

Atherosclerosis as an autoimmune disease: an update

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Immunoinflammatory processes are discussed increasingly as possible pathogenic factors for the development of atherosclerosis. Here, we summarize the data on which we have built our immunological hypothesis of atherogenesis. This concept is based on the observation that almost all humans have cellular and humoral immune reactions against microbial heat-shock protein 60 (HSP60). Because a high degree of antigenic homology exists between microbial (bacterial and parasitic) and human HSP60, the 'cost' of immunity to microbes might be the danger of cross-reactivity with human HSP60 expressed by the endothelial cells of stressed arteries. Genuine autoimmunity against altered autologous HSP60 might trigger this process also.

To date, three main classical theories to explain the initiation of atherosclerosis have been put forward: namely, the 'response to injury', 'altered lipoprotein' and 'clonal proliferation of smooth muscle cells' hypotheses. We will attempt to convince the reader that our autoimmune hypothesis¹⁻³ encompasses these different theories by presenting a common determinant – the humoral and cellular immune reaction against certain stress proteins [heat-shock proteins (HSPs)] – that initiates the inflammatory immunological processes characteristic of the very first stages of atherosclerosis.

The heat-shock protein families
HSPs are expressed by prokaryotic and eukaryotic cells under physiological conditions or in response to stress (e.g. heat, toxins or mechanical stress). HSPs are classified into various families according to their molecular weight; the main families are the 100 kDa, 70 kDa, 60 kDa and low-molecular-weight families, which fulfill a wide variety of physiological functions in protein folding, cellular signaling and protein degradation. Under conditions of stress, some HSPs associate with other cellular proteins and protect them from denaturation (chaperones)⁴.

In the present context, we will focus on the 60 kDa family, members of which have been identified as the culprit autoantigens in the development of early atherosclerosis. The HSP60 family encompasses, for example, HSP60 in mammals, the mycobacterial homolog mHSP65, chlamydial HSP60 (cHSP60) and the *Escherichia coli* homolog GroEL. HSPs of the 60 kDa family appear to be phylogenetically highly conserved between human and bacterial species (Fig. 1), facilitating immunological crossreactions

between pathogen- and self-HSP60. Other authors have provided evidence that other HSPs might be involved in atherosclerosis also – for example, HSP40 (Ref. 5) or HSP70 (Ref. 6) – but, to date, there has been no evidence that atherosclerosis can be induced by injection of these HSPs into experimental animals^{7,8}.

Evidence for the pathogenic role of HSP60 in animal models

Immunization of normocholesterolemic rabbits with Freund's complete adjuvant (normally used to increase the immune response to a given antigen), consisting of mineral oil and heat-killed mycobacteria, leads to the development of atherosclerotic lesions at the known predilection sites in the aorta. The same effect can be observed after immunization with recombinant mHSP65 (Ref. 9), which forms a high percentage of the whole protein content of mycobacteria. Foam cells are still lacking at this early stage. The first autoimmune, inflammatory stage of atherosclerosis induced in normocholesterolemic rabbits by immunization with mHSP65 is still reversible. However, when additional risk factors such as high levels of blood cholesterol are present, the lesions become irreversible¹⁰.

'HSPs of the 60 kDa family appear to be phylogenetically highly conserved between human and bacterial species, facilitating immunological crossreactions...'

Wild-type mice are notoriously resistant to the induction of atherosclerosis by classical means (e.g. a high-cholesterol diet). We were, however, able to show that cholesterol-fed C57BL/6J mice develop aggravated lesions if they are immunized simultaneously with mHSP65 (Ref. 11). Also, we used ApoE-knockout mice, which have very high serum cholesterol levels owing to their inability to remove circulating low-density lipoprotein (LDL). Immunization of these mice with chemically modified LDL (malondialdehyde-LDL) exerts a protective effect against atherosclerosis¹². These experiments compared the two major theories concerning the possible pathogenic antigens involved in atherogenesis: immune reactions against biochemically altered LDL or HSP60.

