Mucosal Administration of Heat Shock Protein-65 Decreases Atherosclerosis and Inflammation in Aortic Arch of Low-Density Lipoprotein Receptor–Deficient Mice

Ruth Maron, PhD; Galina Sukhova, PhD; Ana-Maria Faria, MD; Ethan Hoffmann, BSc; Francois Mach, MD; Peter Libby, MD; Howard L. Weiner, MD

Background—Increasing evidence supports the involvement of inflammation and immunity in atherogenesis as well as the role of autoimmunity to heat shock proteins (HSPs) in the progression of atherosclerosis. Mucosal administration of autoantigens decreases organ-specific inflammation and disease in several models of autoimmunity (diabetes, arthritis, and encephalomyelitis) and is also being tested in human clinical trials.

Methods and Results—We examined the effect of nasal or oral administration of mycobacterial HSP-65 on atherosclerotic lesion formation in mice lacking the receptor for LDL that were maintained on a high-cholesterol diet. Animals were nasally or orally treated for 1 week with HSP-65, and a high-cholesterol diet was started after the last treatment. The mice were mucosally treated once a week for 8 or 12 weeks, at which time pathological analysis was performed. We found a significant decrease in the size of atherosclerotic plaques, a reduction in macrophage-positive area in the aortic arch, increased interleukin-10 expression, and a reduced number of T cells in nasally treated animals compared with control animals. A similar trend was observed in orally treated mice, but it was not significant.

Conclusions—Our results demonstrate that nasal vaccination with HSP reduces the inflammatory process associated with atherosclerosis and provides a new immunologic approach for the treatment of atherosclerosis. (Circulation. 2002;106:1708-1715.)

Key Words: atherosclerosis ■ inflammation ■ immunology

The immune response has an important role in the pathogenesis of atherosclerosis. Inflammation and autoimmunity to heat shock proteins (HSPs) are part of the progression of atherosclerosis. In this regard, immune-based therapies can mitigate the disease process. For example, inhibition of CD40 or monocyte chemotactic protein-1 signaling decreases atherosclerotic lesion formation in mice. The existence of inflammatory cells (macrophages and T cells) and mediators (eg, monocyte chemotactic protein-1, tumor necrosis factor-α, and interferon [IFN]-γ) in the atheromatous plaque and the association of immunity specific for HSP with the progression of atherosclerosis have provided us the opportunity to test a novel antigen-specific immunologic approach for treating the atherosclerosis process.

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Mucosal administration of autoantigens decreases organ-specific inflammation and disease in several models of autoimmunity (diabetes, arthritis, and encephalomyelitis), and it is also being tested in human clinical trials. Mucosal antigen generates tolerance and suppresses proinflammatory (Th1)-type immune responses, in large part by inducing antigen-specific T-cell immune responses, characterized by the secretion of interleukin (IL)-4, IL-10, and transforming growth factor (TGF)-β. This mucosally generated T-cell response then acts systemically to suppress inflammation via bystander suppression at the anatomic location where the fed antigen is expressed. Studies in a viral model of diabetes illustrate this phenomenon well. When mice in which the lymphocytic choriomeningitis virus nucleoprotein is expressed in the pancreas on the rat insulin promoter are infected systemically with lymphocytic choriomeningitis virus, antiviral immune responses cause diabetes. Mucosal administration of insulin induces insulin-specific T cells, which localize to the pancreas, secrete antiinflammatory cytokines (such as IL-10 and TGF-β), and suppress inflammation and diabetes development. Thus, we tested the hypothesis that mucosal administration of HSP could affect the development of atherosclerosis in mice, inasmuch as HSP immunization promotes early atherosclerosis and HSP localizes in human atheroma.

