Role of Heat Shock Proteins in Atherosclerosis

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Abstract—Heat shock proteins (HSPs) are present in most cells, serving as molecular chaperones, and they play a role in cell protection from damage in response to stress stimuli. However, accumulating data indicate the involvement of HSPs in the pathogenesis of diseases. The aim of this article is to update the progress concerning the role of HSPs in atherosclerosis. It has been demonstrated that HSPs are highly expressed in the atherosclerotic lesions of humans, rabbits, and apolipoprotein E–deficient mice. Risk factors for atherosclerosis, eg, infections, oxidized low density lipoprotein, oxidative stress, hypertension, and biomechanical stress, evoke HSP overexpression in endothelial cells, macrophages, and smooth muscle cells via activation of heat shock transcription factor 1. Interestingly, HSPs, normally localized within the cell, have been found as a soluble form in the blood, which is positively correlated with atherosclerosis in humans. Recently, several groups have reported that soluble HSPs specifically bind to the Toll-like receptor 4/CD14 complex, initiating an innate immune response, including the production of proinflammatory cytokines by macrophages and adhesion molecules in endothelial cells via nuclear factor-κB activation. Furthermore, the titers of autoantibodies against HSPs are significantly elevated in patients with atherosclerosis, and T lymphocytes specifically responding to HSPs have been found in atherosclerotic plaques. These proinflammatory responses and autoimmune reactions to HSPs in the vessel wall can contribute to the initiation and perpetuation of atherosclerosis. Thus, HSPs have a general role in the response of the arterial wall to stress and may serve as a mediator/inducer of atherosclerosis in particular circumstances. **(*Arterioscler Thromb Vasc Biol*. 2002;22:1547-1559.)**

Key Words: heat shock proteins ■ atherosclerosis ■ soluble heat shock proteins ■ proinflammatory response ■ autoimmunity

Atherosclerosis is a slowly progressing disease that begins in childhood but does not becoming manifest until middle age or later. Data from different laboratories have recently provided evidence that the first steps of atherosclerosis are inflammatory in nature.[1,2] The discovery of adhesion molecule expression on endothelial cells, including intercellular adhesion molecule-1, vascular adhesion molecule-1, E-selectin, activated T lymphocytes, mast cells and macrophages in atherosclerotic lesions, detection of HLA class II antigen expression, and secretion of several cytokines, has revealed an involvement of immune and inflammatory mechanisms in the pathogenesis of atherosclerosis.[3,4]

Heat shock proteins (HSPs), previously called stress proteins, belong to a group of ≈2 dozen proteins and cognates showing highly homologous sequences between different species, from bacteria to humans.[5] In response to stress stimuli, including heat shock, oxidized LDL (oxLDL), mechanical stress, infections, surgical stress, oxidants, and cytokine stimulation, cells produce high levels of HSPs to protect themselves against these unfavorable conditions.[6,7] HSPs have been found to be highly expressed in cardiovascular tissues and to induce inflammatory responses, and they may be expressed as autoantigens in the development of atherosclerosis.[8,9] The present review will provide an update on the role of HSPs in atherosclerosis, with particular focus on mechanistic studies.

HSP Families and Their Functions

HSPs are multigene families that range in molecular size from 10 to 150 kDa and are found in all major cellular compartments. According to molecular weight, they are divided into following families: HSP10, small HSPs, HSP40, HSP60, HSP70, HSP90, and HSP110. Each family of HSPs contains ≥1 member (Table 1). The distinction between constitutively expressed (eg, Hsc70 and HSP90β) or cognate members of the HSP family and their inducible isoforms (HSP70 and HSP90, respectively) is arbitrary, because accumulating evidence in physiologically relevant in vivo systems now indicates that such relationships depend on cell- and tissue-restricted expression. HSP10, HSP60, and HSP75 are mainly located in mitochondria, whereas others are present in the cytoplasm and nucleus in physiological conditions.[10]

Excellent reviews have been written on the chaperone function of HSPs in general[11,12] and in the cardiovascular system.[7,13] In short, the primary physiological function of HSPs is to fulfill chaperoning activity.[14] Molecular chaper-

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TABLE 1. Heat Shock Protein Families

Family	Members/Other Names	Physiological Function	Pathological Involvement
HSP10	HSP10, HSP17	Promotes substrate release with HSP60	?
Small HSP	HSP22, αA-crystallin HSP23, αB-crystallin HSP27 HSP28	F-actin assembly, molecular chaperones	?
HSP40	HSP32, HO-1 HSP40, Hdj-1 HSP47	Guides protein folding, binding and transport of collagen	Atherosclerosis
HSP60	HSP58, GroEL HSP60, HSP65 Grp58	Assemble polypeptides, translocate proteins across membranes, accelerate protein folding and unfolding	Adjuvant arthritis, rheumatoid arthritis, atherosclerosis, diabetes mellitus, systemic sclerosis, schizophrenia
HSP70	HSP68, Dnak. Hsc70, Hsx70 HSP72 HSP73 HSP75, Grp75 HSP78, Grp78	Molecular chaperone, assemble and transport newly synthesized proteins, fold or unfold polypeptides, remove denatured proteins, bind to specific polypeptides (eg, p53), ATPase activity	Tuberculosis, leprosy, filariasis, atherosclerosis
HSP90	HSP83, HptG HSP87 grp94 HSP90-α HSP90-β	Bind to specific polypeptide, receptors (eg, glucocorticoid receptor)	Schistosomiasis, systemic lupus, erythematosus
HSP110	HSP94 HSP104, HSP105 HSP110, Apg-1 Grp170	Only in yeast (?)	–

ones have been defined as a nonrelated class of proteins that mediate the correct folding of other proteins but do not take part in the final assembly of new structures.[15] HSP27 is active in assisting the assembly of macroglobular protein complexes, such as F-actin polymerization. However, this function is highly dependent on the phosphorylation state and monomeric or multimeric state of HSP27. In the nonphosphorylated monomeric state, HSP27 inhibits F-actin polymerization via specific binding to the plus end of the filaments.[16] HSP60 forms a large (970-kDa) hetero-oligomeric protein complex called the TCP1 ring complex (containing TCP1 and several other proteins), which is essential for protein assembly. As a chaperone, HSP70 plays a role in the assembly and transport of newly synthesized proteins within cells, as well as in the removal of denatured proteins.[17] HSP90 binds steroid receptors, protein kinases, intermediate filaments, microtubules, and actin microfilaments in a specific manner. HSP90 is an essential component of the glucocorticoid receptor, assembled in a complex of several proteins.[18] Thus, HSPs appear to be important in preventing damage and in cellular repair processes after injury. Indeed, increased production of HSPs has been shown to protect cells against apoptosis induced by oxidative stress, toxins, heat shock, ethanol, and cellular damage after ischemia or sepsis-induced injury.[19–24]

