Temporal Expression of Heat Shock Proteins 60 and 70 at Lesion-Prone Sites During Atherogenesis in ApoE-Deficient Mice


Abstract—In the study, we investigate whether the expressions of heat shock protein (hsp)60 (a potential autoantigen) and the stress-inducible form of cytoprotector hsp70 are correlated with the development of atherosclerotic lesions in the aortic tree of apolipoprotein E-deficient (apoE−/−) mice. The apoE−/− mouse model is advantageous because the stress-inducible form of hsp70 is not constitutively expressed in mice, unlike primates; hence, tissues under stress can be clearly defined. Both mammalian hsps were detected newly expressed (before mononuclear cell infiltration) on aortic valves and endothelia at lesion-prone sites of 3-week-old apoE−/− mice. In 8- and 20-week-old mice, they were strongly and heterogeneously expressed in early to advanced fibrofatty plaques, with levels correlating with lesion severity. Expression was markedly downregulated in advanced collagenous, acellular, calcified plaques of 40- and 69-week-old mice and was absent in control aortas of normocholesterolemic wild-type (apoE+/+) mice. Western blot analysis of tissue homogenates confirmed the temporal expression of the hsps. Double immunostaining revealed that both hsps were expressed by lesional endothelial cells, macrophages, smooth muscle cells, and CD3+ T lymphocytes. This study provides evidence that hsp60 and hsp70 are temporally expressed on all major cell types in lesion-prone sites during atherogenesis, suggesting that few cells escape the toxic environment of the atherosclerotic plaque. (Arterioscler Thromb Vasc Biol. 2001;21:1991-1997.)

Key Words: atherosclerosis ■ heat shock proteins 60 and 70 ■ apoE-deficient mouse ■ immunohistochemistry ■ aorta

Heat shock proteins (hsps) have been implicated as antigenic targets for the activation of lesional T cells in atherosclerotic plaques.1,2 Hsps are a family of highly conserved ubiquitous proteins that function as molecular chaperones and aid cells in coping with stressful environments.3 Atherosclerosis is a multifactorial disease characterized by inflammation and endothelial injury arising from infection, hemodynamic forces, oxidized LDL, dietary factors, toxins, and chemical insults, all of which can activate the stress response and induce hsps.4 Expression of mammalian hsp605,6 and hsp707–9 is increased in atherosclerotic lesions of humans. Antibodies against hsp60 are increased in the serum of patients with atherosclerosis and are associated with disease severity.10 Because of their inducibility, strong immunogenicity, and high interspecies homology, mammalian hsp60 and hsp70 have been incriminated in triggering autoimmune disease.11

Given the widespread acceptance of the apoE-deficient (apoE−/−) mouse12–15 as a model of human atherosclerosis, the present study sought whether atherogenesis in apoE−/− mice correlates with arterial expression of mammalian hsp60 and hsp70 (stress-inducible form).

Methods

Mice and Diets
ApoE−/− mice were the progeny of breeding colonies described earlier13,16; they were maintained on a Western-type diet (No. TD88137, Harlan Teklad). Retired breeders (69 weeks old) and normocholesterolemic wild-type (apoE+/+) mice were maintained on a normal mouse chow diet (3.8% fat, diet 86, NRM Auckland). Experimental protocols were approved by the University of Auckland Animal Ethics Committee.

Tissue Sample Preparations and Histology
After euthanasia of mice by CO2 asphyxiation and in situ fixation of tissues by perfusion with 4% paraformaldehyde (pH 7.4), the heart and ascending aorta up to iliac bifurcation were dissected. Aortic segments were embedded in OCT compound (Tissue Tek) and snap-frozen, and cryosections (8 μm) that were collected onto poly-L-lysine–coated slides were stored at −80°C.