Methods

Treatment and Analysis of Mice

Five- to 6-week-old C57BL/6 LDL receptor–deficient (LDLr−/−) female mice (Jackson Laboratory, Bar Harbor, Me) consumed a...
high-cholesterol diet (Research Diets; 1.25% cholesterol, 0% cholate) and received PBS or ovalbumin (OVA) (Sigma Chemical Co) orally or nasally as a control or Mycobacterium HSP-65 (Stressgen). Nasal PBS treatment was no different from nasal OVA treatment in terms of plaque formation, inflammation, and immune responses. Thus, results are presented with OVA as the control; OVA controls for the effect of exposure of a non-relevant antigen to mucosal surfaces.

Orally, the mice (n=8) received 8 µg per treatment, 5 times on consecutive days before the start of the diet and once a week thereafter. Nasally, the mice (n=8) received 3 treatments every other day at 0.8 µg per treatment before the start of the diet and once a week thereafter. Animals were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and were kept under strict pathogen-free conditions. After 8 or 12 weeks of treatment, the mice were euthanized, and their aortas, spleens and sera were analyzed. The aortic arches were perfused with PBS and snap-frozen in OCT (Tissue-Tek). To measure plaque size, longitudinal sections of the aortic arch were analyzed microscopically for all mice. The longitudinal sections allowed us to compare sections from all mice at the same level of the aortic arch. The curvature of the aortic arch was chosen for our measurements because lesions are distributed throughout the arterial tree in the aortic root as well as in the curvature of the aortic arch.14 The maximal plaque area of each mouse was used to compute averages per group. For this purpose, a 3-mm segment of the lesser curvature of the aortic arch was defined proximally by a perpendicular axis dropped from the right side of the innominate artery origin, and the aortic arch wall area subtended by this 3-mm stretch of intima was calculated for each section of all mice by computerized image analysis. For immunohistochemistry, cryostat sections (5 µm) of the aortic arch were cut, air-dried, fixed in acetone, and stained with anti–MAC-3 for macrophages, anti–CD4 for T cells, and anti–IL-10 (Pharmaning), anti–IFN-γ, and anti–TGF-β (R&D) for cytokine staining, as described.15 Areas that stained positively for macrophages were measured by use of computer-assisted image quantification (IPLAB Spectrum P, 3.1). Lympocytes identified by anti–CD4 or anti-cytokine antibody staining were counted microscopically by 4 observers blinded to the study protocol.

In Vitro Analysis
C57BL/6 mice were treated nasally with OVA as a control or with mycobacterial HSP-65; 3 days after the last treatment, they were immunized in the foot pads with 8 µg mycobacterial HSP-65 emulsified in complete Freund’s adjuvant (CFA). Ten days after immunization, lymphocytes from the draining lymph nodes were harvested and tested in vitro for proliferation and cytokine production by [3H]thymidine uptake and ELISA, respectively.

To exclude endotoxin contamination in the HSP-65 batches that we used, we employed a biological assay for endotoxin by testing the in vitro proliferative response of splenocytes from naive C57BL/6 mice stimulated with HSP. Proliferation responses are observed with 1 µg LPS. We observed no proliferation in vitro at concentrations of HSP that were 10 times the amount used for nasal treatment. Splenocytes were harvested from mice that were treated nasally with no immunization. For proliferation assays or cytokine analysis, lymphocytes were cultured with 10 µg/mL HSP-65 in 96-well plates at 5 × 10⁶ cells/ml or 10×10⁶ cells/ml, respectively, in X-Vivo (BioWhittaker). For proliferation assays, cells were pulsed with [3H]thymidine 72 hours later, and radioactivity was determined 16 hours later. For cytokine assays, culture supernatants were collected at 40 hours for IL-10 and IFN-γ and at 72 hours for TGF-β. Quantitative ELISAs for IL-10, IFN-γ, and TGF-β were performed by using paired monoclonal antibodies specific for corresponding cytokines per the manufacturer’s recommendations as described.15

Results
Nasal Tolerance to HSP-65 in C57BL/6 Mice
Before our hypothesis was tested in C57BL/6 LDLr⁻/⁻ mice, pilot experiments were performed to evaluate the tolerization model. A group of C57BL/6 mice was treated nasally with OVA before foot-pad immunization with CFA-OVA. The draining popliteal lymph node cells were tested in vitro against OVA. OVA-treated mice manifested reduced proliferation (4000±950 versus 13 580±1880 cpm in PBS treated mice, P=0.002) and IFN-γ production (0.8±0.2 versus 1.9±0.22 ng/mL in the PBS group, P=0.009). IL-10 production was increased in OVA-treated mice (3.3±0.42 ng/mL) compared with PBS-treated mice (1.65±0.2 ng/mL, P=0.0006).