Given the high degree of amino acid sequence homology between HSPs of different species, the immune response to HSPs derived from pathogens may cross-react with host HSPs.[25] Thus, HSPs may be autoantigens in some circumstances. The HSP60 family has been shown to be involved in the development of many diseases, such as adjuvant arthritis in rats, rheumatoid arthritis in humans, insulin-dependent diabetes mellitus in mice, and systemic sclerosis in humans,[26] whereas HSP47, HSP60, and HSP70 have been identified as being involved in the pathogenesis of atherosclerosis.[6,27]

HSP Expression in Atherosclerotic Lesions

Berberian et al[28] first reported the elevated expression of HSP70 in human and rabbit arteries and its distribution in relation to necrosis and lipid accumulation, as well as vascular smooth muscle cells and macrophages, in human atherosclerotic plaques. HSP70 was mainly concentrated in the central portions of more thickened atheromas around sites of necrosis and lipid accumulation.[29] In contrast, patches of smooth muscle cells were observed in the most complex plaques but without consistent association with necrosis or increased HSP70.[30] HSP70 was expressed even in dendritic

cells in the arterial wall.[31] The results indicated that elevated HSPs in plaque cells, particularly macrophages, were more stressed within the depth of the atheroma, especially in association with necrosis.

To study HSP60 expression in human atherosclerotic lesions, Kleindienst et al[32] demonstrated that HSP60 was detected on endothelium, smooth muscle cells, and/or mononuclear cells of all carotid and aortic specimens. Whereas vessels of smaller diameter, serving as reference specimens for the normal intima without atherosclerotic lesions and mononuclear infiltration, showed no detectable expression of this HSP. The intensity of HSP60 expression correlated positively with the atherosclerotic severity.[33,34] Interestingly, the expressions of HSP60 and the stress-inducible form of HSP70 were correlated with the development of atherosclerotic lesions in the aortic tree of apoE-deficient mice.[35] Both mammalian HSPs were detected newly expressed (before mononuclear cell infiltration) on aortic roots and endothelia at lesion-prone sites of apoE−/− mice. Both HSPs were expressed by lesional endothelial cells, macrophages, smooth muscle cells, and CD3+ T lymphocytes.[35] This study provided evidence that HSP60 and HSP70 were temporally expressed on all major cell types in lesion-prone sites during atherogenesis.[35]

HSP47 acting as a chaperone for procollagen has been also found to be involved in atherosclerosis.[36] Strong focal expression was evident in atherosclerotic, but not normal, arteries and was prevalent in the collagenous regions. All cells expressing type I procollagen also expressed HSP47.[37] Heat shock and oxLDL stimulated the expression of HSP47 mRNA by smooth muscle cells. These findings identify HSP47 as a novel constituent of human coronary atheroma, and selective upregulation by stress raises the possibility that HSP47 may be a determinant of plaque stability.[37]

Infections and HSP Expression

During the past decade, it has been noted that infections might contribute to the pathogenesis of atherosclerosis.[38,39] Seroepidemiological studies suggest an association between several microorganisms and coronary heart disease, including *Chlamydia pneumoniae*, *Helicobacter pylori*, and herpes viruses, although controversial reports exist.[39–42] These microbes may directly promote a proinflammatory, procoagulant, and proatherogenic environment, and HSP might serve as a link between infections and the atherosclerotic process. Support for this notion was the fact that a prospective population-based study provided strong evidence of a correlation between immune reactions to HSP65 and bacterial infections in atherogenesis,[43] indicating the impact of infections in HSP induction.

In this respect, the life cycle of chlamydiae, an obligate intracellular pathogen, appears particularly interesting. During its normal cycle generating infectious progeny, chlamydiae express basal levels of HSP and in the presence of interferon-γ, a product of activated T cells within atheroma, certain chlamydiae can achieve a state of intracellular chronic persistent infection, in which they remain viable but metabolically quiescent and do not replicate.[44] During such chronic and persistent infections, HSP60 production is abundant. Interestingly, Kol et al[45] demonstrated that chlamydial HSP60 colocalized with human HSP60 within macrophages in atherosclerotic lesions. Nonatherosclerotic samples contained neither HSP. These findings suggest that chlamydial infections might exert their role in atherogenesis via HSP production.

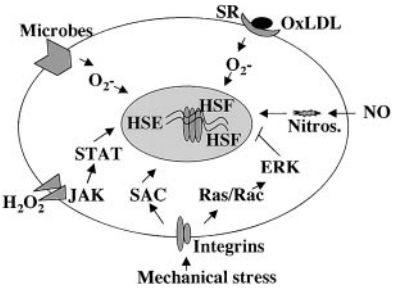

Figure 1. Signaling pathways of HSP expression in vascular cells induced by stresses. SR indicates scavenge receptor; HSF, heat shock transcription factor; HSE, heat shock element; Nitros, nitrosylation; and SAC, stretch-activated channel.

Atherosclerosis is largely viewed as a chronic inflammatory disease, to which chronic infections could contribute via elevated lipopolysaccharide (LPS) or endotoxin.[46] Endotoxin induces local inflammation and systemic toxicity during Gram-negative infections and results in aortic endothelial injury with or without cell death and replication, followed by increased leukocyte adhesion. Seitz et al[47] reported that increased levels of HSP60 were found in aortic endothelial cells of rats in response to *Escherichia coli* LPS. These authors also demonstrated endothelial expression of HSP60 with in vitro administration of LPS.[47] These observations may be significant for understanding the role of HSP in atherosclerosis related to chronic infections.

