Expression of Secretory Group IIA Phospholipase A₂ in Relation to the Presence of Microbial Agents, Macrophage Infiltrates, and Transcripts of Proinflammatory Cytokines in Human Aortic Tissues

Mario Menschikowski, Andrea Rosner-Schiering, Rolf Eckey, Erich Mueller, Rainer Koch, Werner Jaross

Abstract—Recent seroepidemiological and immunohistochemical studies have demonstrated an association between microbial infections and atherosclerosis. However, the mechanisms underlying this association are widely unknown. In the present study, arterial specimens obtained at autopsy after sudden death were analyzed concerning (1) the presence of Chlamydia pneumoniae, cytomegalovirus, herpes simplex virus, and Helicobacter pylori; (2) the expression of secretory group IIA phospholipase A₂ (sPLA₂-IIA) and of proinflammatory cytokines; and (3) the stage of atherosclerosis. Genomic DNA of microbial pathogens was determined by the polymerase chain reaction technique. The expression of sPLA₂-IIA was studied immunohistochemically by using monoclonal antibodies against human sPLA₂-IIA. Transcripts specific for sPLA₂-IIA, interleukin-1β, tumor necrosis factor-α, and interferon-γ were identified by reverse transcription–polymerase chain reaction. In 18 of 102 analyzed specimens, DNA of microbial pathogens was found. Thirteen sections were positive for C pneumoniae, whereas 2 specimens were positive either for cytomegalovirus or for herpes simplex virus. One section contained genomic DNA of all 3 pathogens simultaneously. None of the analyzed tissues exhibited nucleic acids specific for H pylori. In addition to macrophage infiltrates, the presence of microbial DNA was closely associated with the occurrence of transcripts specific for proinflammatory cytokines and sPLA₂-IIA. Pathogens as well as sPLA₂-IIA and cytokines were found to be present not only in advanced but also in early stages of atherosclerosis. In tissues negative for sPLA₂-IIA and cytokine expression, none of the pathogens could be identified. Because macrophages exposed to phospholipase A₂–treated lipoproteins are transformed into foam cells in vitro, the results of this study suggest an alternative mechanism by which microbial infections may act in a proatherogenic fashion in vessel walls. (Arterioscler Thromb Vasc Biol. 2000;20:751-762.)

Key Words: infections ■ secretory phospholipase A₂ ■ inflammation ■ atherosclerosis

From previous histopathologic studies, it is known that atherosclerotic lesions share many features with those of inflamed tissues. The causes of inflammatory reactions in the vessel wall, however, are still under discussion. Several hypotheses have been proposed, including the theory of infection as a cause of coronary artery diseases, which was first described by Osler at the beginning of this century. Although there is now a growing body of evidence suggesting an association between infections and atherosclerosis, the mechanism by which microbial pathogens may act in a proatherogenic manner is still unresolved.

In a previous study, we identified the expression of a secretory phospholipase A₂ (sPLA₂), which is identical to group IIA isozyme (sPLA₂-IIA) according to the new group type classification of phospholipases A₂ proposed by Dennis and Murakami et al., in human atherosclerotic plaques but not in normal arteries. From in vitro studies, it is known that phospholipase A₂ can hydrolyze the phospholipids of lipoproteins, resulting in varied physicochemical properties of modified particles. These alterations apparently lead to an enhanced degradation of modified lipoproteins by macrophages, transforming them into foam cells, a hallmark of early atherosclerotic lesions. These data led to the hypothesis that sPLA₂ could be a new factor in the pathogenesis of atherosclerosis. In recent years, further studies have been published involving the analysis of sPLA₂ expression in human vascular tissues. Bobryshev et al. performed a study on carotid arteries and aortas and found sPLA₂-specific immunostaining in atherosclerotic plaques but not in areas of the adjacent normal-appearing arterial wall. Another 2 studies...
have been published by Hurt-Camejo et al.\textsuperscript{11} and Elinder et al.\textsuperscript{12} respectively, showing sPLA\textsubscript{2} immunoreactivities not only in atherosclerotic arteries but also in nonatherosclerotic arteries. The physiological and/or pathophysiological relevance of sPLA\textsubscript{2} expression in arterial tissues remains to be elucidated. In vitro studies indicated a substrate preference of sPLA\textsubscript{2} for Gram-negative bacterial membranes, such as from Escherichia coli, in the presence of bactericidal/permeability-increasing protein.\textsuperscript{15} Recently, bactericidal activity of sPLA\textsubscript{2} against Gram-positive bacteria has been observed in human tears.\textsuperscript{16} Based on these data, which suggest microbialic properties of sPLA\textsubscript{2}, the question arises of whether the expression of sPLA\textsubscript{2} in atherosclerotic lesions could represent a part of the host defense mechanism against microbial pathogens. This assumption was supported by further in vitro and in vivo data showing that (1) the synthesis and secretion of proinflammatory cytokines, including interleukin (IL)-1\textbeta, tumor necrosis factor-\alpha (TNF-\alpha), and interferon-\gamma (IFN-\gamma), are induced after exposure to bacterial endotoxins;\textsuperscript{17,18} (2) cytokines such as IL-1\textbeta, IL-6, and TNF-\alpha are able to stimulate the synthesis and secretion of sPLA\textsubscript{2} in smooth muscle cells;\textsuperscript{19,20} and (3) proinflammatory cytokines are expressed in atherosclerotic plaques.\textsuperscript{21}