In other experiments, we tested the response of C57BL/6 wild-type mice to nasal administration of mycobacterial HSP-65, followed by immunization with CFA. Mice were nasally treated with PBS, 0.8 µg OVA, or 0.8 µg HSP-65 three times every second day and immunized 3 days later with 8 µg HSP in CFA. The popliteal draining lymph nodes were excised 10 days after immunization and stimulated in vitro with 0.1, 1, and 10 µg/mL HSP. There was no difference between animals tested with PBS versus OVA as a control (not shown). Nasal treatment of these animals decreased proliferative responses and IFN-γ production, whereas there was an increased secretion of IL-10 (Figure 1). Splenocytes from these mice showed a similar pattern of response: specifically, proliferation to HSP in OVA-treated mice (11 500±1000 change in cpm [Δcpm]) versus nasally treated mice (7740±710 Δcpm, P=0.039). However, splenocytes from nonimmunized mice did not exhibit any proliferative response or IFN-γ production on in vitro stimulation with HSP-65. Similar immunologic trends were seen when HSP was given orally, although they were not statistically significant. Neither nasal nor oral administration of HSP-65 changed TGF-β secretion (not shown). We also tested the induction of tolerance to HSP-65 before immunization in incomplete Freund’s adjuvant, because CFA contains HSP and because it has been reported that different adjuvants might direct Th1 or Th2 immunity.16 We did not find any major differences in the Th1/Th2 responses between the 2 adjuvants, although the strength of the response measured by proliferation or cytokine production was weaker in mice immunized with HSP in incomplete Freund’s adjuvant compared with mice immunized with HSP in CFA. Specifically, proliferation was reduced from 9232±1620 to 1341±156 Δcpm (P=0.02). IFN-γ was reduced from 3.2±0.2 to 1.05±0.2 ng/mL in tolerized animals (P=0.001), and IL-10 increased from 210±40 to 1000±260 pg/mL in tolerized mice (P=0.007). Taken together, the data described here and in Figure 1 established the validity of the tolerization model, and we found that mucosally administered HSP induced tolerance associated with decreased proliferation and IFN-γ and also increased IL-10, an immunomodulatory cytokine. The presence of cells secreting an antiinflammatory cytokine such as IL-10 may inhibit the proliferation of inflammatory cells.

Reduction of Atherosclerotic Lesion Size, Macrophage, and T-Lymphocyte Content by Mucosal HSP-65 Treatment
After the demonstration of induction of mucosal tolerance to HSP-65 in these mice, female LDLr⁻/⁻ mice were nasally
treated with HSP-65 a total of 3 times (on days 1, 3, and 5) or orally treated with HSP 5 times (on days 1 through 5). On the last day of treatment (day 5), a high-cholesterol diet lacking cholate was started. Animals were then mucosally treated once per week for 8 weeks, at which time the mice were euthanized and analyzed pathologically. Mice treated by nasal or oral HSP for 8 weeks had a smaller plaque area than did OVA-treated mice (Figure 2A), and there were lower levels of macrophages and CD4^+ T cells in the nasal HSP group compared with either the OVA-treated or oral HSP–treated animals (Figure 2B, 2C). In association with the cellular infiltrates, the intima of atherosclerotic plaques contains IFN-γ. Thus, we examined cytokine expression in the intima of atherosclerotic plaques, both for IFN-γ and for antiinflammatory cytokines IL-10 and TGF-β. Animals treated nasally with HSP had less IFN-γ and an increased expression of IL-10 compared with OVA-treated mice (Table 1). To identify the cells labeled for IL-10, we examined serial sections of the same plaque stained for macrophages and smooth muscle cells in experimental mouse.
Aortic Arch Staining and In Vitro Immunologic Analysis After 12 Weeks of High-Cholesterol Diet and Mucosal HSP Treatment

