Heat Shock Protein 60 and Immune Inflammatory Responses in Atherosclerosis

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Abstract

Hallmarks of inflammation in various cardiovascular diseases, notably atherosclerosis, have been observed for a long time. However, evidence for an (auto)antigen-driven process at these sites of inflammation has come forward only recently. Heat shock proteins (HSPs) have been identified as playing either immunologically mediated disease promoting or protective roles. HSP60 has been shown to trigger innate and adaptive immune responses that initiate the earliest still reversible inflammatory stage of atherosclerosis. HSP60 is structurally highly conserved and abundantly expressed by prokaryotic and eukaryotic cells under stressful conditions. Beneficial protective immunity to microbial HSP60 acquired by infection or vaccination and bona fide autoimmunity to biochemically altered autologous HSP60 is present in all humans. In vitro and in vivo experiments have demonstrated that classical atherosclerosis risk factors can act as endothelial stressors that provoke the simultaneous expression of adhesion molecules and of HSP60 in mitochondria, in cytoplasm, and on the cell surface, where it acts as a "danger signal" for cellular and humoral immune reactions. Hence, protective, preexisting anti-HSP60 immunity may have to be "paid for" by harmful (auto)immune cross-reactive attack on arterial endothelial cells maltreated by atherosclerosis risk factors. These experimentally and clinically proven findings are the basis for the autoimmune concept of atherosclerosis.

Keywords

Typical cellular hallmarks of chronic inflammation and infections, notably infiltration by mononuclear cells, are also present in the cardiovascular system, as has been known for more than 150 years. However, until quite recently, it was not clear whether these inflammatory immunologic processes are primary or secondary in nature.[1] One of the reasons for this uncertainty may have been the fact that most investigations in humans were conducted on surgical or autopsy specimens representing very advanced stages of cardiovascular disease (CVD) and thus did not provide information on the initial mechanisms triggering these processes. In complex situations, such as in atherosclerosis, it was also difficult to appreciate that different, clinically well-proven risk factors may

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provoke a similar, or even identical, pathophysiological outcome. The main thrust to resolve this dilemma was to delineate the array of nonspecific and specific humoral and cellular inflammatory reactions taking place within the afflicted vascular territories. The availability of animal models that, at least partly, mimic human CVD was of utmost importance for this progress. In recent decades, the aim has been to identify exogenous or autologous antigens that may induce the local cardiovascular immune reactions. Among the candidates for such antigens are infectious agents, such as *Chlamydia pneumoniae,* as well as autoantigens, such as biochemically altered ones (eg, oxidized low-density lipoprotein [oxLDL], phospholipids, and heat shock proteins [HSPs]). In this review, we will focus on the role of HSPs in atherosclerosis.

HSPs

Under physiological conditions, HSPs fulfill important intracellular tasks with regard to protein folding and transport. Under stress, HSPs may act as chaperones preventing protein denaturation and loss of function. As the name implies, the expression of HSPs was first demonstrated as a response to increased temperature.[2] Recently, a first attempt for a consistent and clear nomenclature for the HSPs and related chaperone genes in the human database has been achieved.[3] HSPs are classified according to their molecular mass into families ranging from less than 5 kDa to more than 100 kDa.[4] HSPs are structurally highly conserved from prokaryotic to eukaryotic cells. HSP60 of different bacterial species display greater than 95% sequence homology at both DNA and protein levels. An overall 55% homology exists between human and bacterial HSP60 that can even reach 72% at certain domains of the 573-amino-acid-long molecule. All animals and humans show protective innate and adaptive immunity against HSP60. Because of the antigenic similarity between prokaryotic and eukaryotic HSPs, the body is confronted with the dilemma that HSP60 should be recognized and reacted against to fight infection, but autologous HSP60 should still be tolerated to avoid autoimmunity.[5]

Under physiological conditions, animals and humans seem to be tolerant against autologous HSP60. How this state of tolerance is activated and maintained and under what circumstances it may be broken (eg, by an accumulating life-long infectious load or by immunization together with adjuvants) is still a matter of debate. With respect to central tolerance, it has been shown that HSPs are among the molecules that are promiscuously expressed by thymic epithelial cells.[6] In addition, extracellular sampling by circulating dendritic cells migrating into the thymus can be considered.[7] Also, postthymic peripheral failsafe mechanisms must be operative to prevent the onset of overt autoimmunity against HSP60.[8]

Among the CVDs, the role of HSP60 has mainly been studied for atherosclerosis. HSP60 is a mitochondrially expressed stress protein that can be translocated to the cytosol and, later, transported to the cell surface and shed to the environment.[9] Hence, the plasma concentration of soluble HSP60 (sHSP60) shows a wide range related to genetic, biological, and psychological factors in CVD, and patients with borderline hypertension[10] and patients with early CVD show elevated levels of sHSP60.[11] Translocation of HSP60 to the cell surface is a significant stress response that correlates with apoptosis and exacerbation of the disease state.[12,13] HSP60 can activate both the innate immune system (via toll-like receptor 4) and the adaptive immune system.[14]