The objective of the present study was to investigate the expression of sPLA\textsubscript{2}-IIA in relation to the stage of atherosclerosis and the presence of microbial agents and signs of inflammatory reactions in human vascular walls. For this purpose, abdominal and thoracic aortic segments obtained at autopsy after sudden death were analyzed by immunohistochemistry, polymerase chain reaction (PCR), and reverse transcription (RT)-PCR methods.

### Methods

#### Tissues

Human arterial human (n=102) obtained at autopsy from 51 persons (38 males and 13 females ranging in age from 1 to 94 years, mean±SEM 41.7±17.1 years) were taken from subjects whose death was sudden and who did not show signs of severe acute diseases. Most common causes of death were accidents, homicides, or suicides. The time between death and autopsy, during which the subjects were kept at 4°C, ranged from a few hours to 6 days.

#### Histopathology and Immunohistochemistry

Immediately after autopsy, the removed aortic segments were washed in PBS (pH 7.2), fixed in neutral-buffered formalin, and embedded in paraffin. Subsequently, the samples were coded, and all further investigations were performed with observers blinded to the details of the study protocol. To determine the stage of atherosclerosis, sections were stained by hematoxylin-eosin staining. Goldner’s trichrome staining was applied to visualize extracellular components. The immunohistochemical investigations and polymerase chain reaction (PCR) methods.

#### Immunohistochemistry

Histopathology and Immunohistochemistry

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#### Identification of Nucleic Acid Specific for C. pneumoniae, H. pylori, CMV, and HSV

Nucleic acids from paraffin-embedded aortic sections (10×5 μm-thick sections) were prepared by using the DNA isolation kit Puregene (Genentra Systems). For amplification of genomic DNA specific for Chlamydia pneumoniae, Helicobacter pylori, cytomegalovirus (CMV), and herpes simplex virus (HSV), primer pairs were applied as described\textsuperscript{24–27} (Table 1). The buffers and reagents used in the PCR were those supplied in the Gene Amp Kit (Perkin-Elmer, ABI). The conditions of PCR were as follows: an initial denaturing step for 2 minutes at 94°C; 35 cycles at 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute; and, finally, an extension step at 72°C for 10 minutes (with the exception of H pylori, for which 62°C was applied in the extension step). The products of amplification were analyzed on ethidium bromide–stained 2% agarose gels under UV light. In cases of negative results, amplified samples were reamplified by a second PCR under the same condition as applied in the first PCR. Tissues that showed negative results after the second amplification were defined as pathogen-free specimens.

To verify the efficiency of DNA isolation and amplification, genomic DNA specific for pyruvate dehydrogenase was determined in parallel with the microbial pathogens in every aortic specimen. PCR reagents without templates and with nucleic acids isolated from C. pneumoniae, H pylori, CMV, and HSV were used as negative and positive controls, respectively. The C. pneumoniae prototype strain TW-183 and H pylori were kindly provided by Dr Pressler (Friedrich-Schiller-University, Jena, Germany). CMV and HSV of type 1 were obtained from Dr Wischmann (Technical University, Dresden, Germany). Isolated DNA from HSV of type 2 was purchased from Advanced Biotechnology. At the beginning of the present study, investigations on DNA from HSV of type 1 and type 2 proved the specificity of applied primers to identify both types of HSV.

#### Identification of mRNA Specific for sPLA\textsubscript{2}-IIA, IL-1\textbeta, TNF-\alpha, and IFN-\gamma

To evaluate the stability of sPLA\textsubscript{2}-IIA mRNA in situ, 2 aortic segments obtained in surgery were analyzed after storage in humidified chambers at 4°C or 24°C for 1, 2, 4, 5, and 6 days before fixing. A segment of each biopsy was immediately fixed after the removal of tissues for use as a control. After they were embedded in paraffin, 5-μm-thick and 10×5-μm-thick serial sections were prepared for immunohistochemical investigations and for extraction of RNA, respectively.

