Prevalence and Pathology of Amyloid in Atherosclerotic Arteries

Christoph Röcken, Jörg Tautenhahn, Frank Bühlung, Daniela Sachwitz, Steffi Vöckler, Andreas Goette, Thomas Bürger

To the Editor:

Apolipoprotein AI (ApoAI)–associated amyloidosis is characterized by the deposition of apolipoprotein AI (apoAI) and occurs as a hereditary and a nonhereditary form. Hereditary ApoAI amyloidosis is a systemic disease leading to the deposition of amyloid in various organs and tissues and is caused by germline mutations in the ApoAI gene. Nonhereditary ApoAI amyloid is far more prevalent and characterized by deposits of nonvariant protein in atherosclerotic arteries.1–3 Despite being linked to the most common cause of morbidity and mortality in Western societies, nonhereditary ApoAI amyloid has achieved only little attention.1,4,5 It shares several striking similarities with secondary or reactive AA amyloidosis. Nonhereditary ApoAI amyloid occurs in the background of a local chronic inflammatory reaction, it originates from an apolipoprotein that largely associates with high-density lipoproteins (HDLs), and apoAI-containing HDL is endocytosed and retroendocytosed by macrophages, which, in themselves, are able to form amyloid in vitro. Finally, ApoAI amyloidosis is characterized by the deposition of proteolytic fragments of the precursor protein, leading us to speculate that proteolysis is involved in the pathogenesis of ApoAI amyloid.1,3 The aim of this study was to gain further insights into the pathology of nonhereditary ApoAI amyloid.

See cover

The prevalence and spatial distribution of amyloid, macrophages, cathepsin B (CathB), cathepsin K (CathK), cathepsin L (CathL), and carboxy methyl lysine (CML) was studied using carotid artery specimens obtained from a consecutive series of all 225 patients undergoing carotid endarterectomy with polyester patch angioplasty (Table I, available online at http://atvb.ahajournals.org) during the period from 1997 to 2003. All patients were scheduled for elective therapeutic endarterectomy and gave written informed consent in the surgical procedure. Patient characteristics were retrieved from hospital records. This study was in accordance with the guidelines of the ethics committee of the University of Magdeburg. Tissue samples were formalin fixed and paraffin embedded. Deparaffinized serial sections were stained with histochemical staining with anti–apoAI– and anti–SAA- (SAA), and transthyretin as described previously.5–7 In vitro degradation experiments with apoAI-enriched HDL apolipoproteins (purchased from Cathbiotech) were performed using recombinant human CathB (1.5 μmol/L final concentration), Cath K (3.0 μmol/L), and CathL (0.15 μmol/L and 30 μmol/L).5–7 Degradation was performed at 37°C for 10, 30, 120, or 240 minutes at pH 5.5. Proteins were resolved in polyacrylamide gels and visualized by Coomassie blue staining.3 Enzymatic activity was studied in 6 unfixed carotid artery specimens (2 with and 4 without intimal amyloid).