HSP in Atherosclerosis

Early Human Atherosclerotic Lesions

The predominantly inflammatory nature of initial atherosclerotic lesions is supported by both clinical and experimental facts that are, however, still often neglected. Although hypercholesterolemia is a proven atherosclerosis risk factor, more than 60% of patients are normocholesterolemic. Furthermore, activated T cells (mainly CD4^{+}) are the first invaders of the arterial intima in *early* human atherosclerotic lesions,[15-17] only later followed by macrophages and smooth muscle cells (SMCs), the latter 2 often transformed into foam cells prevailing in late, complicated plaques and in early xanthoma. When healthy babies, children, and young adults (aged 8 months to 16 years) were studied, mononuclear cell infiltration at predilection sites again started with T cells, followed by macrophages, SMCs, and a few scattered mast cells. In a cohort of very young children (8 months to 8 years), macrophages did not show the characteristics of foam cells, and extracellular lipid deposits were not yet detectable in the intima.[18] In another study on young subjects (15 to 34 years old) collected from the American Pathobiological Determinants of Atherosclerosis in Youth study,[19] the same phenotypic observations summarized above could again be confirmed with the additional feature of an intricate network of dendritic cells present in the arterial intima providing the prerequisites for a local immune reaction.[16]

The Autoimmune Concept of Atherosclerosis

Experimental and clinical facts summarized below have provided the basis for the development of the autoimmune concept of atherosclerosis[20] that is schematically depicted in Figures 1 and 2.

All humans develop protective, beneficial adaptive immunity against the phylogenetically highly conserved microbial HSP60 antigen via infection or vaccination in addition to the immunity against organism-specific epitopes. Under physiological conditions, vascular endothelial cells (ECs) do not express HSP60. However, when stressed by classical atherosclerosis risk factors, the simultaneous expression of adhesion molecules and HSP60 by ECs leads to a (cross)reaction against and destruction of these target cells by preexisting cellular and humoral immunity against HSP60, entailing intimal infiltration by mononuclear cells. If atherosclerosis risk factors persist, these early, still reversible inflammatory stage of atherosclerosis proceeds to plaque formation with deleterious consequences. Supplemental Table I (available online at http://atvb.ahajournals.org) lists the most important contributions from various laboratories on the association of CVD with anti-HSP antibodies, sHSPs, and HSP-specific T cells that appeared since our last extensive review on this topic in 2004.[20]

Self-HSP-Reactive T Cells and B Cells

Because upregulation of HSP60 is part of an inflammatory response, self-HSP60-reactive T cells with a regulatory phenotype should be part of the physiological termination of the inflammatory response. T cells reacting to self-HSP epitopes have been found in both human and animal studies. An important prerequisite for stimulation of HSP-reactive T cells is the presentation of the corresponding peptides by major histocompatibility complex (MHC) molecules. Self-HSP peptides have access to both MHC class I and class II molecules.

As already mentioned, we have shown that CD4^{+} T cells are the first T-cell population in *early* human lesions. Many of the CD4^{+} cells are HLA-DR and CD25 positive, and a majority of intima infiltrating T cells carry the T-cell receptor α/β, but an unexpectedly high proportion of these T cells are T-cell receptor γ/δ positive. Both SMCs and ECs are HLA-DR positive when activated, interferon-γ-producing T cells are present in the vicinity,[15-17]

suggesting that SMCs and ECs may participate as antigen presenting cells, in the perpetuation of the autoimmune reaction. Interestingly, the T-cell reaction against human HSP60 (hHSP60) is significantly increased in intralesional T cells (mainly displaying an oligoclonally restricted T-cell receptor α/β repertoire) compared with peripheral T cells (polyclonal repertoire) of the same individual.[21] Moreover, carotid plaques of atherosclerosis patients harbor in vivo–activated $CD4^+$ T cells that react specifically to self-HSP60 peptides.[22] In addition to cross-reactive T cells, cross-reactive B cells epitopes have also been shown to serve as autoimmune targets in incipient atherosclerosis.[23]

MHC class II antigen expression on ECs overlying atherosclerotic lesions has been found in low-density lipoprotein-receptor (LDLr)/interferon-γ double knockout mice.[24] In mice immunized with heat-killed *Mycobacterium tuberculosis*, around 20% of all mycobacterium-reactive $CD4^+$ α/β T cells were specific for HSP60,[25] and immunization with mycobacterial HSP60 (mHSP65 as a paradigmatic example of bacterial HSP60) induced cross-reactive $CD8^+$ α/β T cells.[26] Both α/β- and γ/δ-positive HSP60-reactive T cells may play a role in the pathogenesis of human autoimmune diseases (ie, rheumatoid arthritis, multiple sclerosis, Behcet disease, and atherosclerosis).[27] For example, $CD8^+$ α/β T cells recognize HSP60 peptides on MHC class I in vitro, and after transfer to α/β T-cell-deficient mice, these T cells can induce autoimmune disease with severe damage of the gut epithelium.[27] After stimulation with mHSP65 peptides in vitro, HSP60-specific $CD8^+$ T cells recognize and lyse host cells exposed to external stress factors. However, recognition and lysis are prevented when HSP60 expression is blocked in target cells with antisense nucleotides.[28]