Total RNA was prepared after deparaffinization and rehydration from 10×5-μm-thick autopsy sections by acid guanidinium thiocyanate–phenol–chloroform extraction as described by Chomczynski and Sacchi.\textsuperscript{28} Isolated RNA was converted to cDNA by using the GeneAmp RNA-PCR Kit (Perkin-Elmer). A portion of the RT reaction products was then amplified for identification of sPLA\textsubscript{2}-IIA and TNF-\alpha mRNA by using nested PCR and identification of IL-1\textbeta and IFN-\gamma mRNA by using conventional PCR. The applied primer pairs, which are respectively designed to span splicing sites between 2 exons, are summarized in Table 1. For the analysis of sPLA\textsubscript{2}-IIA
mRNA, oligonucleotides were synthesized according to the published nucleotide sequence of human placental sPLA₂-IIA cDNA. In the nested PCR, extrinsic and intrinsic primer pairs were applied in a final concentration of 0.1 μmol/L and 0.8 μmol/L, whereas in the conventional PCR, the final concentration averaged 0.8 μmol/L. The conditions for amplification were as follows: 15 cycles at 94°C for 30 seconds and at 60°C for 50 seconds, followed by 40 cycles at 94°C for 30 seconds and at 72°C for 1 minute. The buffers and reagents used were the same as supplied in the GeneAmp Kit (Perkin-Elmer). After amplification, products were analyzed by electrophoresis on agarose gels. As control, GAPDH mRNA was determined in every autopsy sample.

### Statistical Analysis

The prevalence of the expression of sPLA₂ in relation to the stage of atherosclerosis in autopsy tissues was compared with the stage of atherosclerosis in aortic specimens with and without the analyzed microbial agents by using the χ² test and, if the expected frequencies were low, by the Fisher exact test. The confidence intervals for odds ratios were determined by using the Cornfield method. The Student t test was used to compare the average age of individuals positive and negative for C pneumoniae, CMV, or HSV (Table 2).

### Results

#### Identification of Microbial Agents in Autopsy Aortas

By use of PCR, 18 of the 102 tissue samples investigated showed the presence of DNA fragments of C pneumoniae, CMV, or HSV (Table 2). In 5 individuals, nucleic acids of C pneumoniae were found in both sections of the aorta, whereas in 8 individuals, microorganisms were found to occur in only 1 location, ie, either in the abdominal or the thoracic aorta. Samples with early signs of atherosclerotic changes (types I and II) or preatheromas (type III) were found to be up to 8.5% positive for analyzed microorganisms, whereas the tissues with advanced atherosclerotic lesions (types IV to VI) yielded up to 36% microbial agents. Tissue samples without atherosclerotic lesions were free of analyzed microorganisms (Table 3). Genomic DNA of H pylori was not observed in any of the tissues investigated.

### Identification of sPLA₂-IIA Expression and of Transcripts Specific for Proinflammatory Cytokines in Autopsy Aortas

Preliminary investigation of 2 arterial tissue samples obtained during surgery showed that sPLA₂-IIA and GAPDH mRNA were detectable by means of RT-PCR up to 4 days after removal of the tissue, provided the samples were stored at 4°C before fixing. After the day 5, there was a reduction of mRNA, and on day 6, no mRNA specific for sPLA₂-IIA and GAPDH could be determined (Figure 1A). Compared with tissue samples stored at 4°C, the tissue samples stored at 24°C before fixing exhibited an accelerated decomposition of the mRNA, oligonucleotides were synthesized according to the published nucleotide sequence of human placental sPLA₂-IIA cDNA. In the nested PCR, extrinsic and intrinsic primer pairs were applied in a final concentration of 0.1 μmol/L and 0.8 μmol/L, whereas in the conventional PCR, the final concentration averaged 0.8 μmol/L. The conditions for amplification were as follows: 15 cycles at 94°C for 30 seconds and at 60°C for 50 seconds, followed by 40 cycles at 94°C for 30 seconds and at 72°C for 1 minute. The buffers and reagents used were the same as supplied in the GeneAmp Kit (Perkin-Elmer). After amplification, products were analyzed by electrophoresis on agarose gels. As control, GAPDH mRNA was determined in every autopsy sample.

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Furthermore, no differences in the intensity of sPLA₂-IIA mRNA, however, the immunohistochemical identification of sPLA₂-IIA was not significantly impaired by unfixed storage in the adventitia also exhibited sPLA₂-IIA immunostainings. In shown in Table 2. It can be seen that along with the intima, in 5 samples, the media was specifically stained for sPLA₂-IIA.

TABLE 2. Overview of Specimens Exhibiting Nucleic Acid Fragments Specific for *C pneumoniae*, CMV, and HSV