Table I summarizes the patients’ characteristics. Amyloid was found in the intima and in atherosclerotic plaques in 122 (54%) patients (Figure; Table I). Patients with amyloid were significantly older than patients without amyloid (P<0.001). The presence of amyloid correlated only with triglyceride levels. Fifty-one amyloid-containing specimens were subjected to immunohistochemical staining with anti–apoAI– and anti–SAA-antibodies. Extracellular apoAI immunoreactivity was most prominent as diffuse staining in atherosclerotic plaques and rarely in the arterial media. In addition, macrophages and foam cells commonly stained for apoAI. In 45 (88%) arteries, amyloid deposits clearly stained with the anti-apoAI antibody (Figure). In six (12%) specimens, amyloid could no longer be discerned in the anti–apoAI–immunostained sections. SAA was not detected in any specimen. The presence and distribution of CML was studied in 20 specimens with amyloid and was detected in every specimen. CML was not found within amyloid. However, it was interesting to note that amyloid deposits were always surrounded by CML immunoreactivity. Twenty-four resection specimens were studied for CD68-immunoreactive macrophages and the spatial distribution of cysteine proteases, including 16 specimens with and 8 without amyloid. CD68-immunoreactive macrophages and CathB were found in every specimen. CathB was present in macrophages (83% of the patients), multinucleated histiocytic giant cells (MGCs; 13%), endothelial cells (4%), and myocytes (4%). Extracellular immunostaining was also commonly observed. CathK was found in 11 (69%) amyloidotic and 6 (63%) nonamyloidotic arteries and was the least commonly found cysteine protease. CathK was also present in macrophages (46%), MGCs (13%), and myocytes (54%). CathL was expressed in all arteries, being present in macrophages (100%), MGCs (17%), and extracellularly (Figure). Almost all macrophages stained for CathL, whereas only a small fraction was immunoreactive for CathB and CathK. CathL was the most abundant cysteine protease in macrophages of atherosclerotic arteries. The pattern of immunostaining for cysteine proteases did not show any differences between amyloidotic and nonamyloidotic arteries. Next, we studied enzymatic activity by fluorospectroscopy (excitation 345 nm, emission 440 nm; Spectramax Gemini Dual-Scanning Microplate Spectrofluorometer, Molecular Devices Cooperation) using a Z-R-R-AMC (CathB), Z-G-P-R-AMC (CathK), or Z-F-R-AMC (CathL; all Bachem) fluorogenic substrate. Extracellular apoAI immunoreactivity was most prominent as diffuse staining in atherosclerotic plaques and rarely in the arterial media. In addition, macrophages and foam cells commonly stained for apoAI. In 45 (88%) arteries, amyloid deposits clearly stained with the anti-apoAI antibody (Figure). In six (12%) specimens, amyloid could no longer be discerned in the anti–apoAI–immunostained sections. SAA was not detected in any specimen. The presence and distribution of CML was studied in 20 specimens with amyloid and was detected in every specimen. CML was not found within amyloid. However, it was interesting to note that amyloid deposits were always surrounded by CML immunoreactivity. Twenty-four resection specimens were studied for CD68-immunoreactive macrophages and the spatial distribution of cysteine proteases, including 16 specimens with and 8 without amyloid. CD68-immunoreactive macrophages and CathB were found in every specimen. CathB was present in macrophages (83% of the patients), multinucleated histiocytic giant cells (MGCs; 13%), endothelial cells (4%), and myocytes (4%). Extracellular immunostaining was also commonly observed. CathK was found in 11 (69%) amyloidotic and 6 (63%) nonamyloidotic arteries and was the least commonly found cysteine protease. CathK was also present in macrophages (46%), MGCs (13%), and myocytes (54%). CathL was expressed in all arteries, being present in macrophages (100%), MGCs (17%), and extracellularly (Figure). Almost all macrophages stained for CathL, whereas only a small fraction was immunoreactive for CathB and CathK. CathL was the most abundant cysteine protease in macrophages of atherosclerotic arteries. The pattern of immunostaining for cysteine proteases did not show any differences between amyloidotic and nonamyloidotic arteries. Next, we studied enzymatic activity by fluorospectroscopy (excitation 345 nm, emission 440 nm; Spectramax Gemini Dual-Scanning Microplate Spectrofluorometer, Molecular Devices Cooperation) using a Z-R-R-AMC (CathB), Z-G-P-R-AMC (CathK), or Z-F-R-AMC (CathL; all Bachem) fluorogenic substrate. CathB (Vmax=6.83±4.99) and CathL (Vmax=6.83±4.99) were found in every carotid artery specimen. CathK activity (Vmax=6.73±3.79) was detectable in 3 carotid artery specimens, including 1 with intimal amyloid deposits. We then examined whether native human apoAI obtained commercially is susceptible to degradation by cysteine proteases. All 3 proteases were found to be potentially able to degrade apoAI at concentrations of 30 nmol/L (CathL), 0.15 μmol/L (CathL), 1.5 μmol/L (CathB), and 3 μmol/L (CathK) generating differently...
AGEs (RAGE). Yan et al. have shown that canceling out the activation biological effect of AGEs is mediated, at least partly, by the receptor of immunoreactivity outside the amyloid deposits. Formation. In support of this notion, we found abundant apoAI translocation, which, on its own, is known to increase the risk of amyloid enrichment in atherosclerotic arteries leading to a high local concentration of cell surface apoAI. Thus, the presence of apoAI and its degradation by cellular processes may contribute to the pathology of amyloidosis.