Concerning virus-induced HSP expression, evidence indicates that infectious virus treatment of myocardial cells increases HSP expression.[48] UV irradiation of the virus prevents virus replication and fails to elicit HSP production in heart cells.[48] It has also been found that chronically HIV-infected lymphomas show an increased expression of HSP70.[49] Furthermore, BiP (GRP78) and endoplasmin (GRP94) are specifically upregulated in rotavirus-infected cells. Thus, virus infections can lead to substantial HSP expression in the infected cells[50] (Figure 1).

OxLDL and Free Radicals Induce HSP Expression

OxLDL is believed to be crucial in the development of atherosclerosis. OxLDL possesses several proatherogenic properties, including interactions with several receptors, leading to the engorgement of cells with lipids, inhibition of endothelium-dependent vascular relaxation, cytotoxicity to proliferating cells, stimulation of chemoattractant secretion, and induction of adhesion molecules that mediate the interaction of leukocytes with the endothelium.[51,52] It has recently been reported that oxLDL also triggers in vitro the expression of HSPs (eg, HSP23 and HSP32 [or heme oxygenase]) in mouse peritoneal macrophages,[53] HSP60 in monocytes/macrophages,[54] and HSP70 in human endothelial[55] and smooth

muscle cells.[56] Additionally, Zhu et al[57] demonstrated that oxLDL induced HSP70 expression mainly in growing endothelial cells, indicating that those cells, present in lesion-prone areas, are more sensitive to oxLDL than are quiescent cells.

Accumulating evidence supports a critical role for oxidative stress in the pathogenesis of atherosclerosis.[58] It has been reported that high levels of reactive oxygen species present in atherosclerotic lesions induce DNA damage and inactivate proteins.[59,60] Modulation of growth or apoptotic responses by free radicals has been demonstrated in a number of cell types, including endothelial and smooth muscle cells.[58] Stimulation of cell apoptosis and proliferation by free radicals is thought to be a critical step in atherosclerotic lesion formation. The potential role of oxidative stress conditions in the induction of HSPs has been reported.[61] Treatment of endothelial cells with H_2O_2 or xanthine oxidase has been shown to increase HSP70 mRNA levels.[62] Nuclear runoff transcription data and kinetics of mRNA decay have indicated that the observed increase in HSP70 mRNA levels in H_2O_2-treated cells is mainly due to a transcriptional induction. Furthermore, a similar effect of H_2O_2 on HSP expression in smooth muscle cells has been observed.[63] It has been demonstrated that H_2O_2 activates the HSP70 promoter via enhanced binding of signal transducers and activators of transcription (STAT) to cognate binding sites in the promoter.[63] Because Janus kinase (JAK)2 is activated rapidly in smooth muscle cells treated with H_2O_2, STAT1 and STAT3 were tyrosine-phosphorylated and translocated to the nucleus in a JAK2-dependent manner. Inhibition of JAK2 activity with AG-490 partially inhibited H_2O_2-induced HSP production.[63] Thus, regulation of HSP70 expression via activation of the JAK/STAT pathway suggests that this pathway is responsible for HSP70 induction in response to oxidative stress (Figure 1).

Current data suggest that NO is a double-edged sword that could result in relaxation and/or cytotoxicity of vascular smooth muscle cells via cGMP-dependent or -independent signaling pathways.[64] NO can stimulate the S-nitrosylation of numerous proteins and also binds to the nonheme iron of ribonucleotide reductase to inhibit DNA synthesis. In vivo, increased production of NO has been observed in response to hemodynamic stress, sepsis shock, and endotoxin.[64] It is not yet clear whether NO increases the expression of HSP in smooth muscle cells or whether HSP acts in conjunction with NO. A recent report has demonstrated that NO leads to the induction of HSP70 protein and mRNA in cultured smooth muscle cells[65] and other cells.[66,67] Induction of HSP70 mRNA was associated with the activation of heat shock transcription factor 1 (HSF1). HSF1 activation was completely blocked by hemoglobin, dithiothreitol, and cycloheximide, suggesting that the protein damage and nascent polypeptide formation induced by NO may initiate this activation.[65] Thus, NO induces HSP70 expression in smooth muscle cells via protein nitrosylation–initiated HSF1 activation.

Biomechanical Stress Induces HSP Expression

In vivo, the vessel wall is exposed to 2 main hemodynamic forces or biomechanical stress: shear stress (the dragging frictional force created by blood flow) and mechanical stretch (a cyclic strain stress created by blood pressure).[68,69] Shear stress stimulates endothelial cells to release NO[70] and prostacyclin,[71] resulting in vessel relaxation and protection of vascular cells, whereas smooth muscle cells are stimulated by cyclic strain stress.[72] In humans, atherosclerotic lesions occur preferably at bifurcations and curvatures[73] where hemodynamic force is disturbed; ie, there is lower shear stress and higher mechanical stretch.[74] Although veins do not develop spontaneous atherosclerosis-like lesions, accelerated atherosclerosis occurs rapidly in venous bypass grafts, which bear increased biomechanical forces that are due to alterations in blood pressure, ie, vein (0 to 30 mm Hg) versus artery (120 mm Hg). Another typical example for mechanical force involvement is hypertension-induced arteriosclerosis. Therefore, mechanical stress could be a crucial factor in the pathogenesis of atherosclerosis.

Udelsman et al[75] have reported that restraint (immobilization stress) results in the selective induction of HSP70 mRNA and HSP70 protein in rat aortas. In addition, HSP70 expression has been induced in rat arteries by treatment with the α_1-adrenergic agonist phenylephrine. Restraint-induced expression of the HSP70 gene in the aorta could be blocked by administration of the α_1-adrenergic antagonist prazosin. These results suggest that the vascular HSP70 induction in restrained animals may be mediated via α_1-adrenoceptors.[75] However, studies by other investigators have indicated that this vascular response can also be mimicked, at least to some extent, by in vivo administration of vasopressin, dopamine, or cocaine,[76–78] each of which exerts its effect via interaction with receptors distinct from the α_1-adrenoceptor. Because phenylephrine, dopamine, cocaine, and air-jet stress can all elevate blood pressure, we[79] have demonstrated that the induction of HSP70 in rat aorta by various agents is secondary to acute hypertension. This mechanism is consistent with earlier observations demonstrating that stretching of the myocardium induces HSP70 expression in isolated perfused rabbit hearts[80] and that increased pressure in the heart (as a result of restricting efflux of blood into the aorta) is sufficient to elicit HSP70 induction in the heart,[81] in which stretch-activated ion channels, ie, L-type calcium channels, are important signal transducers.[82] These findings support the hypothesis that induction of HSP70 expression in the arterial wall occurs as a physiological response to acute hypertension, ie, hemodynamic stress or biomechanical stress.[83]