<table>
<thead>
<tr>
<th>No. of Case</th>
<th>Aortic Segment</th>
<th>Age/Sex</th>
<th>Pathogen</th>
<th>sPLA₂-IIA mRNA</th>
<th>sPLA₂-IIA–Specific Immunostaining</th>
<th>CD68-Positive Immunostaining</th>
<th>Cytokine mRNA</th>
<th>Stage of Atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Abdominal</td>
<td>39/M</td>
<td><em>C pneumoniae</em></td>
<td>+</td>
<td>0 0 1+</td>
<td>1+ 0 1+</td>
<td>IL-1β, TNF-α</td>
<td>Type II</td>
</tr>
<tr>
<td>2</td>
<td>Thoracic</td>
<td>45/M</td>
<td>HSV</td>
<td>+ 1+ 0 0</td>
<td>2+ 0 0</td>
<td>–</td>
<td>–</td>
<td>Type III</td>
</tr>
<tr>
<td>3</td>
<td>Thoracic</td>
<td>35/M</td>
<td>CMV</td>
<td>+ 0 0 1+</td>
<td>2+ 1+ 1+</td>
<td>–</td>
<td>–</td>
<td>Type IV</td>
</tr>
<tr>
<td>4</td>
<td>Abdominal</td>
<td>64/M</td>
<td><em>C pneumoniae</em>, HSV, CMV</td>
<td>+ 3+ 0 2+</td>
<td>3+ 1+ 1+</td>
<td>IL-1β, TNF-α</td>
<td>Type VI</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Thoracic</td>
<td>31/F</td>
<td>HSV</td>
<td>+ 1+ 0 1+</td>
<td>1+ 0 1+</td>
<td>IFN-γ</td>
<td>Type II</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Abdominal</td>
<td>77/F</td>
<td><em>C pneumoniae</em></td>
<td>+ 0 2+ 1+</td>
<td>3+ 1+ 2+</td>
<td>IFN-γ</td>
<td>Type VI</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Thoracic</td>
<td>75/F</td>
<td><em>C pneumoniae</em></td>
<td>+ 0 0 3+</td>
<td>3+ 1+ 3+</td>
<td>–</td>
<td>Type V</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Abdominal</td>
<td>15/F</td>
<td><em>C pneumoniae</em></td>
<td>+ 2+ 3+ 3+</td>
<td>2+ 1+ 3+</td>
<td>IL-1β</td>
<td>Type III</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Thoracic</td>
<td>39/M</td>
<td><em>C pneumoniae</em></td>
<td>+ 2+ 0 3+</td>
<td>3+ 1+ 1+</td>
<td>–</td>
<td>Type IV</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Thoracic</td>
<td>94/F</td>
<td>CMV</td>
<td>+ 1+ 2+ 3+</td>
<td>3+ 2+ 2+</td>
<td>IL-1β, TNF-α</td>
<td>Type IV</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Thoracic</td>
<td>29/M</td>
<td><em>C pneumoniae</em></td>
<td>+ 0 0 3+</td>
<td>3+ 2+ 1+</td>
<td>TNF-α</td>
<td>Type IV</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Abdominal</td>
<td>62/M</td>
<td><em>C pneumoniae</em></td>
<td>+ 0 0 3+</td>
<td>3+ 0 1+</td>
<td>TNF-α</td>
<td>Type VI</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Abdominal</td>
<td>20/M</td>
<td><em>C pneumoniae</em></td>
<td>+ 3+ 2+ 2+</td>
<td>3+ 2+ 3+</td>
<td>TNF-α</td>
<td>Type V</td>
<td></td>
</tr>
</tbody>
</table>

Immunostaining scores are defined in Methods. The stage of atherosclerosis was classified according to the recommendation published by Stary et al. Immunostaining scores are defined in Methods. The stage of atherosclerosis was classified according to the recommendation published by Stary et al. indicates presence; –, absence.

sPLA₂-IIA and GAPDH mRNA (Figure 1A). In contrast to mRNA, however, the immunohistochemical identification of sPLA₂-IIA was not significantly impaired by unfixed storage up to 6 days after the intraoperative release of tissue. Furthermore, no differences in the intensity of sPLA₂-IIA immunostainings were observed in aortas fixed in Bouin’s solution, ethanol, or formalin (not shown).

All the autopsy aortic samples in which DNA sequences of pathogens were identified exhibited immunostaining and transcripts specific for sPLA₂-IIA. The distributions and intensities of the sPLA₂-IIA immunostaining and macrophage infiltrates in the individual vessel layers of the aorta are shown in Table 2. It can be seen that along with the intima, the adventitia also exhibited sPLA₂-IIA immunostainings. In 5 samples, the media was specifically stained for sPLA₂-IIA. Transcripts of IL-1β, TNF-α, or IFN-γ were identified in 11 of the 18 pathogen-positive tissues. In addition, macrophage infiltration was found in the intima and, with the exception of 1 sample, in the adventitia as well (Table 2).

Together with the tissues in which pathogens were detected, a further 61 samples in which no DNA of the investigated microorganisms occurred revealed sPLA₂-IIA–specific immunostaining. Of these samples, 52 simultaneously exhibited sPLA₂-IIA–specific transcripts. Of the 52 samples, in which sPLA₂-IIA expression was determined both on the protein level and on the mRNA level, 16 samples yielded transcripts of proinflammatory cytokines. All samples without sPLA₂-IIA expression at the protein and the mRNA levels (n=23) contained none of the investigated pathogens and were also free of transcripts of IL-1β, TNF-α, and IFN-γ. Some of the results of these PCR and RT-PCR investigations are shown in Figure 1B.