Much less is known about the pathogenic role of B cells and anti-HSP60 antibodies in atherogenesis than about the role of T cells. Immunohistological analyses of early and more advanced human atherosclerotic lesions revealed only low numbers of B cells compared with T cells. Furthermore, B cells can mostly only be found in the media and in the adventitia of atherosclerotic plaques but not within early lesion.[16,17,29] However, studies on atherosclerotic aneurysms have demonstrated the presence of plasma cells and occasional lymphoid follicles.[30] Also, aged apolipoprotein E–null (ApoE$^{-/-}$) mice showed adventitial formation of inflammatory follicle-like structures that contain proliferating B cells and plasma cells,[31] indicating that the adventitia may be a site of local adaptive immune reactions during atherogenesis. Nevertheless, recent studies of the effect of B-cell depletion on the development of atherosclerosis have provided contradictory results. In most instances, B-cell depletion entailed an aggravation of the disease,[32-34] whereas several other reports described an amelioration of atherosclerosis.[33,35] This discrepancy may be explained by an increased formation of atheroprotective (auto)antibodies (eg, anti-oxLDL)[32] in the former or of atherogenic (auto)antibodies (eg, anti-HSP60) in the latter case.

hHSP60 can induce naíve mouse B cells to proliferate and secrete interleukin-10 and interleukin-6. hHSP60-treated B cells can upregulate the expression of MHC class II and accessory molecules CD69, CD40, and B7-2. In addition, sHSP60 can activate B cells via toll-like receptor 4–MyD88 signaling.[36] Furthermore, hHSP60 can inhibit mouse B-cell apoptosis, spontaneous or induced, via MyD88 signaling (toll-like receptor 4 is not required in this instance). Inhibition of apoptosis by hHSP60 is associated with upregulation of the antiapoptotic molecules Bcl-2, Bcl-x_L, and survivin. Importantly, B cells incubated with hHSP60 manifested prolonged survival following transfer into recipient mice.[37] In conclusion, these findings show that both T and B cells can regulate HSP60 immune responses and thus induce autoreactive T and B cells recognizing self-HSP60 peptides, but with respect to primary atherogenic mechanisms, the balance seems to be tilted toward T cells.

Experimental Observations

Up to now, a pathogenic role in atherogenesis is proven only for HSP60, and the induction of atherosclerosis in experimental animals has so far been achieved only by immunization with prokaryotic and eukaryotic HSP60. In the present context, mHSP65 is always used as a paradigmatic and potent representative of bacterial HSP60. An induction of atherosclerosis was successfully demonstrated in normocholesterolemic rabbits after immunizations with mHSP65.[38] T-cell depletion in mHSP65 immunized rabbits leads to a significant decrease of atherosclerotic lesions.[39] This is also the case for nonimmunized hypercholesterolemic atherosclerosis-prone (LDLr^{−/−}) mice crossed with lymphocyte-deficient (RAG-1^{−/−}) mice. In the progeny of these double knockout mice, early lesion development was reduced by 54%, indicating that lymphocytes play an important role in early atherosclerosis.[40] Immunization of rabbits with mHSP65 had an even more pronounced effect when these were simultaneously fed a cholesterol-rich (Western) diet.[41] Although the early inflammatory stage of atherosclerosis in normocholesterolemic mHSP65-immunized rabbits was still reversible during a 32-week observation period, the lesions persisted in a similarly immunized hypercholesterolemic group.[41] This was later corroborated in atherosclerosis-resistant wild-type C57BL/6J mice fed a Western diet combined with mHSP65 immunization and in LDLr^{−/−} mice fed normal chow diet together with mHSP65 immunizations (Figure 3).[42,43]

Lesion-derived T cells from normocholesterolemic mHSP65-immunized rabbits show a significantly increased reactivity against mHSP65 as compared with autologous peripheral blood T cells.[44] Passive transfer of T cells from mHSP65 immunized atherosclerotic LDLr^{−/−} mice into non-immunized synergeneic LDLr^{−/−} recipients leads to the development of disease in the latter.[45] Interestingly, passive transfer of a mouse monoclonal antibody (II-13) recognizing an epitope consisting of amino acid residues 288 to 366 of HSP60, or of affinity polyclonal anti-hHSP60 antibodies purified from the serum of atherosclerotic patients can also induce atherosclerosis in ApoE^{−/−} recipient mice.[46] Importantly, immunization of hypercholesterolemic ApoE^{−/−} mice with homologous malondialdehyde–low-density lipoprotein had the opposite effect, ie, a disease ameliorating effect.[47]