Using an in vitro mechanical stress model, we[84] have provided evidence that mechanical forces evoke rapid activation of HSP70 expression in smooth muscle cells. Elevated protein levels were preceded by HSP70 mRNA transcription, which was associated with HSF1 phosphorylation and activation stimulated by mechanical forces. Although mitogen-activated protein kinases (MAPKs), including extracellular signal–regulated kinases (ERK), c-Jun NH_2-terminal protein kinases (JNKs)/stress-activated protein kinases (SAPKs), and p38 MAPKs,[85–87] were also highly activated in response to cyclic strain stress, inhibition of ERK and p38 MAPK activation by their specific inhibitors did not influence HSF1 activation.[84] Interestingly, smooth muscle cell lines stably expressing dominant-negative rac (rac N17) abolished HSP protein production and HSF1 activation induced by mechan-

ical forces, whereas a significant reduction of HSP70 expression was seen in ras N17–transfected cell lines. Therefore, mechanical stretch–induced HSP70 expression is mediated by HSF1 activation and regulated by rac/ras GTP-binding proteins[84] (Figure 1).

Signal Transductions Leading to HSP Transcription

The heat shock response is primarily regulated at the level of transcription and is mediated by ≥1 of a family of HSFs that interact with a specific regulatory element, the heat shock element, present in the promoters of HSP genes.[88,89] At the present, 4 different HSFs have been identified, ie, HSF1, HSF2, HSF3, and HSF4.[13] HSFs are products of the transcription of 4 different genes. HSF1, HSF2, and HSF4 have been identified in human tissues. At present, HSF3 has been described only in the chicken, in which it is involved in the development of various tissues. HSFs are present constitutively in the cell in a non–DNA-binding state; they are activated in response to various stresses to a DNA-binding form. This activation process appears to involve the oligomerization of HSF from a monomeric to a trimeric state and is associated with HSF hyperphosphorylation.[88,89]

The kinases or enzymes responsible for HSF hyperphosphorylation are unknown. However, there is evidence indicating that HSF1 activity can be inhibited through the phosphorylation of HSF1 serine residues by ERKs.[90] In fact, 3 families of MAPKs (ERK, JNK/SAPK, and p38 MAPK) are activated in vascular cells stimulated by heat shock,[91] free radicals,[92] LDL and oxLDL,[93] arachidonic acid,[94] hyperlipidemia,[95,96] and mechanical stress.[97] This indicates a possible relationship between MAPK activation and HSP expression in vascular cells in atherosclerosis. Furthermore, a different stimulus seems to activate different signal pathways, leading to HSF activation. Figure 1 schematically illustrates signal pathways initiated by a variety of stresses that lead to HSP expression. For instance, the mechanical stress–stimulated integrin-rac pathway results in HSP induction in smooth muscle cells, whereas H_2O_2 activates the JAK2-STAT pathway, leading to HSP expression. Most signal transducers or pathways are not fully elucidated, and further studies will be needed to clarify the mechanism of HSP expression at a molecular level.

Surface Expression and Release of HSPs

Although it is assumed that HSP must be located in the cytoplasmic compartment to exert its function, evidence is emerging that surface expression of HSP60 in mononuclear cells may occur as well.[49,98] We[99] showed that aortic endothelial cells express HSP60 on their surface after cytokine stimulation or heat shock treatment. Fluorescence-activated cell sorter analyses revealed that >80% of living endothelial cells stressed by cytokines or 42°C were positively surface-stained with the antibody against HSP60. In that study,[99] several monoclonal antibodies against various epitopes of HSP60 were used to stain stressed endothelial cells. Surface staining of endothelial cells was obtained by antibody II-13, recognizing amino acid residues 288 to 366 of HSP60, but not by ML-30, recognizing residues 315 to 318, and LK1 and LK2, recognizing residues 383 to 447.[99] Subsequently, several groups[100–102] confirmed the surface expression of HSP60 in different types of cells. For instance, Khan et al[102] demonstrated that HSP60 and histone 2B can localize in the T-cell plasma membrane, in which HSP60 functions as a molecular chaperone for histone 2B, and that protein kinase A–catalyzed phosphorylation of HSP60 and histone 2B appears to regulate the attachment of histone 2B to HSP60. Physiologically, HSPs can maintain certain polypeptides in an unfolded state, thus facilitating their translocation across membranes. In this process, a portion of the HSP molecule might extend beyond the cellular plasma membrane to the surface. Alternatively, membrane-expressed HSPs may have physiological functions; eg, HSP17 stabilizes cell membranes antagonizing the stress-induced damages of the membrane.[103]

Interestingly, HSP90 induced in smooth muscle cells stimulated by free radicals can be released into cultured medium as identified by capillary chromatography, electrospray ionization tandem mass spectrometry, and Western blot analysis.[104] These results show that brief oxidative stress causes sustained release of HSP90 from vascular smooth muscle cells that, in turn, can stimulate ERK activation. In addition, cells from a variety of primary human tumors contained considerably higher levels of HSP than did their normal autologous tissue counterparts. Analysis of supernatants of transformed cell lines showed them to be enriched in HSP70 and gp96. On exposure to lysates or supernatants of transformed cell lines, human dendritic cells underwent maturation, which was abrogated by treatment with boiling, proteinase K, and geldanamycin, an inhibitor of HSPs, suggesting that HSPs rather than endotoxin or DNA were the responsible factors.[105] Furthermore, glial cells also release a variety of molecules that support neuronal function, of which HSP70 was shown to be exported into the culture medium whether under normal conditions or subjected to heat shock. The amount of glial HSP70 released ranged from 5 to 15 pg per 10^6 cells per day, being greater after heat shock.[106] Thus, HSPs were expressed not only within the cells but also on the surface, which can be released into the cultured medium or intercellular space in certain circumstances.