Immunohistochemistry
Aortic hsp60 and hsp70 expression was detected by staining frozen sections with 2 different mouse monoclonal antibodies (mAbs) specific for either mammalian hsp60 (SPA-806, clone CK-1; 1:150 dilution) or the stress-inducible form of mammalian hsp70 (SPA-810, clone C92F3A-5; 1:200 dilution; Stressgen, which can be accessed online at http://www.stressgen.com). The SPA-810 mAb does not react with the constitutively expressed form of hsp70.
were not detected on the free aortic wall distant to the valves. They enzymously expressed in aortic valve commissures, extending to the immediate free aortic wall in aortic root sections. They were subsequently incubated for 90 minutes with an alkaline phosphatase–conjugated anti-α-SMC actin antibody (1:100, Sigma), and antibody binding was visualized with the use of Vector red (Vector Laboratories). To determine hsp expression by endothelial cells (ECs), sections from 3-week-old apoE−/− mice were immunostained with the anti-hsp mAbs, as described above, and then with a rabbit polyclonal anti–human factor VIII–related antibody (1:10, Biomeda). Immunoreactivity was visualized by use of a Vector ABC–alkaline phosphatase kit (rabbit IgG) and Vector red substrate.

**Double Immunohistochemical Staining of SMCs and ECs**

To characterize hsp expression by smooth muscle cells (SMCs), sections were immunostained with either SPA-806 or SPA-810 with the use of a Vector mouse-on-mouse kit and Sigma fast 3,3′-diaminobenzidine (Sigma Chemical Co) and counterstained in Gill’s hematoxylin.

**Double Immunofluorescence Staining and Confocal Microscopy**

Double immunofluorescence staining of aortic arch sections from 3- and 20-week-old apoE−/− mice was used to colocalize hsps with markers of ECs (CD31), monocyte/macrophages (MOMA-2), and T lymphocytes (CD3). The antibodies used were biotinylated rat anti-mouse CD31 mAb (1:50, Pharmingen), FITC-conjugated rat anti-mouse MOMA-2 mAb (1:10, Serotec), and FITC-conjugated rat anti-mouse CD3 (1:10, Serotec). Immunoreactivity of anti-hsp mAbs SPA-806 and SPA-810 was detected with Texas red–conjugated horse anti-mouse IgG (1:200, Vector Laboratories). Biotinylated anti-mouse CD31 was detected with streptavidin-FITC (1:150, Sigma). Fluorescently stained sections were examined with a Leica TCS 4D confocal microscope equipped with an argon/krypton laser.

**Western Blotting**

Common aortic segments were pooled and homogenized in protein lysate buffer at 4°C, and after centrifugation at 10 000 g, protein supernatants (100 µg per well) were resolved on 10% SDS-polyacrylamide gels. Proteins were transferred to Hybond C Extra (Amersham Life Science England) and Western-blotted with SPA-806 (1:500) and SPA-810 (1:1000) mAbs.

Supplementary information for the Methods section is available online at http://atvb.ahajournals.org.

**Results**

**Hsp Expression Becomes Extensive as Atherogenesis Progresses in 8-Week-Old ApoE−/− Mice**

The earliest cellular change in 8-week-old apoE−/− mice was the adherence of mononuclear cells to endothelia throughout the arterial tree at lesion-prone sites. Expression of hsp60 and hsp70 became intense at aortic valve commissures and on endothelial, intimal, and adventitial regions of early fatty streaks of the free aortic wall (Figure 1e, 1g, and 1h) in the sinus region. Oil red O staining of the latter regions revealed multiple foam cell deposits on valve commissures and pock-ets and also in the subendothelial region in the form of multilayered foam cell deposits in the wall of the aortic root region (Figure 1i). No obvious cellular changes in the medial layers of the free aortic wall were observed. There was no staining when primary antibodies were omitted (Figure 1f). Nascent plaques/early fatty streaks strongly expressing hsp60 were beginning to develop (Figure 1j), and recently developed intimal thickening in the proximal ascending aorta had attached mononuclear cells highly expressing hsp70 (Figure 1k) and hsp60 (data not shown). Neighboring ECs and medial layers occasionally expressed both hsps. The majority of hsp-positive mononuclear cells adhering to aortic endothelium were monocytes/macrophages (Figure 1l).