TABLE 3. Autopsy Aortic Specimens Free of Atherosclerotic Lesions

<table>
<thead>
<tr>
<th>No. of Case</th>
<th>Aortic Segment</th>
<th>Age/Sex</th>
<th>Pathogen</th>
<th>Cytokine-Specific mRNA</th>
<th>sPLA₂-IIA mRNA</th>
<th>sPLA₂-IIA–Specific Immunostaining</th>
<th>CD68-Positive Immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Abdominal</td>
<td>23/M</td>
<td>–</td>
<td>–</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>2</td>
<td>Thoracic</td>
<td>10/M</td>
<td>–</td>
<td>–</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>3</td>
<td>Thoracic</td>
<td>18/M</td>
<td>TNF-α, IFN-γ</td>
<td>+ 0 2+ 3+</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>4</td>
<td>Thoracic</td>
<td>7/F</td>
<td>IL-1β, IFN-γ</td>
<td>+ 3+ 1+</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>5</td>
<td>Thoracic</td>
<td>12/M</td>
<td>–</td>
<td>–</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

The causes of death were homicide (case 1), accidents (cases 2, 3, and 5), and carbon monoxide intoxication (case 4). Immunostaining scores are defined in Methods. + indicates presence; –, absence.
Table 4 shows the frequency of sPLA2-IIA expression as a function of the stage of atherosclerosis in the autopsy tissues. Because no significant differences concerning the stage-dependent enzyme expression between thoracic and abdominal sections of the aorta were apparent by multivariate analysis (not shown), the data of both segments were considered together. In Table 5, the results of the univariate analysis are summarized. A significant association could be established between the presence of C pneumoniae, CMV, or HSV DNA sequences and the expression of proinflammatory cytokines and sPLA2-IIA transcripts. Furthermore, CD68-positive cell infiltrates and sPLA2-IIA immunostainings were more frequently detectable in the adventitia of aortas with microbial agents compared with specimens free of agents.
numerous CD68-positive macrophages, which was not the case in the other sPLA₂-IIA–free nonatherosclerotic specimens. Comparable findings were made in a further sample taken from a thoracic aorta of a 10-year-old boy who died after an accident (Table 3).

**Discussion**

There is increasing evidence that inflammation and immunologic mechanisms play a major role in atherogenesis, but exactly what stimuli are responsible for the initiation of these reactions remains to be determined. In the present study, aortic specimens obtained at autopsy after sudden death were analyzed for the presence of microbial agents and the expression of sPLA₂-IIA and proinflammatory cytokines. The data show that 14% of the analyzed specimens exhibited nucleic acid fragments specific for *C pneumoniae*, whereas 3% were positive either for CMV or for HSV. Recently, Maass et al.³⁸ published a study demonstrating that 15% to 26% of analyzed specimens obtained from different vascular regions were positive for *C pneumoniae* DNA by using a nested PCR protocol. In aortic wall samples, the prevalence of *C pneumoniae* averaged 18%, a value that closely parallels the frequency observed in the present study. Compared with frequencies observed in other studies, the frequencies observed in the present study and in the study of Maass et al.³⁸ are relatively low. This could be due to differences in the incidence of *C pneumoniae* infections, dependent on the respective geographic regions in which the studies were carried out. Another reason might be that many surgical samples were examined for the presence of *C pneumoniae*, from which it can be expected that the samples will show a preponderance of atherosclerotic lesions. If this is taken into account and the prevalence of *C pneumoniae* in aortas with advanced atherosclerotic lesions (types IV to VI) is determined, the data in the present study yield a value of 31%. Indications of the occurrence of *H pylori* in aortic tissues

**TABLE 4. Expression of sPLA₂-IIA in Relation to Stage of Atherosclerosis in Autopsy Aortas (n=102)**

<table>
<thead>
<tr>
<th>Stages of Atherosclerosis</th>
<th>I and II</th>
<th>III</th>
<th>IV</th>
<th>V and VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>29</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>sPLA₂-IIA specific immunostaining, (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score 0</td>
<td>5 (71)</td>
<td>14 (47)</td>
<td>4 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Score 1</td>
<td>0 (0)</td>
<td>7 (23)</td>
<td>11 (38)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Score 2 or 3</td>
<td>2 (29)</td>
<td>9 (30)</td>
<td>14 (48)</td>
<td>13 (87)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>...</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Odds ratios (95% CI)</td>
<td>1.0</td>
<td>1.1 (0.1–10.0)</td>
<td>2.3 (0.3–21.0)</td>
<td>16.4 (1.3–341)</td>
</tr>
<tr>
<td>sPLA₂-IIA–specific mRNA, (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5 (71)</td>
<td>17 (57)</td>
<td>6 (21)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Positive</td>
<td>2 (29)</td>
<td>13 (43)</td>
<td>23 (79)</td>
<td>12 (80)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>...</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Odds ratios (95% CI)</td>
<td>1.0</td>
<td>1.9 (0.3–17.0)</td>
<td>9.6 (1.2–98.0)</td>
<td>10.0 (0.9–149)</td>
</tr>
</tbody>
</table>

Stage of atherosclerosis²³ and immunostaining scores are defined in Methods. NS indicates not significant.