Soluble HSPs

As described above, HSPs are released into cultured medium in vitro. The question is whether HSPs exist in human blood, which may be correlated with atherosclerosis. Currently, we[107] performed a population-based study (n=826) to determine serum-soluble HSP60 (sHSP60), carotid atherosclerosis, and risk factors. We demonstrated that sHSP60 levels were significantly elevated in subjects with prevalent/incident carotid atherosclerosis and were correlated with intima-media thickness independent of age, sex, and other risk factors. Interestingly, sHSP60 was also correlated with anti-LPS, anti-*Chlamydia*, and anti-HSP60 antibodies, inflammation markers, and chronic infections. The concentration of sHSP60 in some patients is >1 μg/mL, which is high enough to produce extracellular-based signaling (see below). Affinity chromatography and Western blot analysis confirmed the molecular weight of sHSP60, ie, 60 kDa (Figure 2). Because the antibodies used for sHSP60 measurement are reactive to

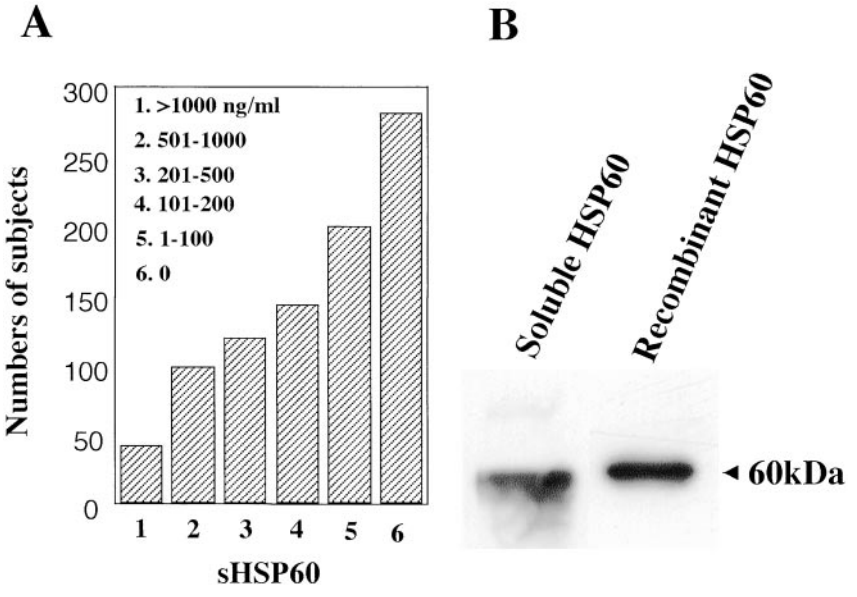

Figure 2. A, Concentrations of sHSP60 in blood. sHSP60 was measured by sandwich ELISA, as described.[107] B, Chromatography and Western blot analysis for soluble HSP. Monoclonal antibody II-13 was coupled to supports by using an immunoglobulin-coupling kit. sHSP60 was isolated from serum with higher sHSP60 levels. Isolated proteins were visualized by Western blot.[107]

human and bacterial HSP60, it is unknown whether these 60-kDa bands are originated from human and/or bacteria. Nevertheless, these data provide the first evidence of a strong correlation between sHSP60 and atherosclerosis, suggesting a role of sHSP60 in the development of atherosclerosis.

Concomitantly, Pockley et al[108] measured sHSP60 and sHSP70 in subjects with borderline hypertension. A major novel observation in their report was findings that sHSP60 was present at a significantly enhanced level in patients with borderline hypertension, which was associated with intima-media thickness and early atherosclerosis. These data provide additional support for the role of sHSPs in the induction/progression of hypertension and atherosclerosis.

Where and how sHSPs are released into the blood is currently unknown. Given that all types of tissues highly express HSPs in response to stress, there are several possibilities. First, infectious agents may be the major factor contributing to sHSP60 release from the organisms and from human cells. For example, chlamydiae, during the life cycle, undergo both phases of nonlytic infection, in which they remain viable but do not replicate, and phases of lytic infection.[109] During the lytic phases, host cells release their own HSP60, produced during a chronic phase of infection, and also chlamydial HSP60, which has been produced by bacteria. Support for this theory is that sHSP60 levels are significantly correlated with anti-chlamydial antibodies,[107] and that chlamydial and human HSP60s exist at high levels in human atherosclerotic lesions.[45] Second, sHSP60 could be released from the dying cells of tissues during chronic inflammation and from atheroma, as earlier studies have shown the occurrence of cell death within atheroma.[110,111] Recent data have demonstrated that open-heart surgery results in the release of sHSP70 into the blood of patients,[112] which may be due to cell damage and inflammatory responses. Finally, surface-expressed HSPs in the cell undergoing apoptosis may be released into blood via the formation of microparticles, which have been identified in the circulating blood of patients with acute coronary syndromes and in nonischemic patients.[113] These microparticles generated in vitro from activated platelets or leukocytes stimulate cultured endothelial cells to produce prostacyclin and cytokines and to express adhesion molecules.[113–116] The microparticles circulating in the peripheral blood of patients with acute myocardial infarction affect endothelium-dependent responses in normal blood vessels.[114] sHSPs may be present in the microparticles and serve as active components exerting their role in these processes. Therefore, HSP60 release into the circulation could be the result of different pathways.

