Anti–Heat Shock Protein 60 Autoantibodies Induce Atherosclerosis in Apolipoprotein E–Deficient Mice via Endothelial Damage

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Background—Accumulating evidence established a positive association of anti–heat shock protein 60 (HSP60) autoantibodies and the presence of atherosclerosis in humans. However, whether these autoantibodies play a causal role in the development of atherosclerosis is unknown.

Methods and Results—In the present study, anti-HSP60 autoantibodies from blood of patients with coronary heart disease were isolated by affinity chromatography and injected into the tail vein of apolipoprotein E–deficient mice. Atherosclerotic lesions in aortas were significantly increased 8 weeks after injection. Furthermore, administration of a specific mouse monoclonal antibody (II-13) recognizing amino acid residues 288 to 366 of HSP60 effectively induced atherosclerotic lesions in apolipoprotein E–deficient mice. II-13 injection resulted in endothelial cell damage, followed by increased leukocyte attachment and accumulation of macrophages and smooth muscle cells in lesions. Interestingly, II-13–induced atherosclerosis was blocked by pretreatment of animals with F(ab)2 segments derived from the antibody, but not mouse IgG F(ab)2.

Conclusions—Autoantibodies recognizing amino acid residues 288 to 366 of HSP60 induce atherosclerosis via the mechanisms of autoimmune reactions to HSP60 expressed on arterial endothelial cells, which can be prevented by F(ab)2 segments derived from these antibodies. (Circulation. 2005;112:1206-1213.)

Key Words: antibodies ■ antigens ■ atherosclerosis ■ endothelium ■ pathology

Atherosclerosis is a main cause of morbidity and mortality, in which inflammation and autoimmunity have been associated with pathogenesis of the disease.1–4 Different autoantigens, eg, oxidized LDL,5 glycoproteins,6 and heat shock proteins (HSPs),7 have been implicated by induction of an autoimmune process in the development of atherosclerosis. HSPs are a family of ∼24 proteins with high sequence homology among species ranging from bacteria to humans.8 Physiologically, they protect the cells from the damage induced by different stimuli. HSP60, a member of this family, is highly expressed in vitro in endothelial cells. Although HSP60 is considered intracellular, surface overexpression is observed in response to various types of stresses, eg, heat shock and cytokine stimulation.9,10

Accumulating evidence from human seroepidemiological studies shows that HSP60 autoantibodies are associated with the progression and severity of atherosclerosis.11–16 These antibodies can cross-react with bacterial and human HSP60 and induce cytotoxic damage of stressed endothelial cells.17,18 Epitope mapping indicates that anti-HSP60 autoantibodies bind mainly to several regions of HSP60,19 including amino acid 288 to 366. Bason et al20 demonstrated that antibodies against cytomegalovirus are able to cross-react with human HSP60 and cause apoptosis of endothelial cells, which is judged a primary event in the pathogenesis of atherosclerosis. Mucosal administration of HSP60 leads to a reduction in atherosclerosis in hyperlipidemic mice, suggesting possible tolerance or vaccination to HSP60.21,22

We aimed to identify whether anti-HSP60 antibodies from humans and mice play a causal role in the induction of atherosclerosis in animal models and to describe a novel mechanism by which autoantibodies against HSP60 induce endothelial cell damage in apolipoprotein (apo) E–deficient mice.