A hallmark of atherosclerosis is the post-translational modification of cellular RAGE delayed the onset of reactive amyloidosis in mice, thus describing a putative pathophysiological pathway by which AGEs may influence amyloid formation. In this study, we show that amyloid-containing arteries are rich in CML, a distinct, chemically characterized type of AGE. Thus, AGEs may also be involved in the pathology of non-amyloidotic AapoAI amyloid.

Apart from the primary structure, local or systemic protein concentrations, and the presence of AGEs, other factors contribute to the pathology of amyloid and amyloidoses, including proteolysis of the precursor protein and amyloid deposition, as well as macrophages. We believe that the first to show the presence of proteolytically active CathB, CathK, and CathL in atherosclerotic arteries, which have been shown previously to also be potentially involved in the pathology and pathogenesis of AA- and immunoglobulin-derived AL amyloid. Interestingly, and sharing another similarity with other forms of amyloid, we also found CathK-immunoreactive MGCs in amyloidotic arteries. CathK belongs to the most active human elastases described until now and probably represents an enhanced specific proteolytic capability of histiocytic cells. Furthermore, we show that all 3 proteases are able to degrade apoAI, generating intermediate-sized fragments, some having a molecular weight similar to AapoAI amyloid proteins.

With an increasing knowledge about conformational diseases, it has become evident that protein misfoldings and aggregates can be pathogenic. Amyloid in atherosclerotic plaques might be just the tip of an iceberg. Large amounts of aging proteins in the plaque are prone to a multiplicity of conformational changes and formation of supramolecular structures, not all of which necessarily have to form amyloid to gain a pathologic function. In this respect, atherosclerosis may share similarities with Alzheimer disease. Further studies into this topic are warranted.

Acknowledgments

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References


Figure 1:

Carotid artery specimens from a patient with symptomatic carotid artery stenosis. Green birefringent amyloid deposits were found in Congo red-stained specimens (Congo red), which were immunoreactive for apoAI (arrow). Note abundant apoAI immunostaining in the surrounding non-amyloidotic plaque area. CathL was found in all arteries, being the most abundant cysteine protease in macrophages of atherosclerotic arteries, and was also found extracellularly. Congo red staining in polarized light; immunostaining with anti-apoAI and anti-CathL antibodies; hematoxylin counterstain. Original magnifications ×200. In vitro degradation experiments using native apoAI-enriched HDL apolipoproteins were performed with CathL (30 nmol/L) for 10 minutes, 30 minutes, 2 hours, and 4 hours. Incubation for 4 hours at 37°C in the presence of proteases and E64 served as a control. NP denotes no protease added. SDS-PAGE and Coomassie blue staining.
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Methods

Patient population

Carotid artery specimens were obtained from a consecutive series of all 225 patients undergoing carotid endarterectomy with polyester patch angioplasty at the Department of General Surgery, University Hospital Magdeburg during the period from 1997 to 2003. All patients were scheduled for elective therapeutic endarterectomy. All patients gave written consent in the surgical procedure and their baseline characteristics are shown in Table I. This study was in accordance with the guidelines of the Ethics Committee of the University of Magdeburg.