sHSPs Having Proinflammatory Activities

Kol et al[45] provided the first evidence that chlamydial HSP60 and human HSP60 can act as extracellular agonists and induce tumor necrosis factor-α (TNF-α) and matrix metalloproteinase-9 production by macrophages. Subsequently, they demonstrated that chlamydial or human HSP60 activates human endothelial cells, smooth muscle cells, and monocyte-derived macrophages.[117] Chlamydial and human HSP60 induce E-selectin, intercellular adhesion molecule-1, and vascular adhesion molecule-1 expression on endothelial cells similar to levels induced by LPS. Each HSP60 also significantly induces interleukin (IL)-6 production by endothelial cells, smooth muscle cells, and macrophages. Heat treatment abolishes all these effects but does not alter the ability of *E coli* LPS to induce these functions. Therefore, chlamydial and human HSP60s activate human vascular cell functions relevant to atherogenesis and lesional complications. Similarly, Chen et al[118] have demonstrated that autologous HSP60 serves as a danger signal to the innate immune system, which results in proinflammatory responses, including the production of TNF-α, IL-12, and IL-15. Asea et al[119] have demonstrated that exogenous HSP70 also acts as a cytokine to human monocytes by stimulating a proinflammatory signal transduction cascade that results in an upregulation of IL-1, IL-6, and TNF-α expression. Furthermore, it has been shown that HSP60 mediates monocyte adhesion to endothelial cells in vivo and in vitro via CD14. Poston et al[120]

found that monocytes efficiently adhere to sHSP60-coated plates; this adherence was specifically inhibited by CD14 antibodies, suggesting that sHSP60 itself behaves as an adhesion molecule through its affinity to CD14. Hence, autologous sHSPs may alert innate immunity[121] via the same recognition as for microbial pathogens, resulting in proinflammatory responses in the vessel wall.

HSP-Initiated Signal Transductions

The finding that sHSPs have a cytokine-like activity evoking proinflammatory responses in vascular cells leads to searching "specific" receptors of sHSPs and related signal pathways in a variety of cells. Surprisingly, several groups reported at approximately the same time that the Toll-like receptor (TLR)4/CD14 complex is a soluble HSP receptor.[122–127] So far, >10 human TLRs have been identified. A variety of bacterial and fungal components are known TLR ligands, including peptidoglycan for TLR2, LPS for TLR4, flagellin for TLR5, and unmethylated CpG motifs in bacterial DNA for TLR9.[128] It is possible that TLRs may collectively be responsible for detecting a large repertoire of microbial pathogens. TLRs are evolutionarily conserved innate immune receptors that are shared by IL-1 receptor signaling to activate the nuclear factor (NF)-κB pathway and release inflammatory cytokines.[128] Xu et al[129] investigated the expression of these receptors in murine aortic and human coronary atherosclerotic plaques and demonstrated preferential expression of TLR4 in lipid-rich and macrophage-infiltrated murine and human atherosclerotic plaques. Other studies in vitro demonstrated basal expression of TLR4 by macrophages, which was upregulated by oxLDL. Concurrently, Edfeldt et al,[130] using a semiquantitative polymerase chain reaction and immunohistochemical analysis, demonstrated that of 9 TLRs, the expression of TLR1, TLR2, and TLR4 was markedly enhanced in human atherosclerotic plaques. A considerable proportion of TLR-expressing cells was also activated, as shown by the nuclear translocation of NF-κB. Very recently, Kiechl et al[131] demonstrated that a polymorphism or mutation of TLR4 was strongly correlated with the incidence and development of atherosclerosis in a large population study (Bruneck Study). Thus, TLRs could be involved in the pathogenesis of atherosclerosis.

As mentioned above, several groups reported that soluble HSPs specifically bind to the TLR4/CD14 complex, initiating different signal pathways in different types of cells.[122–127] In human mononuclear cells, human sHSP60 binds to TLR4/CD14, leading to p38 MAPK activation,[122] whereas in smooth muscle and epithelial cells, chlamydial and human sHSP60 stimulates ERK42/44 activation.[132,133] ERK42/44 is a central component of signaling via growth factors. Sequential activation of Ras and Raf activates MAPK kinase. MAPK kinase then activates ERK by dual phosphorylation of key threonine and tyrosine residues, and MAPK, in turn, phosphorylates serine and threonine residues on several transcription factors, including c-Myc, activator protein-1, NF–IL-6, activating transcription factor-2, and Elk-1, leading ultimately to cell growth and differentiation.[134] This indicates that chlamydial and human HSP60 are potent inducers of human smooth muscle cell proliferation and that these effects

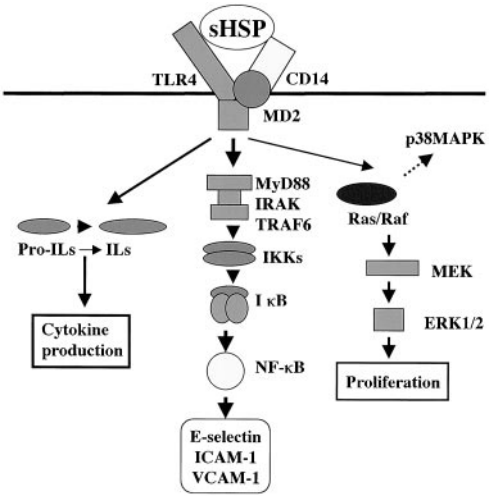

Figure 3. sHSP-initiated signal transduction pathways.

are mediated, at least in part, by rapid TLR4-initiated activation of ERKs.[133] However, it should be pointed out that data from most of the studies mentioned above are derived from usage of recombinant HSPs produced from *E coli*, indicating a possibility that HSPs might be contaminated with LPS.

In macrophages and endothelial cells, the binding of sHSP60 to the TLR4/CD14 complex leads to the activation of MyD88–NF-κB pathways. In 293 cells, chlamydial sHSP60–mediated NF-κB activation required TLR4 and MD2. A dominant-negative MyD88 construct also inhibited sHSP60-induced NF-κB activation, indicating a MyD88-dependent signaling pathway.[123] In addition, it has been demonstrated that soluble HSP70 and mycobacterial soluble HSP65 have a binding activity similar to that of TLR4/CD14, which initiates the MyD88–NF-κB signaling pathways.[126] These findings suggest that TLR4/CD14 is a receptor for several soluble HSPs and have partially clarified the signal pathways after soluble HSP–TLR4/CD14 binding (Figure 3).

Antibodies Against HSP60

In the early 1990s, we[135] first reported an association of anti-HSP65 antibodies with atherosclerosis. Serum antibodies against mycobacterial HSP65 were significantly increased in subjects aged 40 to 79 years with carotid atherosclerosis compared with those without lesions, and increased antibody concentration was independent of age, sex, and other established risk factors. A follow-up study confirmed that HSP65 antibody titers in the population emerged as highly consistent over a 5-year observation period.[136] Titers were significantly elevated in subjects with progressive carotid atherosclerosis and were correlated with intima/media thickness. Again, multiple linear regression analysis documented these associations to be independent of age, sex, and other risk factors. Finally, HSP65 antibody titers significantly predicted the 5-year mortality.[136] Subsequently, Mayr et al[43] demonstrated that anti-mycobacterial HSP65 antibody titers were positively correlated with human IgA to *C pneumoniae* and with IgG to *H pylori*, indicating a role for infections in the production of anti-HSP antibodies.