**Hsp60 and Hsp70 Expression Is Heterogeneous in Advanced Plaques of 20-Week-Old ApoE−/− Mice**

In mice aged 20 weeks, lesions had progressed from intermediate fibrofatty plaques containing multiple layers of lipid-filled macrophages and SMCs to advanced lesions displaying a heterogenous pattern of hsp60 and hsp70 expression. Hsp60 and hsp70 expression patterns were identical. Both hsps were strongly expressed in the subendothelial region, fibrous caps, and areas surrounding necrotic cores, with expression extending to the medial layer underlying necrotic cores and shoulder regions of advanced plaques in the aortic sinus (Figure 2a; data for hsp60 are not shown). Lesional expression in the remainder of the aorta was similar to that of the aortic root. Staining of tissue sections detected extracellular hsp60 and hsp70 components (Figure 2a, 2c, and 2d), particularly in necrotic regions, in agreement with the fact that hsps can be secreted by cells and that human hsp70 has extracellular and intracellular locations in human plaques. Staining was specific; there was no staining after omission of the primary antibody (Figure 2b). The medial layer of the abdominal aorta was not always positive for hsps (Figure 2c and 2d). Advanced lesion areas, characterized by calcification foci and osteoclast-like cells, lacked hsp expression, whereas hsp positivity was seen in adventitia (Figure 2e; data for hsp60 are not shown).

**Downregulation of Hsp Expression in Advanced Plaques of 40- and 69-Week-Old ApoE−/− Mice**

The aortas of Western diet–fed 40-week-old and chow diet–fed 69-week-old apoE−/− mice had advanced complicated plaques containing large necrotic cores and acellular areas that were collagenous and highly calcified. Hsp60 and hsp70 could not be detected in the developed calcium foci of 40-week-old mice (data not shown), and there was only weak and patchy expression in lesions of the aortic sinus and remainder of the aorta (Figure 2f and 2g). The aortic sinus
lesions of 69-week-old mice similarly lacked hsp expression (Figure 2h and 2i), and there was only weak to patchy expression in lesions of the abdominal aorta (Figure 2j; data for hsp60 are not shown). There was a general geographic coincidence between intimal/plaque lipid accumulation and hsp60 and hsp70 expression in the aortic lesions of 20-, 40-, and 69-week-old mice. Rarely was there intraplaque necrosis or lipid accumulation without hsp immunostaining (data not shown).

Control aortae from normocholesterolemic, wild-type apoE\(^{+/−}\) mice were lesion free, had a normal histology, and lacked expression of hsp60 and hsp70 at all ages examined, as illustrated for 20-week-old mice in Figure 2k.

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Homogenates of heat-shocked (1 hour at 42°C) EL-4 cells (lanes a), pooled lesion-prone and lesioned aortic segments from 3- and 8-week-old mice (lanes c), non-lesion-prone aortic segments from 3- and 8-week-old mice (lanes d), unstressed EL-4 cells (lanes e), and pooled aortic segments from 3-, 8-, and 20-week-old apoE–/– mice (lanes f) were Western-blotted with SPA-806 (top) or SPA-810 (bottom) mAbs.

**Western Blotting of Aortic Tissue Homogenates Confirms Hsp Levels**

Western blot analysis of aortic tissue homogenates of pooled lesion-prone and lesioned sites of 3-, 8-, and 20-week-old apoE–/– mice revealed strong single bands of 60 and 70 kDa (Figure 3, lanes b; top and bottom panels, respectively) after staining with the hsp-specific mAbs. In contrast, neither hsp60 nor hsp70 was detected in nonlesioned distal abdominal aortic tissue of 3- and 8-week-old mice (Figure 3, lanes d). In accord, expression of hsp60 and hsp70 was downregulated in the lesions of 40- and 69-week-old mice (Figure 3, lanes c). A lysate of heat-shocked EL-4 T-lymphoma cells served as a positive control, generating strong bands of 60 and 70 kDa (Figure 3, lanes a), whereas unstressed EL-4 cells lacked expression (Figure 3, lanes e). Hsp60 and hsp70 were not detected in aortic tissue homogenates pooled from the aortas of normocholesterolemic wild-type apoE–/– mice (Figure 3, lanes f).