*Exact 1-tailed Fisher test of the prevalence of sPLA₂-IIA–specific immunostainings with a score of 2 or 3 and of the identification of sPLA₂-IIA mRNA related to the prevalence found in nonatherosclerotic aortas.

**TABLE 5. Comparison of Data Observed in Autopsy Aortas With and Without *C pneumoniae*, CMV, or HSV**

<table>
<thead>
<tr>
<th>Genomic DNA of Pathogens</th>
<th>Present (n=18)</th>
<th>Absent (n=84)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SEM age, y (range)</td>
<td>49.3±20.1 (15–94)</td>
<td>40.1±16.7 (1–94)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>12/6</td>
<td>64/20</td>
<td>NS</td>
</tr>
<tr>
<td>Abdominal aorta, n</td>
<td>7 (39%)</td>
<td>44 (52%)</td>
<td>NS</td>
</tr>
<tr>
<td>Thoracic aorta, n</td>
<td>11 (61%)</td>
<td>40 (48%)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β, TNF-α, and/or IFN-γ mRNA, n</td>
<td>11 (61%)</td>
<td>16 (19%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>sPLA₂-IIA mRNA, n</td>
<td>18 (100%)</td>
<td>52 (62%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>sPLA₂-IIA–immunostainings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intima, n</td>
<td>11 (61%)</td>
<td>44 (52%)</td>
<td>NS</td>
</tr>
<tr>
<td>Media, n</td>
<td>5 (28%)</td>
<td>29 (35%)</td>
<td>NS</td>
</tr>
<tr>
<td>Adventitia, n</td>
<td>17 (94%)</td>
<td>44 (52%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adventitial CD68-positive cell infiltrates, n</td>
<td>17 (94%)</td>
<td>50 (60%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
could not be found in the present study, which is consistent with results published by Blasi et al. 39

To determine whether the presence of bacteria or viruses is accompanied by inflammatory reactions, markers of inflammatory activities such as infiltrates of CD68-positive macrophages and expressions of proinflammatory cytokines and sPLA₂-IIA were investigated. In the present study, it was determined that all of the tissue samples in which genomic DNA of pathogens was found had strong macrophage infiltrates and sPLA₂-IIA immunostainings in the intima and in the adventitia. Furthermore, in 11 of these specimens, transcripts specific for IL-1β, TNF-α, or IFN-γ could be identified, which is significantly more frequent (P<0.001) than in specimens free of C pneumoniae, CMV, or HSV DNA sequences. These results are in accord with in vitro data showing that the infection of mouse macrophages or human alveolar macrophages with C pneumoniae resulted, along with the generation of reactive oxygen species, in a dose- and time-dependent release of TNF-α and IL-1β into the culture supernatant. 40,41 Similar results concerning the production of TNF-α and IL-1β were obtained in human peripheral blood mononuclear cells after infection with C pneumoniae. 42 In CMV-infected mononuclear cells, an elevated level of IL-1β activity was determined. 43 The reason that cytokine-specific transcripts were not found simultaneously in all pathogen-containing and sPLA₂-IIA-positive tissues may, on the one hand, be due to the concentration of mRNA being too low. On the other hand, it is also possible that along with the cytokines IL-1β, TNF-α, and IFN-γ investigated in the tissues examined, other proinflammatory mediators, such as IL-6, that can also induce sPLA₂-IIA expression were present. 44

sPLA₂-IIA is known to exhibit a highly alkaline pI, leading to a strongly positively charged protein under physiological conditions. 45,46 For this reason, it can be assumed that the enzyme will interact with negatively charged proteoglycans and, for that reason, bind itself, independent of the location of synthesis, to cell surfaces and extracellular matrix structures and be able to remain there for extended periods of time, as recently analyzed by Romano et al. 13 Increased sPLA₂-IIA activities in the vessel wall may act in a proatherogenic manner in different ways. Besides the direct cytotoxicity due to the hydrolysis of membrane phospholipids, through which the maintenance of plaque structures can be disturbed, and besides the generation of proinflammatory lipid mediators, including eicosanoids and platelet-activating factor, a series of indirect effects may be associated with the expression of sPLA₂-IIA. Lysophosphatidylcholine, which is produced in the hydrolysis of phospholipids, was shown to exhibit cytolytic properties, 47 to induce the synthesis of heparin-binding epidermal growth factors through macrophages, 48 to express...
vascular cell adhesion molecules in endothelial cells, and to be chemotactic for human monocytes and T lymphocytes. Furthermore, phospholipids of lipoproteins retained in the subendothelial space can be hydrolyzed by the enzyme identifiable at the same sites. It has been shown in vitro that the exposure of macrophages to LDL and HDL treated with phospholipase A2 results in a considerable cholesterol accumulation in the cells, transforming macrophages into foam-like cells.