Methods

Animals

All animal experiments were performed according to protocols approved by the Institutional Committee for the Use and Care of Laboratory Animals. ApoE-deficient mice13 and transgenic TIE2-LacZ mice expressing β-gal under the control of the endothelium-specific protein TIE2 promoter24 were purchased from the Jackson Laboratory. An endothelium-specific enhancer was introduced into the first intron of the mouse TIE2 gene. A combination of the TIE2 promoter with the intron fragment containing this enhancer allows...
gene expression specifically and uniformly in virtually all vascular endothelial cells throughout embryogenesis and adulthood. ApoE−/− mice were crossed with TIE2-LacZ mice in our laboratory, and heterozygous offspring were mated to produce apoE-deficient mice expressing β-gal in endothelial cells (TIE2-LacZapoE−/−). Three genotypes of LacZ−/−, +/−, and +/+ mice were identified using the Jackson Laboratory’s polymerase chain reaction protocol (primers: 5′-ATC TGC ATG GTC AGG TC3′ and 5′-CTG GCC CTG ATT CAT TCC-3′). For apoE−/− mice genotyping, a similar protocol was used with these primers: oMLR180 (5′-GCC TAG CCG AGG GAG AGC CG)-, oMLR181 (5′-TGT GAC TTG GGA GCT CTG CAG C)-, and oMLR182 (5′-GCC GCC CCG ACT GCA TCT-3′). The mice were maintained on a light/dark (12/12 hour) cycle at 22°C and received food and water ad libitum. The genetic constitution of all mice used in the present study was C57BL/6 bred in our laboratory.

**Antibody Preparation**

Purification of serum anti-HSP antibodies was performed following an established method. Briefly, immunoglobulins (IgGs) of pooled high-titer sera from patients were precipitated by a standard (NH₄)₂SO₄ procedure and incubated in a chromatography column with 2 mL agaroose gel beads (Affi-Gel Kit, Biorad) coupled with 3 mg recombinant HSP60 (StressGen). Specific IgGs were recovered by 20 mmol/L HCl acid elution, pooled, and equilibrated with PBS, pH 7.2. Anti-HSP antibody titers of purified IgGs were similar to original sera (1:1280).

Hybridoma cell lines producing mouse monoclonal antibody II-13 (oHSP60288 to 366; IgG2a) and ML-30 (IgG1) were provided by Drs R.S. Gupta (McMaster University, Hamilton, Canada) and J. Ivanyi (MRC, London, UK), respectively. The cell lines were cultivated in serum-free medium, and IgG was isolated with standard (NH₄)₂SO₄ procedure. The antibody titers and protein concentrations were measured by ELISA and Bio-Red protein assays, respectively.

**ELISA**

Antibodies against HSP60 were determined by ELISAs according to an established protocol. In brief, microtiter plates were coated with 1 µg/mL PBS with recombinant HSP60 overnight at 4°C and incubated with 100 µL human serum (diluted with PBS from 1:10 to 1:5160) or II-13 for 1 hour. A serum dilution was considered positive for antibodies to HSP60 if the optical density at 410 nm exceeded 1:5160 or II-13 for 1 hour. A serum dilution was considered positive for antibodies to HSP60 if the optical density at 410 nm exceeded 1:5160.

**Antibody Intraocular Administration**

Ten-week-old mice were treated with 4 intravenous injections every 8 for both groups). The third group (n=11) was treated with II-13 (100 µg) or intraperitoneally with lipopolysaccharide (LPS) (5 µg/kg), which is a known reagent to induce endothelial damage. Mice received intravenous Evans blue dye injection 48 hours after treatment; their aortas were harvested and opened; and blue areas were measured with AxioVision.

**Lipid Measurement**

Blood from the mice was collected and centrifuged, and the serum was kept for analysis. Serum cholesterol and triglycerides were measured with the Infinity Kit (Sigma) for cholesterol and triglycerides, respectively.

**Endothelial Cytotoxicity Assays**

Human umbilical vein endothelial cells were cultured and identified as described previously. For antibody-mediated cytotoxicity, endothelial cells were heat stressed at 42°C for 30 minutes to induce HSP60 expression, whereas control cells remained at 37°C. The assay was performed following an established protocol. Supernatant was analyzed for 51Cr radioactivity (Amersham) in a gamma counter (Wallac-Wizard Automatic Gamma Counter). Specifically, released radioactivity was determined by calculation according to the following formula: 51Cr release in the presence of antibodies minus spontaneous release divided by maximal release minus spontaneous release. Maximal release was obtained by adding 5% Triton X-100 to the cultures. Supernatant release was determined in the presence of complement without antibodies and did not exceed 15% of maximal release.