**Lesional ECs, SMCs, Monocytes/Macrophages, and CD3+ T Lymphocytes Express Hsp60 and Hsp70**

**Endothelia**

Double staining of aortic sections of 3-week-old mice with the different anti-hsp mAbs and an anti-CD31 mAb revealed that hsp70 (Figure 4a and 4b) and hsp60 (data not shown) were expressed by ECs. This result was confirmed by using a mAb against factor VIII–related antigen [Figure 4c; data for hsp70 are not shown]. As evident from Figure 4c, endothelial hsp expression precedes cellular attachment/infiltration. Lesion-prone sites such as the lesser curvature of the aortic arch displayed strong endothelial hsp60 expression, whereas non–lesion-prone sites of the distal abdominal aorta lacked hsp expression (Figure 4d).

**SMCs**

Migratory SMCs were occasionally detected at developing lesion sites of 20-week-old mice, and some of these cells (stained orange) expressed hsp70 (Figure 4; compare panels e and f) and hsp60 (data not shown). Small numbers of SMCs were detected in and around the necrotic core of advanced lesions, with several (stained orange) expressing hsp60 (Figure 4g and 4h) and hsp70 (data not shown). Certain SMC-rich areas underlying the necrotic core and fibrous cap regions of advanced lesions exhibited increased expression of hsp60 and hsp70.

**Monocytes/Macrophages**

Monocytes/macrophages expressing hsp70 (Figure 5a through 5c) and hsp60 (data not shown) were the most prominent cell type in lesions. Cells doubly positive for the hsp60 and MOMA-2 were seen distributed around the necrotic core, shoulder, fibrous cap, and subendothelial regions of advanced plaques.

**T Cells**

Small numbers of hsp60 (Figure 5d through 5f) and hsp70 (Figure 5g through 5i) expressing CD3+ T cells were seen...
levels of microbial challenge. The apoE/H11001 resident monocyte/macrophages, CD3 were infiltrated with leukocytes. Aortic ECs, infiltrating and old normocholesterolemic apoE lesions of aged mice. In contrast, the aortas of 3- to 69-week-mice and then dramatically downregulated in the chronic atherosclerosis is an immune-based disease, and in accord, lesions of hsp expression often correlated with oil red O–detectable lipid deposits on aortic valves, it also extended to the apparently lipid-free aortic wall adjacent to valve commissures. That lipid deposition necessarily precedes hsp upregulation cannot be discounted without sensitive methods to colocalize lipids with hsps. Rarely was there lipid accumulation without intense hsp60 and hsp70 staining; however, there were plaque areas displaying intense hsp60 and hsp70 staining without notable lipid deposition. Both hsps were heterogeneously expressed in intermediate fibrofatty and advanced necrotic fibrous-capped plaques of 20-week-old apoE/H11001 mice. Large, relatively acellular, collagenous areas of the lesions stained lightly for both hsps, whereas calcified foci and occasionally the medial layer adjacent to developing plaques lacked hsp expression. Lesions at various stages of evolution were observed in a single 69-week-old mouse, in which lesion development started at the aortic root and progressed distally. Hence, hsps represent disease markers that can be used to trace the disease as it progresses within the aortic tree. The mechanisms responsible for the decline of hsp expression in late-stage aortic lesions of aged mice are unknown. The acellular nature of large necrotic cores due to apoptosis and the cytolytic effects of anti-hsp65/60 and hsp70 autoantibodies provide an obvious explanation. Hsp70 expression also diminishes with aging in acute hypertension.

The cellular stressors responsible for the induction of hsp60 and hsp70 in the aortas of apoE/H11001 mice are not known, but the initial insult is likely to be lipid deposition. ApoE/H11001 mice lack apoE, a glycoprotein ligand that mediates LDL receptor clearance of serum lipoproteins, and spontaneously develop hyperlipidemia, as in humans expressing dysfunctional apoE. LDL oxidized in the arterial wall is chemotactic for and enhances the adhesiveness of monocytes, activates T cells, and is cytotoxic. It induces the expression of hsp60 and hsp70 in several cell types.