Exerting endothelial stress in rats or rabbits, eg, by intravenous injection of bacterial lipopolysaccharide, leads to the simultaneous expression of adhesion molecules and HSP60 at known predilection sites for the later development of atherosclerotic lesions in ECs at arterial branching points subjected to turbulent blood flow conditions.[48] This process can be visualized in vitro and in vivo by molecular imaging techniques (Supplemental Figure I).[49]

In Vitro Observations

HSP60 is encoded in the nucleus but is expressed in mitochondria. Under stressful conditions, HSP60 is transported into the cytosol and then appears on the cell surface, where it acts as a "danger signal" for innate and adaptive immunity. The latter has been proven with immunofluorescence,[50] atomic force microscopy,[9] and metabolic labeling.[51] In our hands, all classical atherosclerosis risk factors studied so far first lead to the simultaneous endothelial expression of adhesion molecules and HSP60, allowing for an interaction of HSP60-specific T cells and (auto)antibodies with the target cells, in this case the stressed ECs. Interestingly, the threshold for adhesion molecule and HSP60 expression on confrontation with such stress factors is lower in arterial as compared with venous ECs because the former have been subjected to lifelong "prestress" by the higher arterial blood pressure. This has been demonstrated in an arterio-venous carotid bypass model. These venous conduits subjected to the new arterial blood flow and pressure conditions developed neointimal lesions within 1 to 2 weeks after surgery, finally leading to complete restenosis.[52] The first event in these pathological processes is the ECs expression of HSP60

and adhesion molecules and T cells infiltrating the intima.[20] Stressed ECs, but not unstressed ones, are lysed by anti-HSP60 monoclonal or affinity purified polyclonal human anti-HSP60 antibodies in a complement-mediated fashion or via antibody-dependent cellular cytotoxicity.[53] Moreover, HSP60 may also function as an inducer of anti-EC antibodies able to trigger cytotoxic and apoptotic responses when recognized by the related autoantibodies. Depending on the HSP60 epitope specificity, it appears that anti-EC antibodies with HSP60 reactivity may differ in their functional effects.[54]

EC Stressors

The role of classical atherosclerosis risk factors has been proven in abundant numbers of clinical, experimental, and in vitro studies. Because the autoimmune concept of atherosclerosis in essence relies on 2 assumptions—(1) preexistent immunity to HSP60 and (2) pathological stress-induced transformation of ECs to HSP60-expressing targets for this immunity—it was deemed of interest to scrutinize the role of different risk factors with respect to the latter phenomenon. This stressor effect was first observed for high (blood) pressure in vivo and in vitro in rats,[55] as well as for lipopolysaccharide in vivo in rabbits[48] and in vitro on human ECs.[56] These latter data are also in agreement with the correlation of the lifelong infectious load with anti-HSP60 immunity and the appearance of atherosclerosis.[20]

Recently, we have obtained in vitro evidence that oxLDL, ie, an inducer of foam cell formation, and advanced glycation end products as a surrogate for diabetic metabolic conditions, also act as endothelial stressors. oxLDL showed a more pronounced HSP60-inducing capacity compared with advanced glycation end products (Figure 4). For a considerable period of time, cigarette smoke extract appeared to be our most potent endothelial stressor. Using cigarette smoke extract in vitro and calibrating its concentration via the nicotine content mimicking blood levels in mild, intermediate, or heavy smokers, we were able to achieve abundant expression of HSP60, adhesion molecules, proinflammatory cytokines, and at higher concentrations autophagy and necrosis.[57] As a final step in this series of experiments on the mode of action of endothelial stressors, we have just recently finished a study on the potential stressor effect of an infection with *C. pneumoniae* and found this to be the most potent EC stressor that we have observed so far, even exceeding the effect of cigarette smoke extract. *C. pneumoniae* also induces excessive expression of adhesion molecules and proinflammatory cytokines. Furthermore, ongoing experiments in our laboratory have also revealed a downregulation of the expression of antioxidant molecules and, most importantly, of autophagy-associated molecules, notably ATG 2, ATG 5, and ATG 12. Thus, for a certain period of time, this intercellular endothelial stressing organism seems to be able to create a subtle equilibrium of the host cell metabolism, providing appropriate conditions for its own survival.

Damage of ECs and their sloughing off into the blood-stream has been demonstrated for several atherosclerotic risk factors (eg, cigarette smoking and hyperlipidemia), especially in areas of turbulent blood flow conditions. This results in increased endothelial turnover with increased EC apoptosis or necrosis. Interestingly, this damage seems to be continuously repaired. This reendothelialization can occur both by out-growing neighboring ECs and by circulating endothelial progenitor cells.[58,59] We hypothesize—but have no experimental proof—that ECs at atherosclerosis predilection sites are especially prone to this sequence of events.