**Endothelial Damage Assays In Vivo**

Evans blue dye stains blue in the areas where endothelium is damaged or dysfunctional. Five mice were treated intravenously with II-13 (100 µg) or intraperitoneally with lipopolysaccharide (LPS) (5 µg/kg), which is a known reagent to induce endothelial damage. Mice received intravenous Evans blue dye injection 48 hours after treatment; their aortas were harvested and opened; and blue areas were measured with AxioVision.

For X-gal staining, TIE2-LacZ mice were used. Three mice were injected with II-13, 2 were injected with LPS, and 2 remained untreated. After 48 hours, the aortas were fixed in situ with 2% formaldehyde plus 2% glutaraldehyde in PBS and then harvested and processed as described previously. A 5-minute wash in PBS was followed by an 18-hour incubation at 37°C with the X-gal mixture. Then, 3% DMSO and PBS washes began for 5 minutes, and the aortas were mounted with glycerol. Blue-stained areas were measured with AxioVision.

**En Face Staining**

Three groups of mice were treated with II-13 or LPS or were untreated controls. The mice were killed after 48 hours; their aortas were harvested, processed, and fixed as described previously and probed for 1 hour at room temperature with either CD45 rat anti-mouse antibodies (BD Biosciences; 1:20 dilution) or MAC-1 (R&D Systems) at 1:50 dilution). Positive-stained cells were enumerated under the microscope.

**Immunostaining**

Cryosections of aortic roots from the II-13- and ML-30–treated groups were used. The sections were stained for smooth muscle cells with a mouse monoclonal antibody against α-smooth muscle actin conjugated with alkaline phosphatase (Sigma Chemicals; 1:20 dilution) or α-smooth muscle actin conjugated with horseradish peroxidase (DAKO Corp; 1:50 dilution). Positive-stained cells were enumerated under the microscope.
**F(ab)_2, Preparation and Administration**

II-13 and normal mouse IgG were digested with pepsin at a ratio of 1:7.5 (pepsin:IgG) at 37°C for 18 hours. The reaction was terminated by adding 2 mol/L Tris buffer. F(ab), fragment was purified by Sephadex G100 (Amersham Biosciences) column fractionating chromatography. The purity of F(ab), proteins was verified by electrophoresis and Coomassie staining. F(ab), was competitively bound to HSP60 in ELISA plates (data not shown). The F(ab)_2 proteins were administered intravenously from the tail vein 3 hours before II-13 administration at a ratio of 3:1 of F(ab)_2:II-13 (200 μg:100 μg per mouse) because F(ab), corresponds to approximately two thirds of the total IgG proteins. Two groups of mice were pretreated with F(ab), derived from II-13 (n=7) or pretreated with F(ab), from normal mouse IgG (n=6). The protocol for harvesting and analysis remained the same as for other treated groups.

**Statistical Analysis**

Statistical analyses were performed with the Mann-Whitney *U* test and ANOVA, respectively. Results are given as mean±SEM. A value of *P*<0.05 was considered significant.

**Results**

**Autoantibodies to HSP60 Induce Atherosclerosis**

Igs isolated from pooled sera of healthy donors with low levels of anti-HSP60 antibodies (titer <1:10; Ig^low^) and of patients with coronary heart disease with high titers of antibodies (titer >1:2000; Ig^high^) were intravenously injected into 10-week-old apoE^−/−^ mice. Eight weeks after the first injection, significant atherosclerosis enhancement was observed in mice treated with high-titer Ig autoantibodies (Figure 1A). Aortic sinus lesion was increased ∼2-fold and 3.5-fold, respectively (*P*<0.05), in these animals compared with the untreated controls (Figure 1B). In contrast, injection of low-titer Ig did not bring about significant lesion increase. The extent of the induced atheromas maintained the same statistical trend even in the aortic lesions (Figure 1C and 1D). To investigate the specific effects of anti-HSP60 antibodies in atherosclerosis, we purified them from high-titer sera including all Ig subtypes using HSP60-coupled column. A single injection of purified anti-HSP60 antibodies resulted in a marked induction of atherosclerotic lesions in both aortic sinus and the surface (Figure 1).