In addition to the tissue sections containing nucleic acids of pathogens, sPLA2-IIA immunostaining could be found in tissues in which there was no indication of the presence of *C pneumoniae*, CMV, or HSV. The mechanisms that induced the sPLA2-IIA transcription in these tissues are still unexplained. A concomitant expression of proinflammatory cytokines in some of these aortas may suggest that other stimuli were present, possibly even other microorganisms. In this connection, it is noteworthy that in 5 pathogen-free, but sPLA2-IIA–positive sections, IFN-γ mRNA was detectable. These data may suggest, at least in IFN-γ–positive tissues, that activated T lymphocytes were present, underscoring the occurrence of immunologic reactions in these specimens.

**Figure 3.** Paraffin serial sections from an abdominal aorta with complicated lesions of type VI. The aorta exhibited nucleic acid fragments from *C pneumoniae*, HSV, and CMV and was positive for mRNA of sPLA2-IIA, IL-1β, and TNF-α (Figure 1B, lane 1). A, Goldner’s staining. B, Stained with anti-CD68 antibodies against macrophages. C and E, Stained with anti-α-actin antibodies. D and F, Stained with anti–sPLA2-IIA antibodies. Two lipid cores covered by a fibrous cap are marked by stars; asterisks indicate the region characterized by heavy cell infiltrates and neovessels. Arrows indicate α-actin–positive (E) and sPLA2-IIA–positive (F) round cells. Mayer’s hematoxylin counterstaining was used. Original magnification ×200 (A through D) and ×1000 (E and F).
The finding of a correlation between the prevalence of sPLA₂-IIA expressions and the stage of atherosclerosis provided further evidence that the induction of the enzyme synthesis and secretion in the aortic wall may be of importance not only for the progress but also for the initiation of atherosclerotic processes. The immunohistochemical investigations demonstrated that sPLA₂-IIA–positive immunoreactivities were present, especially in regions of lipid cores (primarily at the base of lipid cores adjacent to the media) and in areas of extracellular matrix structures. When the sPLA₂-IIA and CD68-specific immunostainings are compared, the enzyme has never been identified in intima free of considerable numbers of macrophages. On the other hand, the presence of macrophages did not correlate strongly with sPLA₂-IIA expression, in view of the fact that macrophage infiltrations with and without evidence of sPLA₂-IIA were detectable, suggesting a different activation state of macrophages in atherosclerotic lesions, which is in accordance with data published by van der Wal. Furthermore, a number of tissues have been found to exhibit adventitia, including the

**Figure 4.** Paraffin sections from thoracic aortas showing the adventitia with vasa vasorum (stars). A through C. Serial sections of an aorta with atherosclerotic lesions of type IV, which exhibited CMV DNA and transcripts of sPLA₂-IIA, TNF-α, and IL-1β (Figure 1B, lane 8). D through F. Serial sections from a nonatherosclerotic aorta, which was free of analyzed pathogen DNA sequences and of transcripts specific for sPLA₂-IIA, IL-1β, TNF-α, and IFN-γ (Figure 1B, lane 2). Panels A and D were stained with anti-sPLA₂-IIA antibodies; panels B and E were stained with anti-CD68 antibodies for macrophages; and panels C and F were stained with anti–α-actin antibodies against smooth muscle cells. Mayer’s hematoxylin counterstaining was used. Original magnification ×200.
media half directed toward it, and to a lesser extent intima, which were positive for sPLA2-IIA. At the same time, massive infiltrations of CD68-positive macrophages were to be found in the adventitia of these tissues. Because sPLA2-IIA–specific immunostainings could be attributed, among other cellular and extracellular structures, to macrophages in the adventitia, the question arises as to whether inflammatory cells may initiate the induction of the sPLA2-IIA gene after they have entered the arterial wall not only from the vascular region but also from the adventitia via the vasa vasorum.

In addition to CD68-positive macrophages, α-actin–positive round cells associated with areas of plaque neovascularization and strong cell infiltrates were identified as sPLA2-IIA positive. The nature of these cells remains to be elucidated. Other α-actin– and sPLA2-IIA–positive cells frequently exhibited a typical stellate-shaped appearance. Recently, Tjurmin et al53 have shown that stellate cells of myxomatous tissue represent a heterogeneous cell population, sharing features of macrophages, smooth muscle cells, and antigen-presenting dendritic cells. The latter cell type is characterized by immunoreactivity with antibodies recognizing α-actin, HLA-DR, and CD1a.53 Bobryshev et al10 have argued that in addition to macrophages, at least in part, vascular dendritic (CD1a-positive) cells may be responsible for the sPLA2-IIA expression in atherosclerotic plaques. These data suggest that in addition to CD68-positive macrophages, smooth muscle cells, and adventitial fibroblasts, stellate cells may be responsible for the expression of sPLA2-IIA identified in atherosclerotic lesions. Interestingly, Tjurmin et al53 hypothesized that in atherosclerotic lesions, stellate cells might be involved in local immune responses, in which they act as antigen-presenting cells.