HSP60 and human atherosclerosis

Within the framework of the Bruneck Study, a prospective population-based survey on the pathogenesis of atherosclerosis, involving almost 900 men and women aged 40–80 years who have been followed for >10 years since 1990, we were able to show that – as expected owing to prior infections or

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CH60_HUMAN MLRLPTVFRQMRPVSRLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIIEQSWGSPKVTKDGVTVAKSIDLKDVKYKNI GAKL
CH60_CHLTR -----VAKNIKYNEEARKKIQKGVKTLAEAVKVTGLGPKGRHVVIDKSFSGSPQVTKDGVTVAKEVELADKHENMGAMQ
CH60_ECOLI -----AAKDVKFGNDARVKMLRGNVVLADAVKVTGLGPKGRNVVLDKSFSGAPTITTKDGVSVAREIELEDKFENMGAMQ
CH62_MYCTU -----AKTIAYDEEARGLERGLNALADAVKVTGLGPKGRNVVLEKKWGAPTITNDGVSIAKEIELEDPEYKIGAEI

CH60_HUMAN VQDVANNITNEEAGDGTATTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDAVIAELKKQSKPVTTPPEIAQVATISANGDKEIGNIISDAMKVKV
CH60_CHLTR VKEVASKTADKAGDGTATTATVLAFAIYTEGLRNVTAGANPMDLKRKIDKAVKVVVDQIRKISKPVQHHKEIAQVATISANNDAEIGNLIAEAMEKVG
CH60_ECOLI VKEVASKANDAAGDGTATTATVLAQAIITEGLKAVAAGMNPMDLKRKIDKAVTAAVEELKALSVPCSDSKAIAQVGTISANSDETGVKGLIAEAMDKVG
CH62_MYCTU VKEVAKTDDVAGDGTATTATVLAQALVREGLRNVAAGANPLGLKRGIEKAVEKVTETLLKGAKEVETKEQIAATAAISAG-DQSIGDLIAEAMDKVG

CH60_HUMAN RKGVITVKGDKTLNDELEIIEGKMFDRGYISPYFINTSKGQKCFQDAYVLLSEKKISSIQSIVPALEIANAHKRPLVIIAEDVDGEALSTLVLNRL
CH60_CHLTR KNGSITVVEAKGFETVLDIVEGMNFNRGYLSSYFATNPETQECVLEDALVLIYDKKISGKDFLPVLQQAESGRPLIIAEDIIEGALATLTVNRI
CH60_ECOLI KEGVITVEDGTGLQDELVDVEGMQFDRGYLSPYFINKPETGAVELESPIFILLADKKISNIREMLPVLAVAKAGKPLIIAEDVEGEALATAVNTI
CH62_MYCTU NEGVITVEESNTFGLQLELTEGMRFDKGYISGYFVTDPERQEAVLEDPYILLVSSKSVTVKDLLPLEKVIKAGKPLIIAEDVEGEALSTLVNKI

CH60_HUMAN KVGLQVAVKAPGFGDNRKNQKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGVEIVTKDDAMLLKKGDKAQIEKRIQEIIEQLDVTTSEYEKE
CH60_CHLTR RGGFRVCAVKAPGFGDRRKAMLEDAIILTGGQLISEE-LGMKLENANLAMLGKAKKIVVSKEDTTIVEGMGEKEALEARCESIKKQIEDSSSDYDKE
CH60_ECOLI RGIKVAVKAPGFGDRRKAMLDIATLGGTVISEE-IGMELEKATLEDLQAKRVVINKDTTTIIDGVGEEAAIQGRVAQIRQIEEATSDYDRE
CH62_MYCTU RGTFFKSAVKAPGFGDRRKAMLDMAIILTGGQVISEE-VGLTLENADLSLLGKARKVVVTKDETTIVEGAGDTDAIAGRAVQIRQIEENSDDYDRE

CH60_HUMAN KLNERLAKLSDGVAVLKVGTSDEVNEKKDRVTDALNATRAAVEEGIVLGGCCALLRCIPALDSLTPANEDQKIGIEIKRTLKIPAMTIAKNA
CH60_CHLTR KLQERLAKLSGGVAVIRVGAATEIEMKEKDRVDDAQHATIAAEEGILPGGTALIRCIPTEAFPLMLTNEDEQIGARIVLKALSAPLKQIAANA
CH60_ECOLI KLQERVAKLGGVAVIKVGAATEVEMKEKARVEDALHATRAAVEEGVAVAGGVALIRVASKLADLR--GQNEQNVGKIKVALRAMEAPLRQIVLNC
CH62_MYCTU KLQERLAKLAGGVAVIKAGAATEVELKERRHRIEDAVRNAKAAVEEGIVAGGVTLLQAAPTLDELK--LEG-DEATGANIVKVALEAPLKQIAFNS