To determine the role of different epitopes recognized by antibodies, 2 murine monoclonal antibodies, II-13 and ML-30, were produced from hybridoma cell lines,25,26 purified, and injected as for human Ig. Atherosclerosis was significantly enhanced in mice treated with II-13 as seen in their aortic sinus and the surface (Figure 2). In particular, there was a 4- and 2.5-fold increase (*P*<0.05) in size of the aortic sinus lesion in mice treated with II-13 for 4 times and only single injection, respectively, compared with untreated mice. An even greater difference was observed when the II-13–injected groups were compared with mice treated with normal mouse IgG injection (*P*<0.05). In contrast, injection with ML-30 did not significantly enhance lesion development (Figure 2). Aortic lesions in II-13– and ML-30–treated mice followed the same trend as the aortic sinus lesions.

Blood cholesterol and triglyceride levels did not significantly differ between antibody injection and untreated controls (the Table). Thus, Ig administration alone does not alter blood cholesterol and triglyceride levels in mice.

**Autoantibodies to HSP60 Induce Endothelial Damage**

To investigate the mechanisms of autoantibody-induced atherosclerotic lesions, vascular endothelial cells labeled with ^51^Cr were treated with a variety of antibodies in the presence of complement. Furthermore, II-13 binding to HSP60 coated on ELISA plates was completely inhibited by the presence of human Ig from high-titer serum (Ig^high^), whereas other Ig or antibodies had no effects, indicating that II-13 shares the same binding epitope with human autoantibodies (Figure 3A). For Cr release assay, a negative control antibody against smooth muscle cell α-actin was used as described previously.9 Significant ^51^Cr release by stressed endothelial cells was induced by purified human Ig from high-titer serum and II-13 but not by low-titer Ig and ML-30 (Figure 3B). These data provide evidence that only Ig from high-titer serum and II-13 have a cytotoxic effect on endothelial cells.

In vivo administration of II-13 to apoE^−/−^ mice resulted in extensive damage of the aortic endothelium as identified by 3 different methods. First, blue areas (percent) on the surface of aortas were scanned and quantified after Evan’s blue injection, revealing extensive damage of the endothelium in
animals treated with II-13, 15 times greater than in untreated controls \((P<0.05)\) (Figure 4A). Second, damage of the aortic endothelium was also observed in II-13–treated mice expressing the Lacz gene in endothelial cells (TIE2-LacZ) with X-gal staining. There was a 5-fold difference between II-13–treated and untreated animals (Figure 4B). Finally, SEM images confirmed the endothelial damage after II-13 treatment (Figure 4C). The aortas of II-13– and LPS–treated animals are characterized by smooth muscle cells pealing off and by platelet accumulation.

Blood Lipids

<table>
<thead>
<tr>
<th>Group</th>
<th>Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>333±39</td>
<td>51.7±14.5</td>
</tr>
<tr>
<td>Ig\textsuperscript{low}</td>
<td>255±22.5</td>
<td>65±15.3</td>
</tr>
<tr>
<td>Ig\textsuperscript{high}</td>
<td>270±34.6</td>
<td>55.4±11.4</td>
</tr>
<tr>
<td>ML-30</td>
<td>307±37</td>
<td>85±24</td>
</tr>
<tr>
<td>II-13</td>
<td>321±37</td>
<td>44.4±12.7</td>
</tr>
<tr>
<td>II-13\textsuperscript{single}</td>
<td>342±33.8</td>
<td>46±23.4</td>
</tr>
<tr>
<td>F(ab\textsubscript{2}), II-13</td>
<td>304±14</td>
<td>58±18.3</td>
</tr>
<tr>
<td>Ig\textsuperscript{G}</td>
<td>320±19</td>
<td>47±9.6</td>
</tr>
<tr>
<td>F(ab\textsubscript{2}), Ig\textsuperscript{G}</td>
<td>296±31</td>
<td>43±13</td>
</tr>
</tbody>
</table>

After endothelial damage by II-13, there was increased leukocyte adhesion to the surface of the aorta of mice treated with II-13 antibodies. In particular, there was 20-fold difference in the number of pan-leukocytes and macrophages in mice treated with II-13 antibodies compared with untreated controls \((P<0.05)\) (Figure 5A and 5B). Immunostaining for the sections of aortas from II-13–treated animals displayed increased neointimal lesions as revealed by the large numbers of macrophages and smooth muscle cells (Figure 5C). There was a 5-fold difference in the numbers of macrophages \((P<0.05)\) between II-13–treated mice and untreated negative controls. ML-30 antibody treatment did not bring about any
particular change in the number of infiltrating smooth muscle cells compared with the untreated animals.

