC/HS versican was evaluated according to Student’s unpaired t-test and indicated in the figure (**p<0.001**).