Clinical Observations

All healthy humans display innate and adaptive anti-HSP60 immunity, the latter induced by infection, by vaccination, or as bona fide autoimmunity against biochemically altered

autologous HSP60, probably derived from damaged or necrotic ECs.[20,60] For example, it has been speculated that in genetically predisposed individuals the same antigen(s), especially mycobacterial antigen(s), eg, HSP60 (Mtb-HSP60), may induce different immune responses, leading to the development of sarcoidosis and tuberculosis, respectively.[61]

The use of circulating sHSP60 concentrations or anti-hHSP60 antibody titers as prognostic biomarkers for the risk of developing CVD has been discussed during the last decade. In a prospective follow-up study of 195 healthy subjects, significantly higher anti-hHSP60 antibody titers were found in individuals with future CVD compared with individuals without cardiovascular events.[62] Similarly, a large population-based study with 826 subjects showed elevated sHSP60 levels in individuals with prevalent/incident carotid atherosclerosis, with serum levels that correlated with common carotid artery intima-media thickness (IMT).[63] These data were later confirmed in a prospective follow-up study.[11] Furthermore, patients with borderline hypertension and coronary heart disease present with elevated levels of sHSP60[64,65] and increased titers of anti-hHSP65 and anti-hHSP70 antibodies are associated with established hypertension.[10] Increased serum levels of sHSP60, sHSP72, and inflammatory markers can be correlated with the extent of cardiac and microvascular dysfunction in patients with angiographically normal coronary arteries.[66]

A significant correlation between the titers of anti-hHSP65 antibodies and sHSP60 with carotid atherosclerosis lesion size has been found in a large prospective longitudinal atherosclerosis prevention study originally comprising 1000 volunteers of both sexes, aged 40 to 79 years (the BRUNECK study).[67,68] In follow-up studies on this cohort, it was then shown that anti-HSP60 reactivity of peripheral blood T cells correlates with increased IMT in clinically healthy male youngsters but not in men aged 50 to 69 from this cohort,[69] indicating a more prominent role of specific cellular immunity to HSP60 in the early stages of atherosclerosis. Furthermore, the anti-hHSP60 antibody titer was not only identified as a new early biomarker for morbidity but also for mortality from atherosclerosis.[70] In Western blots, these anti-hHSP60 antibodies not only react with recombinant bacterial HSP60 but also cross-react with recombinant hHSP60. Interestingly, anti-HSP60 antibody titers drop after myocardial infarction, a phenomenon that is probably due to immune complex formation with hHSP60 released from the damaged myocardial tissue.[71] This possible sequence of events has been suggested and proven later in experiments in rats on induction of cardiac hypoxia, as mentioned above.[72]

A cross-sectional study involving 17 to 18 year-old clinically healthy men (the Atherosclerosis Risk Factors in Male Youngsters study),[73] revealed an unexpected and alarmingly high incidence of increased arterial IMT as a hallmark of the first inflammatory stage of the disease already afflicting 28% of these young men. This parameter showed a significant correlation with cigarette smoking, followed by a significant correlation with HSP60-specific reactivity of peripheral T cells, surprisingly only then followed by a correlation with the diastolic blood pressure and finally, least significantly, with anti-hHSP60 antibody titers.[73] Furthermore, T-cell reactivity against hHSP60 correlates with increased IMT in these youngsters.[69] This indicates a prominent role of specific cellular immunity to HSP60 in very early stages of atherosclerosis. In a study investigating 19- to 21-year-old young healthy female volunteers (the Atherosclerosis Risk Factors in Female Youngsters study),[74] 19% had an increased arterial IMT that correlated significantly with passive smoking and, again similar to the Atherosclerosis Risk Factors in Male Youngsters study, anti-HSP60 reactivity of peripheral T cells. In this group, no correlation between increased IMT and anti-HSP60 antibodies emerged.[74]

Lifelong infectious load has been discussed as correlated with antimicrobal HSP60 antibody titers and with atherosclerosis. More detailed studies on anti-HSP60 antibody reactivity have

revealed a linear correlation of the antibody titer to non-HSP60 antigens of *C. pneumoniae* and *Helicobacter pylori* with the antibacterial HSP60 titer and atherosclerosis.[75,76] Cross-reactivity of plasma anti-GroEL and anti-*Porphyromonas gingivalis* antibodies with hHSP60 have also been demonstrated in atherosclerosis patients.[77] It therefore seems that anti-HSP60 immunity is a common denominator for the association of infectious load with atherosclerosis. In contrast, no association with ECs dysfunction and the presence and severity of coronary artery disease and antibody response to *C. pneumoniae* IgG or human or *Chlamydia* HSP60 has been documented, arguing against the suggestion that infection contributes to disease progression.[78] In our hands, anti-cytomegalovirus antibody titers do not correlate with anti-HSP60 antibody titers.[76] However, this is not surprising, taking into account the fact that viruses do not encode their own HSP60 but that the HSP60 found in viral envelopes originate from the surface of the host cells.