II-13 F(ab)₂ Administration Blocked Atherosclerosis Induction

If II-13–induced atherosclerosis in apoE⁻/⁻ mice was due to II-13 binding to the HSP60 epitope on endothelial cells, it is possible to block lesion formation by masking the epitope. F(ab)₂ fragments derived from II-13 antibodies were prepared and appeared as a single band after purification (Figure 6A). Pretreatment of mice with F(ab)₂ derived from II-13 resulted in a significant decrease in the extent of atherosclerosis induced by II-13 antibody (Figure 6B and 6C). Evidently, in the aortic sinus, there is a 2-fold decrease in the extent of atherosclerosis compared with II-13 treatment alone, and the difference remained significant between the F(ab)₂-pretreated animals and those treated with purified Ig F(ab)₂. The same applies to the aortas of these groups. There is a 3-fold decrease (P<0.05) in oil red O–stained area in the aortas of the F(ab)₂-pretreated mice compared not only with II-13 treatment but also with purified Ig F(ab)₂ control treatment.

Discussion

Previous studies have demonstrated that human anti-HSP60 autoantibodies were associated with the development of atherosclerosis⁷⁻¹⁰ and can cross-reactive with bacterial and mammalian HSP60.¹⁷,¹⁸ In this study, we have demonstrated that human anti-HSP60 autoantibodies can accelerate atherosclerosis in apoE-deficient mice. Induction of atherosclerosis after high-titer Ig treatment was not due to a systemic inflammatory response against a foreign protein because...
low-titer Ig treatment did not induce atherosclerosis. Thus, anti-HSP60 autoantibody–induced atherosclerosis could be due to the recognition of specific epitopes on endothelial cells.

The questions that arise are if and where do these autoantibodies bind to the cell surface and by what mechanism do they accelerate atherosclerosis. Previous studies from our group and others have demonstrated cell surface expression of HSP60 as a result of altered hemodynamic stress and hyperlipidemia. HSP60 is normally a cytoplasmic protein, but because of its physiological role, it could transport peptides across membranes, and sometimes during that stage, a portion of the protein could penetrate the extracellular membrane. Another mechanism is passive transport; HSP60 could become a transmembrane protein after stress. These surface-expressed HSPs can be recognized by circulating anti-HSP antibodies. It has been shown that human anti-HSP autoantibodies bind to several epitopes, of which the 288 to 366 amino acid sequence is shared with αHSP60. We demonstrated that II-13 binding to HSP60 is competed by human anti-HSP60 autoantibodies and has the endothelial cytotoxic effects, whereas another monoclonal antibody, ML-30, which binds to amino acids 315 to 318 of HSP60, does not have cytotxic effects against cells in vitro. Our results demonstrate significant induction of atherosclerosis after II-13 administration, whereas ML-30 did not, highlighting the importance of the 288 to 366 sequence epitope for the process.

Establishing the involvement of anti-HSP60 autoantibodies in the induction of atherosclerosis and demonstrating the epitope binding specificity of these autoantibodies created a need to clarify the mechanism that leads to atherosclerosis. We hypothesize that atherosclerosis results from endothelial cell damage induced by antibody-mediated cytotoxicity. Support for this hypothesis is the notion that we demonstrated significant endothelial damage in the aorta after II-13 treatment, which was verified by Evan’s blue leaking method, TIE2-LacZ marker expressed in endothelial cells, and SEM analyses. Each of these approaches has identified endothelial cell damage after II-13 treatment, suggesting that it is a key response to II-13 recognition.