To understand the possible role of sPLA2-IIA in the pathogenesis of atherosclerosis, the determination of whether this enzyme is expressed in normal arteries is of major importance. In our previous study, sPLA2-IIA–positive immunostainings were visible in arterial and aortic specimens exhibiting atherosclerotic plaques but not in nonatherosclerotic aortas or nonaffected parts of the arterial wall.4 Whereas Bobryshev et al10 found these results to be confirmed in their studies, Hurt-Camejo et al11 and Elinder et al12 described sPLA2-IIA immunoreactivities both in normal nonatherosclerotic arteries and in those with atherosclerotic lesions. In the present study, 7 specimens were classified as nonatherosclerotic. Five of them showed neither sPLA2-IIA–specific immunostainings nor traces of sPLA2-IIA mRNA. Because of the significantly faster decomposition of sPLA2-IIA mRNA in removed aortic tissues after storage at 24°C compared with those at 4°C before fixing, the present study only considered autopsy samples taken from subjects who were delivered to the facility within a few hours of death and were kept at 4°C until autopsy. Furthermore, only those samples were evaluated in which GAPDH mRNA could be detected as a control. This applied also to the 5 sections classified as nonatherosclerotic. Finally, the preliminary investigations showed that when samples were stored at 4°C, sPLA2-IIA levels remained immunohistochemically unchanged up to 6 days post mortem, so that false negatives concerning the sPLA2-IIA immunostaining in the 5 nonatherosclerotic aortas and in further aortic specimens with early stages of atherosclerosis (n=18) can be ruled out.

In 2 nonatherosclerotic thoracic aortas, strong positive sPLA2-IIA immunostainings were evident. These findings were confirmed in both tissue samples by the detection of sPLA2-IIA–specific mRNA. The investigations of proinflammatory cytokines have shown, however, that both specimens simultaneously exhibited transcripts specific for TNF-α and IFN-γ or IL-1β and IFN-γ, which was not the case in the other sPLA2-IIA–free nonatherosclerotic specimens. Such results point out inflammatory reactions as the possible cause of the sPLA2-IIA expressions observed in both samples, although the reason for the inflammation in these tissues is presently unexplained. Therefore, the different results concerning whether sPLA2-IIA is expressed in normal arterial sections4,10–13 are possibly explained with reference to whether inflammatory reactions were ongoing before the removal of tissues.

In summary, the present study has shown that the presence of microbial agents is closely associated with the expression of sPLA2-IIA and macrophage infiltrates in human aortic

Figure 5. Paraffin sections from a thoracic aorta of a 7-year-old female. The cause of death was a carbon monoxide intoxication. The aorta was classified as nonatherosclerotic with adaptive intimal thickening according to Stary et al23 and exhibited transcripts of sPLA2-IIA, IL-1β, and IFN-γ. A, Stained with anti-sPLA2-IIA antibodies. B, Stained with antibodies against α-actin. Arrows indicate examples of smooth muscle cells positive for α-actin (A) and sPLA2-IIA (B); arrowheads on the left mark the position of internal elastic lamina. Mayer’s hematoxylin counterstaining was used. Original magnification ×200.
specialized form of a protective inflammatory response to various forms of insults to the vessel wall. In the progression of this response, a set of proinflammatory cytokines including IL-1β, TNF-α, and IFN-γ may be released by inflammatory cell infiltrates, which in turn induce the synthesis and secretion of sPLA2-IIA. However, when infectious agents persist in the arterial wall, the expression of sPLA2-IIA, possibly triggered as a protective response to microbial invasions, may become excessive. In addition to a series of pathophysiological reactions propagating inflammatory processes in the arterial wall, chronically increased sPLA2-IIA activities may be associated with the hydrolysis of lipoprotein phospholipids. Thereby, the process of foam-cell formation can be forced, especially when high concentrations of lipoproteins are simultaneously present in the subendothelium of the vessel wall. Interestingly, a recently published study involving mice with LDL receptor deficiency provided evidence that the chlamydial proatherogenic effects are dependent on elevated serum cholesterol levels.

Although native lipoproteins proved to be a poor substrate for sPLA2-IIA, an increased susceptibility of lipoproteins to phospholipid hydrolysis after minimal oxidation, which is especially likely to occur under inflammatory conditions in vivo, could recently be shown. This mechanism, if proven in vivo, may inaugurate an alternative strategy for prevention and therapy of coronary artery disease by applying specific sPLA2-IIA inhibitors.

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References


Expression of Secretory Group IIA Phospholipase A2 in Relation to the Presence of Microbial Agents, Macrophage Infiltrates, and Transcripts of Proinflammatory Cytokines in Human Aortic Tissues

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