TABLE 2. Summary of Epidemiological Studies on HSP Antibodies

First Author and Reference	Cases/Controls	Disease	HSPs	Odds Ratio and *P**
Xu et al[135]	867	Carotid AS	HSP65	1.52 (1.09–2.02)
Gruber et al[140]	107/90	Vascular diseases	HSP65	$P<0.001$
Hoppichler et al[141]	203/76	CHD, MI	HSP65	$P<0.05$
Mukherjee et al[143]	28/12	CHD, PTCA	HSP65	$P=0.036$
Frostegard et al[142]	66/67	Hypertension	HSP65	$P=0.034$
Birnie et al[144]	136	CHD	HSP65	$P=0.012$
Prohaszka et al[147]	74	CHD	HSP60	$P<0.0001$
Xu et al[136]	750	Carotid AS	HSP65	1.42 (1.02–1.98)
Chan et al[145]	61/21	Peripheral AS	HSP70	$P=0.0037$
Zhu et al[138]	274/91	CAD	HSP60	1.86 (1.13–3.04)
Burian et al[137]	276/129	CAD	HSP60	2.6 (1.3–5.0)
Gromadzka et al[146]	180/64	C ischemia	HSP65/70	$P<0.0001$
Prohaszka et al[149]	424/321	CHD	HSP60	$P<0.007$
Ciervo et al[148]	179/100	CHD	HSP60	$P<0.05$
Huittinen et al[139]	239/239	CHD	HSP60	2.0 (1.1–3.6)

*P values are shown when odds ratios were not provided in the articles. AS indicates atherosclerosis; CHD, coronary heart disease; CAD, coronary artery disease; MI, myocardial infarction.

Regarding anti-human HSP60 antibodies, 2 independent groups demonstrated that >70% of the study subjects had anti-human HSP60 antibodies.[137,138] The prevalence of coronary artery disease was significantly increased in seropositive compared with seronegative patients. Importantly, HSP60 antibodies were related to disease severity, which persisted after adjustment for traditional risk factors, ie, age, race, sex, smoking, diabetes, hypercholesterolemia, hypertension, and C-reactive protein levels.[137,138] Moreover, Huittinen et al[139] reported that human HSP60 IgA or chlamydial HSP60 antibodies were a significant risk factor for coronary events. When an elevated human HSP60 IgA antibody level was present simultaneously with a high *C pneumoniae* IgA antibody level and an elevated C-reactive protein level, the relative risk was 7.0. In addition, many other groups[140–149] confirmed the elevated levels of HSP antibodies in coronary heart disease, myocardial infarction, stroke, hypertension, and restenosis after angioplasty (Table 2). Therefore, an elevated human HSP60 antibody level may be a risk factor for atherosclerosis and could be a marker of the disease, especially when it is present with *C pneumoniae* infection and inflammation.

Circulating antibodies to HSPs may be induced or maintained by several different mechanisms. First, infection with agents that contain homologous HSP60 proteins could induce an anti–self-response through molecular mimicry in susceptible individuals.[150] Second, the protein could become immunogenic because of structural alteration or posttranslational modification resulting from oxidation or metabolic alterations.[151] Third, other foreign or self-antigens could interact with HSP60 to form immunogenic complexes in which B cells recognize HSP60 and T cells direct their response at the associated antigen.[26] Fourth, soluble HSP might be not recognized as a self-protein by a population of T and B lymphocytes, inasmuch as HSPs being leaked are intracellularly localized in physiological conditions.[152] Finally, genetic variation may also be important for antibody production. Supporting this issue are findings that a strong association between the IL-6 promoter −174 polymorphism and anti-HSP60 antibody level was seen. Carriers of allele C at this position had significantly lower levels of anti-HSP60 and anti-HSP65 antibodies. A lack of association between IL-1β and IL-1α gene polymorphisms and antibody levels has been detected.[153] Therefore, circulating anti-HSP antibody titers could be maintained at higher levels via different mechanisms.

T Cells Specifically Responding to HSP60/65

It has been established that atherosclerotic lesions of humans and rabbits contain a large number of T lymphocytes.[4] About half of these T cells express major histocompatibility complex class II antigens, and some also express IL-2 receptors, indicating a state of activation. Studies have shown that T lymphocytes in human atherosclerotic plaques are mostly Th1 cells expressing the low-molecular-weight form (CD45RO) of the leukocyte common antigen and very late activation antigen-1, an integrin.[4] These T cells are polyclonal in origin and are based on the phenotype of T cell receptors, ie, α/β, and γ/δ.[32,154,155] Most T lymphocytes involved in atherosclerosis bear the α/β T-cell receptor. However, in the earliest stage of atherogenesis, there is an average of 9.7% γ/δ T cells. T-cell receptor $\gamma/\delta+$ cells have been proposed to constitute a first line of defense, and recent results have also indicated a possible participation of T cells in the early stages of atherosclerosis.[156] The presence of T cells in atherosclerotic lesions could be important, because these cells can act as effector cells and secrete factors chemotactic for mast cells, monocytes/macrophages, and smooth muscle cells, and they can also determine the differentiation and function of B cells and monocytes/macrophages.