In summary, the results point to a possible primary pathogenetic role of HSP60-specific T cells in the earliest stages of the disease, whereas HSP60 antibodies seem to lead to later aggravation and perpetuation. Our laboratory has recently confirmed this assumption in mice showing that T cells from draining lymph nodes and spleens in ApoE$^{-/-}$ animals immunized with mHSP65 harbor mHSP65 peptide–specific T cells. In humans, we have isolated intralesional T cells both from late and, more importantly, *early*, clinically still inapparent lesions (Figure 5). The reactivity of intralesional T cells against HSP60 significantly exceeded that obtained with T cells from the peripheral blood from the same donors.

HSP Vaccination

In the last few years, several studies have provoked an increased interest in mucosal tolerization against HSP60 aiming to suppress atherogenesis.[79,80] Both of these studies could successfully demonstrate a reduction in aortic plaques by this approach. However, only full-length mHSP65 (not defined proatherogenic peptides) was applied, therefore not taking into account the importance of bacterial-human cross-reactive epitopes. Full-length HSP60 and a HSP60 peptide (253 to 268) have also been orally administered to LDLr$^{-/-}$ mice before the induction of atherosclerosis, which resulted in a 80% reduction in plaque size in the carotid arteries and in a 27% reduction in plaque size at the aortic root.[81] The reduction in plaque size correlated with an increase in the number of T-regulatory cells,[81] which are known to be protective in atherosclerosis.[82,83] Nasal treatment with mHSP65 can effectively attenuate atherosclerosis in rabbits fed a Western diet.[84] Moreover, a promising cross-reactive B-cell epitope on both mHSP60 and hHSP60 involved in early atherogenesis has recently been demonstrated.[23] However, whether immune reactions against this epitope are involved in the pathogenesis of atherosclerosis remains to be elucidated. oxLDL is another example in which modulation of autoimmunity against atherosclerotic-associated autoantigens represents a novel and promising target for prevention and treatment of CVDs. Similar to HSP60, oxLDL is targeted by both antibody mediated and cellular immune responses. The immune activation is primarily of the proinflammatory Th1-type and inhibition of Th1 immunity reduces atherosclerosis in experimental animals. Atherosclerosis vaccines based on different antigens derived from oxLDL have been developed to modulate these processes.[85]

In conclusion, these studies suggest that vaccination with HSP60, or more preferably with atheroprotective HSP60 peptides, is a promising idea for the prevention and treatment of atherosclerosis. We are now in the process of delineating atherogenic HSP60 peptides in the murine system to use these for the development of an antiatherosclerosis vaccine via the induction of mucosal tolerance. Although the tolerizing approach in mice may form the basis for the subsequent development of such a vaccine in humans, it is rather improbable that the same HSP60 peptide candidates will emerge as atherogenic in both species. Here,

our own data obtained with T cells from early human atherosclerotic lesions will be of crucial importance.