CH60_HUMAN GVEGSLIVEKIMQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIPKEEKDPGMGAMGGMGGMGG--GMF
CH60_CHLTR GKEGAIIFQQVMSRSANEGYDALRDAYTDMLEAGILDPKAVTRSALESASVAGLLTTEALIAEIP--EEKPAAAPAMPGAG--MDY-----
CH60_ECOLI GEEPSVVANTVKGGDNGYNAATEEYGNMIDMGLDPTKVTRSALQYASVAGLMIITTECMVTDLPKNDADLGAAGMGGMGG--MGMGMGM
CH62_MYCTU GLEPGVVAEKVRNLPAGHGLNAQTGVYEDLLAAGVADPVKVTRESALQNAASIAGLFLITTEAVVADKPEKEK--ASVPGGGDMGG--MDF-----

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Fig. 1. Multiple sequence alignment of 60 kDa heat-shock proteins (GenBank®) from human (CH60_HUMAN), *Chlamydia trachomatis* (CH60_CHLTR), *Escherichia coli* (CH60_ECOLI) and *Mycobacterium tuberculosis* (CH62_MYCTU). Identical amino acid positions are highlighted as dark purple boxes, and homolog positions as light purple boxes. The overall amino acid identity is 34% and homology is 71%. Homology is defined as positive scoring in the PAM250 amino acid substitution matrix.

vaccinations – all participants produced anti-bacterial (anti-mHSP65) antibodies (Abs) at levels that gave a significant positive correlation with the presence of sonographically visible atherosclerotic lesions in the carotid arteries¹³. Statistically, these Abs represent a new risk factor that is independent of classical atherosclerosis risk factors, except age. We showed also that Abs specific for mHSP65 in ELISA cross-react with GroEL, cHSP60 and – most importantly – human HSP60 (Ref. 14). In a five-year follow-up study, we found that anti-HSP60 and/or anti-HSP65 titers are very robust parameters, which are not only predictive for morbidity, but also, for mortality due to atherosclerosis¹⁵.

In recent years, chronic infections have been implicated in the pathogenesis of atherosclerosis^{16,17}. We have studied this issue also in the course of the Bruneck Study. In this study, it was shown clearly that chronic infections amplify the risk of carotid atherosclerosis. It emerged also from this study that increased levels of soluble HSP60 (sHSP60) and Abs to bacterial HSP60 seem to constitute the common denominator in these infections and are predictive of an increased risk of atherosclerosis¹⁸. We found that, in addition to their association with chlamydial infections, levels of anti-mHSP65 Abs are correlated

closely with immune reactions to endotoxin in the general population. In this respect, infections with *Chlamydia*, which has been incriminated repeatedly as a pro-atherogenic infectious agent¹⁹, seem to differ from other infections in a quantitative rather than qualitative manner; *Chlamydia* are extremely rich in HSP60 (cHSP60) that is strongly immunogenic, they have an endothelial-cell tropism and chlamydial infections constitute an extreme form of stress for the host cells, reflected by excessive expression of

'...exertion of mechanical stress on ECs *in vitro*...induces simultaneous expression of HSP60 and adhesion molecules, both at the RNA and protein levels.'

eukaryotic HSP60 (G. Millonig, M. Maass and G. Wick, unpublished). In addition to its antigenic properties, bacterial HSP60 product might have direct pro-atherogenic effects by stimulating functions of macrophages considered relevant to atherosclerosis and its complications, such as the production of proinflammatory cytokines [e.g. tumor necrosis factor α (TNF- α)] and matrix-degrading metalloproteinases²⁰. Also, other data suggest an interference with the innate immune system, because HSP60 can bind to CD14, the lipopolysaccharide (LPS) receptor, and activate

monocytes and/or macrophages and endothelial cells²¹.

Prohaszka *et al.* have suggested another possibility for the effect of anti-HSP60 Abs – the presence of natural Abs against human HSP60 that exert their effect in a complement-dependent fashion²². We consider this to be rather a semantic question, because ‘natural’ Abs are, in general, induced by antigenic stimulation also, although the nature of these antigens is rarely known. In any case, the main and important aspect is the reactivity of Abs and T cells to HSP60 expressed on endothelial cells (ECs), which are the first target cells encountered by humoral or cellular effector mechanisms.