To investigate further the effect of mucosal treatment on the development of atherosclerosis, we studied animals followed for a longer period of time (12 weeks). We found that nasal treatment with HSP-65 decreased aortic arch plaque size as well as levels of macrophages and CD4+ T cells (Table 2). Photomicrographs of the atheromatous plaques demonstrate less intimal thickening and macrophage infiltration in animals treated with nasal and oral HSP after 12 weeks of treatment (Figure 4).

We found no immune response to HSP in splenocytes taken from LDLr<sup>−/−</sup> mice as measured by T-cell proliferation or cytokine production before the initiation of the diet or 7 to 8 weeks after the start of the high-cholesterol diet. Splenocytes taken 12 to 14 weeks after the beginning of the diet from HSP-treated mice, compared with those from OVA-treated mice, had decreased in vitro proliferative responses and IFN-γ production with no detectable IL-10 when they were stimulated with HSP in vitro (Table 2, Figure 5). To rule out a total downregulation of the cellular response by the HSP-65 treatment, the spleen cells were stimulated in vitro with anti-CD3. We could not detect any significant difference in proliferation (118 000±9975 cpm for OVA-treated group versus 122 400±12 100 cpm for HSP-treated group) or in IFN-γ secretion (10.4±0.9 ng/mL for OVA-treated group versus 11.8±1 ng/mL for HSP-treated group). However, we did observe increased IL-10 production (1.8±0.2 ng/mL) when cells from HSP-treated mice were stimulated with anti-CD3 versus cells from the OVA control group (1.0±0.12 ng/mL, P=0.0012).

### TABLE 2. Aortic Arch Staining and In Vitro Immunologic Analysis in LDLr<sup>−/−</sup> Mice Nasally Treated With HSP-65 and Maintained for 12 Weeks on High-Cholesterol Diet

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Nasal OVA</th>
<th>Nasal HSP</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage, % MAC-3 staining</td>
<td>20.2±5.9</td>
<td>12.9±6.4</td>
<td>0.032</td>
</tr>
<tr>
<td>T cells (CD4+ cells), n</td>
<td>35.0±7.4</td>
<td>22.3±7.5</td>
<td>0.004</td>
</tr>
<tr>
<td>IFN-γ (score)</td>
<td>1.30±0.53</td>
<td>1.25±0.52</td>
<td>NS</td>
</tr>
<tr>
<td>IL-10 (score)</td>
<td>1.25±0.65</td>
<td>2.20±0.65</td>
<td>0.012</td>
</tr>
<tr>
<td>TGF-β (score)</td>
<td>0.95±0.82</td>
<td>1.15±0.83</td>
<td>NS</td>
</tr>
</tbody>
</table>

Immune response (splenocytes)

<table>
<thead>
<tr>
<th>Proliferation, Δcpm</th>
<th>Nasal OVA</th>
<th>Nasal HSP</th>
<th>0.00015</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ, pg/mL</td>
<td>170±20</td>
<td>25±5</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-10, pg/mL</td>
<td>Not detected</td>
<td>Not detected</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA indicates not applicable. Eight mice in each group were euthanized after 12 weeks on high-cholesterol diet. Spleens were stimulated in vitro with HSP-65, and cytokine secretion was measured by ELISA. Aorta sections were stained for MAC-3, CD4+ T cells, and cytokines. Statistical significance was determined by Student’s t test.
Anti-HSP Antibody Titers and Isotypes After Mucosal HSP Treatment