Open Issues

There are still many other open questions to be answered with respect to the role of adaptive and innate immunity to HSP60 in atherogenesis: (1) Why are healthy people tolerant to autologous HSP60? (2) Does sensitization of HSP60-specific T cells take place in situ or in regional lymph nodes? (3) How can bona fide beneficial anti-HSP60 immunity, ie, for elimination of damaged and dead cells, be distinguished from pathogenetically relevant immune reactions? (4) Which atherogenic HSP60 epitopes are recognized by T cells isolated from early, clinically still inapparent human atherosclerotic lesions? (5) What is the exact sequence of gene expression in early versus late atherosclerotic lesion as assessed by microarray techniques? (6) With respect to the endothelial stressor-effect of classical atherosclerotic risk factors, what is the role of infection with *C. pneumoniae*? (7) Although binding of HSPs to toll-like receptors and trigging of the MyD88 signal-transduction pathway has now been unequivocally demonstrated, what is the possible pathogenic significance of this fact? (8) Finally, because data from clinical studies showed an increasing statistical significance of the reactivity of HSP60-specific T cells in the peripheral blood with decreasing age of the cohorts, we deem it important to perform similar studies in children, ie, revealing the very earliest stages of atherosclerotic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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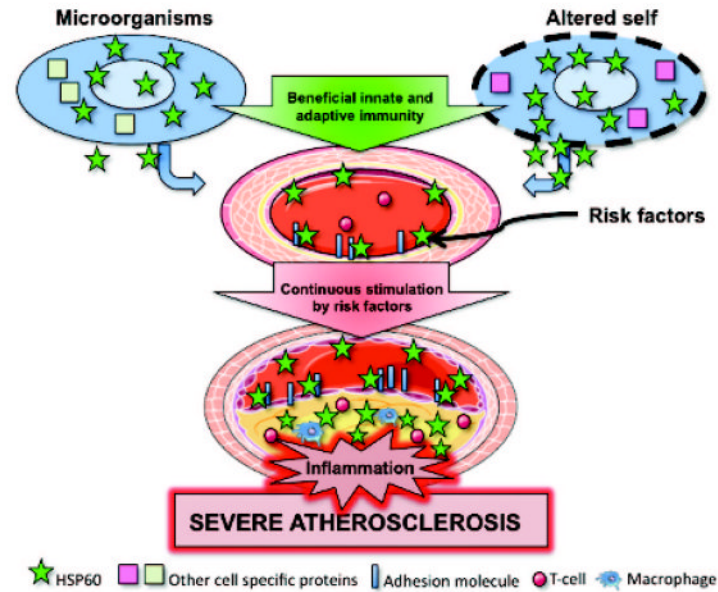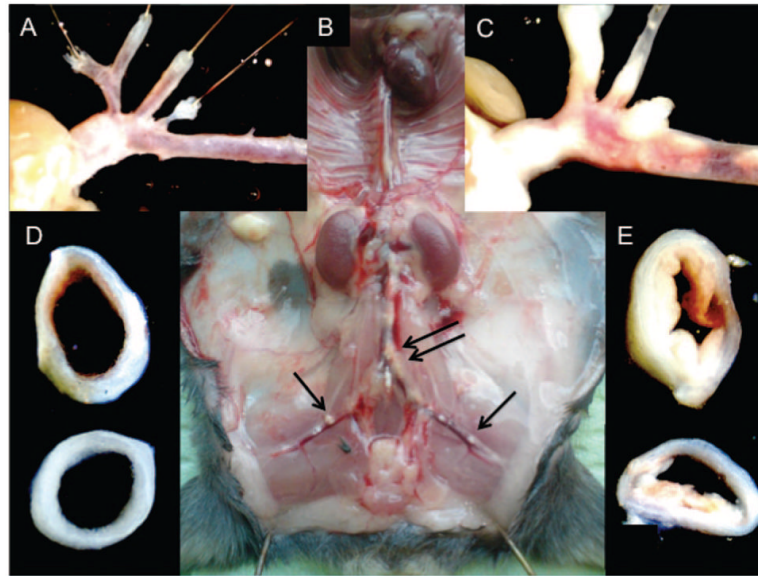
Figure 1.
Schematic overview of the autoimmune concept of atherosclerosis. Under physiological conditions, human vascular ECs do not express HSP60 (green stars) on their surface. However, when ECs are stressed by classical atherosclerosis risk factors, this leads to the simultaneous expression of HSP60 and adhesion molecules. HSP60 expression on the EC surface may serve as a target for preexisting cross-reactive antimicrobial human (cross)reactive HSP60 immunity or bona fide autoimmune reactions induced by biochemically altered autologous HSP60. Continuous stress by persisting risk factors leads to severe atherosclerosis. Partly adapted from Wick et al[20] and Servier Medical Art.
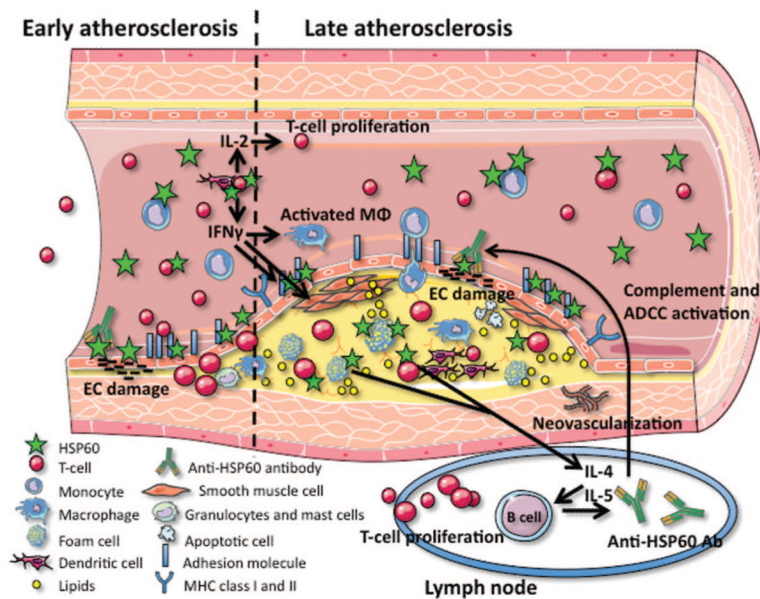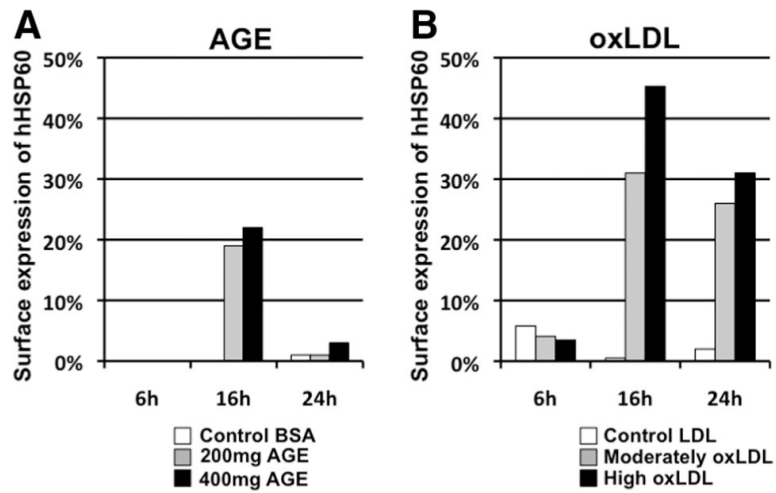
Figure 2.
Schematic overview of HSP60 expression/function in early and late atherosclerosis. Stress-induced EC surface expression of adhesion molecules and HSP60 enables T cells, monocytes, and dendritic cells to adhere to the ECs and transmigrate into the intima. The T cells (mostly CD4^{+}) are the first cells entering the intima during early atherosclerosis. Antigen recognition can be performed both by professional antigen-presenting cells (dendritic