So far, we do not know if Abs or autoreactive T cells specific for HSP60 are the first pathogenic factors in early atherosclerosis. As mentioned previously, we have made considerable progress with respect to the analysis of humoral immunity, but we are still lagging behind in our knowledge of the role of T-cell reactivity against HSP60 in the pathogenesis of human atherosclerosis. Other authors have shown that T-cell clones derived from advanced lesions show a preferential reactivity against human HSP60 (Ref. 23). In our hands, a significant enrichment of HSP60-reactive T cells can be demonstrated in atherosclerotic lesions of cholesterol-fed rabbits compared with T cells from the peripheral blood of the same animal²⁴.

However, as mentioned at the start of this article, our interest is focused on the very early stages of the disease. For obvious technical and ethical reasons it is difficult, or even impossible, to get access to human specimens displaying these clinically undetectable alterations.

HSP60 and *in vitro* experiments

We have shown that a given stressor (e.g. heat, TNF- α or oxygen radicals) induces the simultaneous expression of HSP60 and adhesion molecules [e.g. intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and endothelial-leukocyte adhesion molecule 1 (ELAM-1)] by ECs (Ref. 25). In this respect, it is important to emphasize that arterial ECs have a lower threshold than venous ECs for the effect of various stressors (i.e. classical atherosclerosis risk factors, notably oxidized LDL). We have attributed this characteristic to the fact that arterial ECs are pre-stressed by their life-long exposure to the higher arterial blood pressure compared with that of the venous part of the circulation. As a matter of fact, exertion of mechanical stress on ECs *in vitro*, as well as in the common carotid artery *in vivo*, in a rat model induces the simultaneous expression of HSP60 and adhesion molecules, at both the RNA and protein levels²⁶.

Interestingly, although HSP60 protein is expressed in mitochondria, several functional, phenotypic and biochemical analyses have provided

evidence that HSP60 is localized also on the surface of stressed ECs and macrophages^{27,28}. We have shown that affinity-chromatography-purified anti-mHSP65/hHSP60 cross-reactive human Abs are able to lyse stressed, but not unstressed, human ECs (Ref. 29) and macrophages³⁰ in a complement-mediated fashion or by Ab-dependent cellular cytotoxicity (ADCC).

‘...T cells, rather than macrophages, are the prevailing mononuclear cells in the intima infiltrate at the first stage.’

Attempts in our laboratory to provide a molecular basis for the atheroprotective effect of nonsteroidal drugs revealed that, *in vitro*, aspirin induces the expression of HSP60 by ECs but, simultaneously, is able to suppress the TNF- α -induced expression of adhesion molecules (e.g. ICAM-1, VCAM-1 and ELAM-1), thus, blocking the adhesive potential of HSP60-reactive T cells. Interestingly, the immunosuppressive drug cyclosporin A turned out to be a strong inducer of expression of HSP60, without any additional effects on the expression of adhesion molecules. This might explain the increased incidence and severity of atherosclerosis in cyclosporin-A-treated patients³¹.

Local aspects of the arterial wall – immunohistochemical studies

In fully blown atherosclerotic lesions, granular Ig deposits with co-distributed complement components³², as well as an increased expression of C3b receptors (CR1) and C3bi receptors (CR3) on macrophages, can be demonstrated³³. In contrast to the current dogma^{34,35}, T cells, rather than macrophages, are the prevailing mononuclear cells in the intima infiltrate at the first stage^{2,36,37}. A considerable percentage of these T cells is activated (HLA-DR⁺CD25⁺)³⁸. CD4⁺ T cells prevail over CD8⁺ T cells³⁷ and, among the former, the number of T helper 1 (Th1) cells exceeds the number of Th2 cells³⁹. It does not seem probable that ECs act as antigen-presenting cells (APCs), because, at least in our hands, they only express MHC class II antigens in the presence of subendothelial T-cell infiltrations when interferon γ (IFN- γ) is available locally³⁶. Therefore, we assume that the sensitization of intimal T cells must take place in regional lymph nodes, similar to the situation in the skin. This concept is supported by our recent demonstration of a network of dendritic cells (DCs) in the intima of arteries at those sites that are subjected to increased (turbulent) flow stress (i.e. at the vascular branching points)^{40,41}. It is of special interest, in this context, that this network of vascular-associated DCs is already present in the arteries of healthy children