What are the antigens recognized by these T cells in atherosclerotic lesions? They are possibly HSPs, a notion that

is supported by the finding of T cells cultivated from atherosclerotic lesions by Xu et al.[33] These researchers showed that a population of the T lymphocytes isolated from the atherosclerotic lesions of rabbits specifically responded to HSP65 in vitro. IL-2–expanded T-cell lines derived from atherosclerotic lesions showed a significantly higher HSP65 reactivity than those developed from peripheral blood of the same donor. In human atherosclerotic lesions, Mosorin et al[157] showed that a proportion of T cells responded to chlamydial sHSP60. They found that sHSP60 induced specific proliferation in 71% of the cases and revealed 2 haplotype (DRB1*1502 and DQB1*06) binding motifs in human sHSP60. Another group has obtained a similar result with T cells responding to HSPs in atherosclerotic lesions.[158] They demonstrated that some chlamydia-specific T-cell lines derived from human atherosclerotic plaques responded to HSP60. Those recognizing chlamydial HSP60 did not cross-react with human HSP60, but human HSP60-responsive lines from atherosclerotic lesions were also observed. Taken together, these findings support the presence of antigen-specific T lymphocytes to HSPs in atherosclerotic lesions. An excellent review regarding how these T cells are recruited, activated, and subsequently exert their role in atherogenesis has recently been published in *Arteriosclerosis, Thrombosis, and Vascular Biology*.[4]

Immune Reaction to HSP60 in Atherogenesis

Because of the high sequence homology between chlamydial, other bacterial, and human HSPs (75% at the amino acid level),[25] it is naturally possible that cross-reactions of antibodies and T cells against HSPs between microbes and humans contribute to the development of atherosclerosis. Wick et al[159] first hypothesized that autoimmune reactions to HSPs could be crucial in initiating atherosclerosis. Recently, Wick et al,[160] stating that atherosclerosis is an autoimmune disease, have updated this hypothesis. Although existing data do not allow us to establish that atherosclerosis is an autoimmune disease, autoimmune reactions to HSPs may contribute, at least in part, to atherogenesis. Because their article concerning this issue has been thoroughly reviewed,[160] the present review will briefly summarize the progress in this field.

As demonstrated, human serum anti-mycobacterial HSP65 antibodies react with a recombinant form of human HSP60 and homogenates of atherosclerotic lesions.[161] Human anti-HSP65 antibodies react with human HSP60 present in endothelial cells, macrophages, and smooth muscle cells of atherosclerosis.[161] Schett et al[162] have purified human anti-HSP65 antibodies and have shown that they are cytotoxic to endothelial cells. By Western blotting analysis, they have demonstrated that such antibodies from patients with atherosclerosis react specifically with recombinant mycobacterial HSP65, recombinant human HSP60, chlamydial HSP60, and *E coli* GroEL/HSP60.[163] Heat-stressed endothelial cells could be lysed by these antibodies in the presence of complement via complement-mediated cytotoxicity or in the presence of peripheral blood mononuclear cells via antibody-dependent cellular cytotoxicity.[163] In addition, a population of T cells in atherosclerotic lesions may also play a similar role as auto-

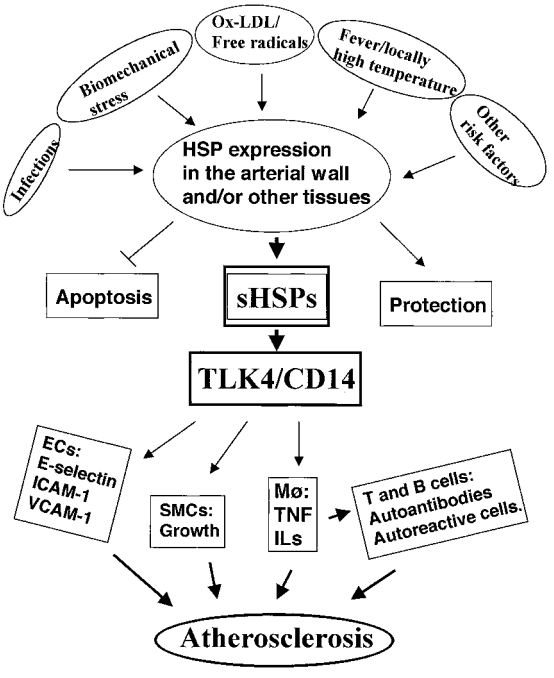

Figure 4. Schematic representation of potential role of HSP60 in atherogenesis. Various stressors, such as infections, biomechanical stress, oxLDL, free radicals, toxins, heat shock, and other stress, induce HSP production in the arterial wall, which is released into blood to form soluble HSPs (sHSPs). sHSPs bind to TLR4/CD14 complex resulting in proinflammatory responses and autoimmune reactions, which contribute to atherosclerosis. ECs are endothelial cells; TLK, Toll-like receptor; SMCs, smooth muscle cells; and Mø, macrophages.

antibodies, suggesting that cell-mediated immune responses to HSP60 are involved in the pathogenesis of this disease. In further support of autoimmunity are findings that rabbits and mice develop atherosclerosis after immunization with HSPs.[164,165] Therefore, serum autoantibodies and T cells react not only with bacterial HSP65 but also with human HSP60 in vascular cells.

Hypothesis and Perspectives

Figure 4 depicts the current model for the role of HSPs in atherogenesis. All risk factors, eg, infections, biomechanical stress, oxLDL, and free radicals, directly stimulate cells of the arterial wall and/or other tissues to express high levels of HSPs. The physiological functions of these HSPs are to protect cells against apoptosis. Pathologically (ie, from stimulation by the risk factors), the cells are dying; this releases intracellular HSPs into intercellular spaces to form sHSPs. sHSPs bind to TLR4/CD14 receptors, resulting in endothelial cells expressing adhesion molecules, in smooth muscle cells leading to proliferation, and in macrophages inducing a range of proinflammatory cytokines. Simultaneously, macrophages present antigens to T and B cells, which produce autoantibodies and autoreactive cells against HSPs. All contribute to the development of atherosclerosis.

The past several years have seen a dramatic increase in the number of studies involving the role of HSPs in the pathogenesis of atherosclerosis. On the basis of these progresses, I have summarized the data and formulated an HSP hypothesis

as shown in Figure 4. However, many outstanding questions have to be answered before the topic can be brought to completion. For instance, what are the molecular mechanisms of risk factor–induced HSP expression (eg, signal transduction pathways between stimuli and HSP induction)? How are intracellular HSPs released? How many signaling pathways in different types of cells are involved after sHSP-TLR4/CD14 binding? To which degree do autoimmune reactions to HSPs contribute to atherosclerosis in humans? I believe that further research into the issues mentioned above will provide a better understanding of the molecular mechanisms in atherosclerosis and result in important clinical applications.

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