Study Protocol

All patients were hospitalized. The following patient characteristics were retrieved from hospital records: patient age and gender, body weight, body height, diabetes mellitus, arterial hypertension, history of myocardial infarction, history of coronary-artery bypass grafting, chronic ischemic heart disease, peripheral arterial disease, symptomatic carotid artery stenosis and laboratory tests including total cholesterol, high-density lipoprotein cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, C-reactive protein-levels, and white blood cell count. Data were encoded to ensure patient protection.

Histology

All resection specimens were fixed in formalin immediately after surgery and embedded in paraffin. Deparaffinized serial sections were stained with hematoxylin and eosin, and Elastic van Gieson stain. The presence of intimal amyloid was demonstrated by Congo Red staining using polarization and fluorescence microscopy\textsuperscript{1,2}. Amyloid effecting the media was disregarded, since this is known to be of different origin and does not correlate with atherosclerosis\textsuperscript{3,4}. 
Immunohistochemistry

Immunostaining was performed with antibodies directed against apolipoprotein AI (apoAI; dilution 1:1500; polyclonal); cathepsin B (CathB; dilution 1:50; Calbiochem, BadSoden, Germany), cathepsin K (CathK; 1:1000), cathepsin L (CathL; 1:50; both Quartett, Berlin, Germany), CD68 (1:50; DAKO, Glostrup, Denmark), CML (1:1000; monoconal; clone 4G9; Roche Diagnostics GmbH, Penzberg, Germany), SAA (dilution 1:400, monoclonal, clone mc1, DAKO), and transthyretin (1:500; polyclonal; DAKO). Prior to immunostaining, the specimens were pre-treated with 10 mM EDTA (12 + 8 min, 450 W microwave oven; CathL, transthyretin), Proteinase K (30 min., 37 °C; CML) or with trypsin (10 min, room temperature; CD68). Immunoreactions were visualized with the avidin biotin complex method applying a Vectastain ABC alkaline phosphatase kit (Biogene-Alexis GmbH, Grünberg, Germany) or the iVIEW DAB Detection kit (Ventana, Illkirch CEDEX, France). Neufuchsin and 3,3-diaminobenzidine-tetrahydrochloride, respectively, served as chromogens. The specimens were counterstained with hematoxylin. The specificity of immunostaining was verified using specimens containing known classes of amyloid (apoAI), using positive controls recommended by the manufacturers (remaining antibodies) and by omitting the primary antibodies.

Enzyme activity

Enzyme activity of CathB, CathK and CathL was studied in 6 patients as described elsewhere with minor modifications. Immediately after surgery the tissue samples were dissected in two pieces. The first half of the sample was immediately frozen at –20°C, the second half was fixed in formalin and embedded in paraffin as described. Deparaffinized serial sections were stained with hematoxylin and eosin, Elastic van Gieson and Congo red. The unfixed tissue sample was lysed in PBS (pH 7.4) on ice and centrifuged. The protein concentration of the
supernatant was adjusted with assay buffer [50 mM citrate buffer (pH 5.5), 4 mM DTT, 0.6% DMSO] to 0.5 mg/ml and incubated for 21 min. with either 5 µM Z-R-R-AMC (for CathB activity), 5 µM Z-F-R-AMC (for CathL activity) or 125 µM Z-G-P-R-AMC (for CathK activity; all Bachem, Heidelberg, Germany) at 37°C. The fluorescence was measured in 11 s intervals by fluorospectroscopy (excitation 345 nm, emission 440 nm; Spectramax® Gemini Dual-Scanning Microplate Spectrofluorometer, Molecular Devices Cooperation, Sunnyvale, CA, U.S.A.).

Production of Human CathB and CathL

The recombinant human CathB, CathK and CathL were produced and purified as described elsewhere 8. The specific activity against the standard Cbz-FR-MCA substrate as expressed by the $k_{cat}/K_M$ value at pH 6.0 was $4.25 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for CathB and $5.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for CathL.