Hsp expression differs between different species, making comparisons complicated. The stress-inducible form of hsp70 investigated in the present study is not constitutively expressed in most species, except primates. In humans, unlike mice, it is homogeneously distributed throughout normal-appearing areas of the intima and media. Hence, studies of aortic hsp70 expression in apoE/H11002 mice are advantageous because they are not complicated by having to distinguish the constitutive versus inducible expression of hsp70. The expression of hsp70 in mice should directly mark aortic cells that are under stress. Nevertheless, the expression pattern of hsp70 in early fatty streaks of 8-week-old mice and in advanced fibroproliferative lesions of 20-week-old apoE/H11001 mice is largely in agreement with the expression pattern of hsp70 in human lesions. In humans, there appear to be major changes in the localization of hsp70 during atherosclerotic evolution rather than quantitative changes. Increased expression in diseased aortic regions is balanced by decreases in regions in which hsp70 is normally found. Hsp70 is poorly

Figure 5. Colocalization of hsp60 and hsp70 with monocytes/macrophages and T cells. Shown are confocal images of an aortic arch section from 20-week-old apoE/H11002 mice, stained by double immunofluorescence for hsp60, hsp70, monocytes/macrophages, and T cells. Aortic intimal and medial SMCs expressed hsps, indicating that all major lesional cell types were susceptible to the toxic effects of hsps. Hsp70 expression also diminishes with aging in acute hypertension. The expression patterns of hsp60 and hsp70 were identical but the initial insult is likely to be lipid deposition. ApoE/H11001 mice lack apoE, a glycoprotein ligand that mediates LDL receptor clearance of serum lipoproteins, and spontaneously develop hyperlipidemia, as in humans expressing dysfunctional apoE. LDL oxidized in the arterial wall is chemotactic for and enhances the adhesiveness of monocytes, activates T cells, and is cytotoxic. It induces the expression of hsp60 and hsp70 in several cell types.

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Discussion
The present study provides the first evidence that expression of hsp60 and hsp70 is strongly upregulated very early at lesion-prone sites in the aortas of young apoE/H11001 knockout mice and then dramatically downregulated in the chronic lesions of aged mice. In contrast, the aortas of 3- to 69-week-old normocholesterolemic apoE/H11001 mice remained free of hsps. The apoE/H11001 mice were double-stained for hsp60 (d, red), hsp70 (g, red), and T cells (e and h, green). f and i, Merging of images d and e (f) and images g and h (i). Small numbers of T-cell clusters expressing hsp60 and hsp70 could be visualized as yellow-colored cells (arrowed). Original magnification ×40. L indicates lumen of the aorta.

preferentially located, often in clusters, in the subendothelial/fibrous cap, necrotic core (macrophage-rich area with intense hsp expression), and shoulder regions of plaques of 20-week-old mice.

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expressed in complicated, acellular, collagenous plaques, in accord with the present data. In humans, expression of hsp60 is correlated positively with atherosclerotic severity, with the highest levels of expression seen in the shoulder regions and around the necrotic core of atherosclerotic plaques, in accord with the present data.

Hsp60 and hsp70 have been inducted in triggering several autoimmune/chronic inflammatory diseases. Data with the present data.

Enhance the survival of arterial SMCs and acting as a novel chaperone and a cytoprotector to stimulate human and mouse macrophages to release proinflammatory cytokines and to activate cell types found in aortic lesions, leading to the upregulation of cell adhesion molecules. Hsp70 potentially plays a dual role in atherosclerosis, serving as a chaperone and a cytoprotector to enhance the survival of arterial SMCs and acting as a novel cytokine to cause monocytes to express interleukin-1 and interleukin-6, and tumor necrosis factor. Hence, as with hsp60, hsp70 released from necrotic lesional cells may stimulate the innate immune response to promote inflammation during atherogenesis. Serum titers of anti-hsp70 antibodies are raised in patients with cardiovascular diseases.

In conclusion, hsp60 and hsp70 are disease markers that can be used to stage the progression of atherosclerosis. Modulating the expression of hsp60 and hsp70 may allow the roles of these hsp in atherogenesis to be identified. In this regard, the present study suggests that the apoE mouse will be a useful model in which such a study could be undertaken.

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