Investigations have suggested that antibodies against HSP-65 may also play a role in atherogenesis. Thus, we measured HSP-65 antibody responses in the serum of nasally or orally treated animals. We found no measurable humoral response to HSP-65 before the initiation of the diet or up to 7 weeks after the start of the high-cholesterol diet. Twelve weeks after the start of the diet, we found that nasally treated mice had decreased anti-HSP antibody levels compared with levels in OVA-treated mice (Figure 6). Furthermore, the antibodies in the HSP nasally treated...
mice showed a Th2-type pattern with significantly increased amounts of IgG1 antibodies, which was consistent with our observation of decreased IFN-γ and increased IL-10 in the intima of the plaque (Table 2). No IgE antibodies to HSP were detected. To rule out differences in the global production of immunoglobulins between the control and mucosally treated groups, we measured nonspecific total IgG in the control mice and in HSP nasally and orally treated mice (1:50 000 dilution). We did not detect a difference between the 3 groups (optical density 0.752±0.0383 for the control treated group, 0.749±0.0356 for the HSP nasally treated group, and 0.733±0.0277 for the HSP orally treated group).

Discussion

Our findings further support the involvement of immune mechanisms in atherosclerosis and affirm the presence of specific immunity to HSP-65. Although many factors may contribute to atherosclerosis, including lipid disorders and hypertension, the pathogenesis of atherosclerosis ultimately includes a major inflammatory component. T lymphocytes and macrophages localize in atheroma from the earliest stages of development. In addition, these leukocytes in atherosclerotic lesions contain IFN-γ. Interruption of IFN-γ signaling reduced atheroma formation in apoE-knockout mice. In the present study, cytokine staining in the aortic arches demonstrated a decrease of this Th1 cytokine in nasal HSP–treated mice at the 8-week time point. In addition, we observed increased amounts of IL-10 in nasally treated animals. We were unable to demonstrate a deviation in the immune response to HSP in splenocytes at 8 weeks because of a lack of sensitivity, inasmuch as the mice were not immunized with HSP. At 12 weeks, although we did not detect IL-10 secretion in the splenocytes stimulated in vitro with HSP from the nasally treated mice, increased IL-10 secretion was observed with anti-CD3 stimulation. IL-10 is an antiinflammatory cytokine produced by activated lymphocytes and monocytes. Pinderski Oslund et al demonstrated that mice with a murine IL-10 transgene under the control of the human IL-2 promoter had decreased lesions compared with control mice on an atherogenic diet, whereas IL-10–null mice had increased lesions.

Substantial evidence points to a role of immunity to HSP-65 in atherosclerosis. HSPs constitute a highly conserved family of proteins found in all prokaryotes and eukaryotes and function in protein folding, protection, and transport. Necrotic cells but not apoptotic cells can release HSPs, which can activate dendritic cells and macrophages. Xu et al originally showed the presence of an HSP-65–specific immune reaction within the atherosclerotic plaque. They also showed that normocholesterolemic rabbits immunized with HSP-65 developed atherosclerosis when fed a normal diet, and they also found increased expression of HSP 60 within atherosclerotic lesions and a humoral and cellular response to HSP in humans with carotid atherosclerosis or coronary artery disease. Since then, the role of HSPs has become further delineated. Afek et al demonstrated that immunization of LDLr–/– mice with HSP-65 promoted earlier atherosclerosis. They also demonstrated enhanced fatty streak formation in C57BL/6 mice by immunization with HSP-65. Kol et al demonstrated that HSP-60 activates the innate immune response and that activation of mononuclear cells by HSP-60 occurs via the CD14 receptor. These observations may relate to recent studies suggesting that chlamydial infection may play a role in atherosclerosis and demonstrating that chlamydial HSP-60 localizes in human atheroma and regulates macrophage tumor necrosis factor-α and matrix metalloproteinase expression.