cells and macrophages) and by ECs and SMCs expressing MHC class I and class II (induced by interferon-γ). In addition, circulating anti-HSP60 antibodies are present. During progression from an early lesion to a severe plaque, EC damage via anti-HSP60 antibodies, an increased number of macrophages and SMCs often loaded with lipids (foam cells), and neovascularization can be demonstrated. The foam cells may rupture and release their contents into the lesional area, leading to the characteristic formation of cholesterol crystals. Also, the concentration of sHSP60 and anti-HSP60 antibodies is elevated in the serum of subjects with severe atherosclerosis. Anti-HSP60 antibodies can lyse the ECs in a complement-mediated fashion or via antibody-dependent cellular cytotoxicity (ADCC). In humans, the pathogenetic role of HSP60-specific T cells and antibodies, respectively, has still not been completely elucidated. However, studies in experimental animals have provided evidence for both mechanisms to contribute to atherogenesis. IL indicates interleukin; IFN, interferon; ADCC, antibody-dependent cellular cytotoxicity. Partly adapted from Servier Medical Art.
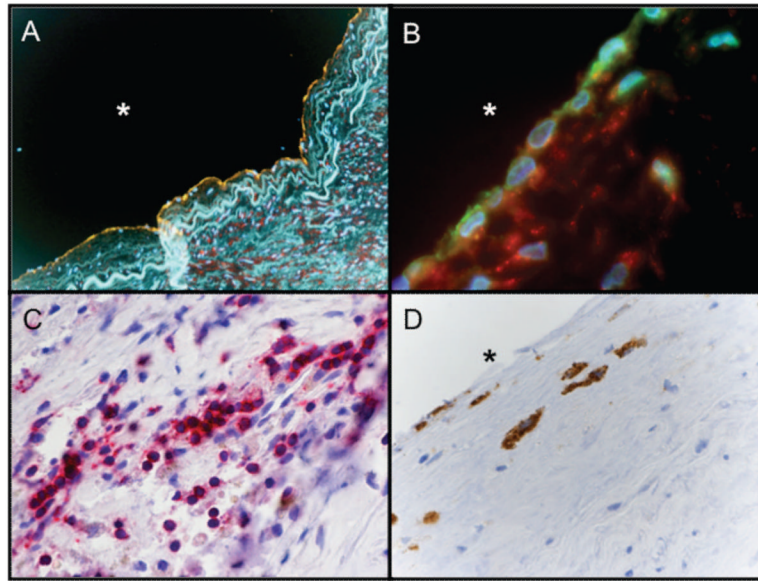
Figure 3.
Plaque formation in an atherosclerosis-prone animal model, the ApoE$^{-/-}$ mouse. A, The aortic arch of a 22-week-old female ApoE$^{-/-}$ mouse fed normal chow diet. B, A 22-week-old female ApoE$^{-/-}$ mouse fed a cholesterol-rich Western diet for 14 weeks and in addition having been injected 4 times with mHSP65 (50 μg/100 mL). Black arrows indicate visible plaque formation. C, A higher magnification of the aortic arch and branching arteries (of B). D, A cross-section of the aortic arch (of A). E, A cross-section of the aortic arch (of B).

Figure 4.
hHSP60 surface expression of human umbilical vein endothelial cells (HUVECs) treated with metabolic stressors. (A) Comparison of the effect of 2 different concentrations of advanced glycation end product (AGE) (200 mg/mL and 400 mg/mL) to native bovine serum albumin (BSA) (400 mg/mL). (B) oxLDL (moderate or high) was compared with native low-density lipoprotein (LDL) (60 μg/mL). Measurements were performed after 6, 16, and 24 hours of stimulation.

Figure 5.
Immunohistochemical staining of early, clinically inapparent human atherosclerotic lesion.
A, Under stress conditions, ECs (green: von Willebrand factor) express hHSP60 (red).
Yellow represents colocalization between vWF and HSP60. Blue indicates DNA. B,
Adhesion molecules (green: vascular cell adhesion molecule-1) and hHSP60 (red)
simultaneously expressed on the EC surface. Blue indicates DNA. The intima of the early
lesion contain a higher number of T cells (C) (red: CD3) compared with macrophages (D)
(brown: CD68). Original magnification: ×250 (A), ×600 (B), and ×400 (C and D). *Lumen.