After II-13 binding to endothelium, we demonstrated significantly increased levels of leukocyte and macrophage attachment to the surface of aortas. This indicates that the mechanism of endothelial damage might be antibody-dependent cellular cytotoxicity after complement activation. Furthermore, there was increased neointimal formation, accompanied by significantly elevated levels of infiltrating macrophages, in the aortic roots of II-13–treated mice. Thus, antibody-induced endothelial damage triggers the inflammatory response in the vessel wall that accelerates atherosclerosis.

The most encouraging observation, however, comes from the II-13 F(ab), blocking treatment in mice. As mentioned, the epitope of HSP60 288 to 366 could potentially be recognized by autoantibodies to HSP60 and cause cell death. We hypothesize that, if this epitope were somehow “hidden” or “masked,” then the anti-HSP60 autoantibodies could not recognize it, resulting in prevention of II-13–induced atherosclerosis. Interestingly, after the epitope was masked in vivo with the F(ab), portion of II-13, which can bind to HSP60 but is not recognized by complement and macrophages, atherosclerotic lesions induced by II-13 administration were prevented or retarded, suggesting that the autoimmune system could be “tricked” by hiding the target epitopes and thus that atherosclerosis could be prevented.

As mentioned, accumulating evidence indicates the impact of HSPs in the pathogenesis of atherosclerosis via different mechanisms, including the involvement of soluble HSPs present in circulating blood in the induction of immune reactions. Several studies showed that HSPs could directly bind to CD14/toll-like receptors to induce innate immune responses. Because the data from these studies are derived from the use of recombinant HSPs isolated from bacteria, Gao and Tsan found that endotoxin contamination could, at least in part, be involved in the experimental
system. In our study, that seems not to be the case, although recombinant HSP60 was used. For antibody formation from human serum, recombinant HSP60 was coupled to the beads using a specific kit for protein binding. Importantly, isolated anti-HSP60 antibodies did not show any binding activity to endotoxin in ELISA assay (K. Mandal et al, unpublished data). Similarly, both mouse monoclonal antibodies (II-13 and ML-30) have no cross-reactivity to endotoxin. Thus, the effect of endotoxin contamination on antibody-induced atherosclerosis in apoE-deficient mice could be excluded in our study.

In summary, although the previous studies have shown an association between anti-HSP60 autoantibodies and atherosclerosis, this is the first study to demonstrate the causal role of anti-HSP60 autoantibodies in atherosclerosis in animal models. We not only demonstrated the induction of atherosclerosis but, most importantly, clarified the mechanism of the endothelial damage induced by the antibodies to particular epitopes, which eventually leads to the formation of atherosclerotic plaque. This study could potentially encourage the prevention or retardation of atherosclerosis via inhibition of autoimmune reactions.

Acknowledgments

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References

CLINICAL PERSPECTIVE

It is widely accepted that atherosclerosis is an inflammatory disease in which (auto)immune responses may play an important role. During the last decade, investigators in the field had searched for candidate antigens that induce or initiate immune and inflammatory reactions in the arterial wall. Previous experimental and clinical data suggest that an early stage of atherogenesis consists of an autoimmune reaction against a stress protein, the 60-kDa HSP (HSP60). In the present study, we demonstrated that anti-HSP60 autoantibodies from patients with coronary heart disease could directly induce atherosclerosis in apoE−/− mice. After several further experiments, we found that autoantibodies against epitope amino acid residues 288 to 366 of HSP60 are responsible for the induction of atherosclerosis. Interestingly, the lesion induction can be blocked by pretreatment with F(ab) fragment of a mouse monoclonal antibody. These findings have 2 implications. First, autoantibody-induced endothelial damage could initiate the pathogenesis of the disease in which the new mechanism of atherogenesis, ie, autoimmune reactions, is revealed. Another implication is that we may develop a new strategy for the treatment of coronary heart disease, ie, a vaccine to prevent atherosclerosis. The first atherosclerosis vaccine symposium was held during AHA scientific sessions 2004, in which a clinic trial for atherosclerosis vaccination was reported. Our findings of autoimmune response to amino acid 288 to 366 provided basic information for designing a vaccine in a future study.
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