A number of studies have demonstrated that antigen-nonspecific immune interventions can modulate atherosclerosis. For example, inhibiting CD40 signaling decreases atheroma, and atherosclerosis has been inhibited by T-cell depletion in normocholesterolemic rabbits. Furthermore, chemokines contribute to atherosclerosis. We have used an approach involving antigen-specific therapy by mucosal administration of HSP given by the nasal route. Others have shown regression of atherosclerotic lesions by immunization with HSP-65 containing material in normocholesterolemic but not hypercholesterolemic rabbits. The effect we observed in LDLr–/– mice occurred in the presence of hypercholesterolemia. Mice fed the high-cholesterol diet for 8 weeks had a cholesterol reading of 1395±255 mg/dL in the control group versus 1439±285 mg/dL in the HSP nasally treated group (P=NS).

Because we were unable to test reactivity to self-HSP, the tolerance we induced was conclusively shown against bacterial HSP, and only by inference was it directed toward self-HSP, via the strong homology between mycobacterial HSP-65 and mammalian HSP. HSP may also participate in other inflammatory and autoimmune processes, and injection with an HSP peptide ameliorates autoimmune diabetes in mice. The results in the present study demonstrate that an antigen-specific approach involving mucosal administration of HSP-65 can specifically affect immune variables and decrease both plaque formation and the inflammatory phenotype. Nasal administration was more effective than oral dosing in this regard. We could not show a significant deviation by oral HSP in our preliminary experiments. Although we saw reduced plaque area by oral feeding, we could not see a significant reduction in macrophage staining because the oral treatment is less robust than the nasal treatment. Consistent with the weaker response seen by the oral treatment, we also could not see an increase in the IgG1 class of anti-HSP antibodies after oral treatment. These differences most probably relate to our observation that nasal HSP induced stronger cellular (IL-10) and Th2-type humoral (IgG1) immune responses than did oral HSP.

A study by Freigang et al, who immunized LDLr–/– mice with different forms of oxidized LDL, reported an antiatherogenic effect by immunization. They hypothesized that the effect seen may be attributed to the activation of cellular immune responses rather than to the induction of high titer antibodies to oxidized LDL. The present study and theirs both used an antigen-specific immune approach.
for treatment, although the mechanisms contributing to the antiatherogenic effect appear to be different.

Serum antibodies against HSP-65/60 of *Escherichia coli* (Gro EL) and *Chlamydia* (HSP-60) cross-react with human HSP-60 and can mediate endothelial cytotoxicity. Thus, a humoral immune response to bacterial HSP may also produce vascular endothelial injury, a key event in atherosclerosis.32 We also found that nasal HSP not only decreased proliferation and IFN-γ secretion in splenocytes from treated mice but decreased total anti-HSP IgG as well, which may have contributed to the beneficial effects that we observed with nasal HSP administration.

Mucosal administration of autoantigens not only has proven effective as a treatment for a large number of animal models of autoimmune diseases8 but also has provided a novel therapeutic approach for treating processes not classically considered to be autoimmune but involving an inflammatory component. For example, oral administration of myelin basic protein decreases infarct size in a rat model of stroke,33 and nasal administration of amyloid Aβ peptide limits decreased amyloid plaque deposition in a transgenic animal model of Alzheimer’s disease.34 The results of the present study suggest similar consideration for the treatment of atherosclerosis. On the basis of our results, we postulate that mucosal treatment with HSP stimulates the development of adaptive immune cells that secrete antiinflammatory cytokines such as IL-10. These cells migrate from mucosal inductive sites to the target organ, the aortic arch, where they are restimulated by HSP to secrete antiinflammatory cytokines. Secretion of IL-10 creates an antiinflammatory milieu in the vascular wall analogous to increased expression of IL-10 in the gut and decreases the proliferation of inflammatory cells secreting IFN-γ. These effects result in the enhanced secretion of IL-10 by macrophages and smooth muscle cells in the vascular wall.

The nasal administration of HSP offers the potential for an antigen-specific therapy that targets regulatory cells to the disease area. Such immunomodulatory therapy appears particularly apt for atheroma, as we increasingly appreciate the importance of functional attributes of the plaque related to inflammation in the clinical consequence of this disease.35 Furthermore, such treatment would be easily administered and clinically applicable for a chronic process such as atherosclerosis, which may require prolonged therapy.

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References


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