Degradation Experiments

*In vitro* degradation experiments with apoAI-enriched HDL-apolipoproteins (purchased from Calbiochem) were performed as follows: 10 µg of apolipoproteins were dissolved in 7.5 µl reaction buffer (200 mM sodium acetate, 10 mM EDTA, and 5 mM DTT). CathB was added reaching a final concentration of 1.5 and 0.3 µM, CathK of 3.0 and 1.5 µM, and CathL of 0.15 µM and 30 nM. The mixture was incubated at 37 °C for 10, 30, 120 or 240 minutes at pH 5.5. Incubation was stopped by adding E64 at a concentration of 1.6 mM. Omission of cathepsins and incubation in the presence of E64 served as controls.

SDS-PAGE

Proteins were resolved in polyacrylamide gels, according to Schägger and von Jagow 9 (4% stacking gel, 10% and 16.5% resolving gels) and visualized by Coomassie blue staining as described previously 10.
References


### Table I: Patient characteristics and univariate analyses

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total n=225</th>
<th>Amyloid not present n=103</th>
<th>Amyloid present n=122</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years±SD)</td>
<td>66.2±8.6</td>
<td>62.4±8.8</td>
<td>69.4±7.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>167/58</td>
<td>76/27</td>
<td>91/31</td>
<td>n.s.</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.03±3.98</td>
<td>27.18±3.98</td>
<td>26.89±3.99</td>
<td>n.s.</td>
</tr>
<tr>
<td>Diabetes mellitus [no. (%)]</td>
<td>143 (64)</td>
<td>69 (67)</td>
<td>74 (61)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hypertension [no. (%)]</td>
<td>179 (80)</td>
<td>81 (79)</td>
<td>98 (80)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Myocardial infarction [no. (%)]</td>
<td>42 (19)</td>
<td>16 (16)</td>
<td>26 (21)</td>
<td>n.s.</td>
</tr>
<tr>
<td>CABG [no. (%)]</td>
<td>30 (13)</td>
<td>14 (14)</td>
<td>16 (13)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Chronic ischemic heart disease [no. (%)]</td>
<td>80 (36)</td>
<td>29 (28)</td>
<td>51 (42)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Peripheral arterial disease [no. (%)]</td>
<td>73 (33)</td>
<td>34 (33)</td>
<td>39 (32)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Symptomatic internal carotid artery stenosis [no. (%)]</td>
<td>59 (26)</td>
<td>31 (30)</td>
<td>28 (23)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Cholesterol [mmol/l±SD; Ref. &lt;5.2]</td>
<td>5.66 ± 1.46</td>
<td>5.74 ± 1.51</td>
<td>5.60 ± 1.42</td>
<td>n.s.</td>
</tr>
<tr>
<td>HDL Cholesterol [mmol/l±SD; Ref. &gt;0.9]</td>
<td>1.21 ± 0.39</td>
<td>1.18 ± 0.32</td>
<td>1.22 ± 0.43</td>
<td>n.s.</td>
</tr>
<tr>
<td>LDL Cholesterol [mmol/l±SD; Ref. &lt;4.0]</td>
<td>3.56 ± 1.21</td>
<td>3.54 ± 1.27</td>
<td>3.58 ± 1.17</td>
<td>n.s.</td>
</tr>
<tr>
<td>White blood cell count [x10^9/l]</td>
<td>8.22 ± 2.20</td>
<td>8.38 ± 2.30</td>
<td>8.09 ± 2.12</td>
<td>n.s.</td>
</tr>
<tr>
<td>Triglycerides [mmol/l±SD; Ref. &lt;2.3]</td>
<td>2.36 ± 1.70</td>
<td>2.66 ± 2.0</td>
<td>2.11 ± 1.34</td>
<td>0.03</td>
</tr>
<tr>
<td>CRP [mg/l±SD; Ref. &lt;5.00]</td>
<td>8.52 ± 9.14</td>
<td>8.92 ± 7.86</td>
<td>8.16 ± 10.2</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

CABG denotes coronary-artery bypass grafting, CRP C-reactive peptide; n.s. indicates not significant