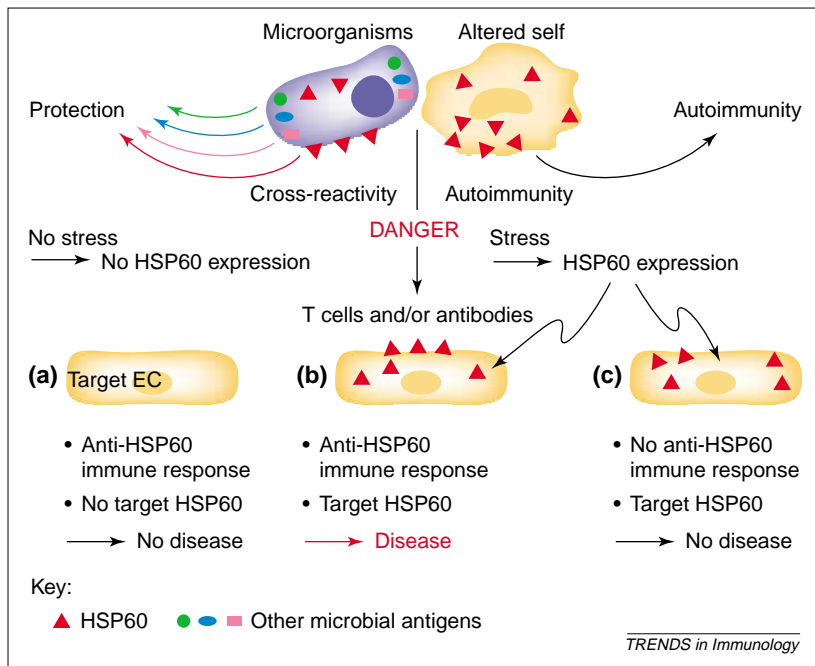


Fig. 2. Atherosclerosis – the price we pay for immunity to heat-shock protein 60 (HSP60).

(a) Protection against microbial infection is mediated by humoral and cellular immunity directed against microbial HSP60 (mHSP60) and non-HSP60 antigens. Under physiological conditions, human vascular endothelial cells (ECs) do not express target human HSP60 (hHSP60) and no disease results from this immune response. (b) The stress-induced expression of HSP60 on target ECs can result in cross-reactivity of the immune response directed against mHSP60 and immune destruction of ECs. This might also result from an autoimmune reaction directed against altered self-antigens. (c) In some cases, autoimmunity might not develop, despite the presence of target hHSP60, because the MHC class I and II grooves of these individuals do not accommodate the pathogenic HSP60 epitopes.

and parasites) display a variety of antigens, including HSP60, which shows a high degree of evolutionary conservation. All animals and humans have humoral and cellular immunity against microbial HSP60 that – together with the immune response against non-HSP60 antigens – confers protection against infection. Therefore, induction of tolerance against atherogenic epitopes represents a therapeutic option only if it does not hamper immune reactivity against other, nonatherogenic HSP epitopes and, thus, immunity is preserved. The main point is, however, humoral and cellular reactivity against HSP60, which is expressed on vascular ECs subjected to various forms of stress. Therefore, we do not deny the importance of proven classical atherosclerosis risk factors, but we assign a new role to them in the initial stages of the disease (i.e. their activity as endothelial stressors). In cases where atherosclerosis risk factors are not present, ECs do not express HSP60 on their surface and, therefore, do not represent targets for autoimmunity. In cases where we maltreat our ECs – particularly, taking into account the pre-stressing, threshold-lowering effect of the high arterial blood pressure – HSP60 is expressed on the endothelial surface and we have to ‘pay’ for our protective anti-HSP60 immunity by endothelial damage and the subsequent development of early atherosclerotic lesions. In occasional instances, atherosclerosis might not develop despite the presence of atherosclerosis risk factors, because the MHC class I and class II grooves in these individuals do not accommodate the potentially dangerous atherogenic HSP60 epitopes. Thus, in summary, autoimmune diseases in general and early atherosclerosis in particular do not depend only on the presence of humoral and cellular autoimmunity against potentially harmful autoantigens, but are governed mainly by a genetically determined or environmentally induced susceptibility of the target cell to the autoimmune attack⁴⁶. This concept of an autoimmune pathogenesis of atherosclerosis opens new avenues for the early diagnosis, monitoring and possible therapy of this disease, which represents a major cause of mortality in developed countries.

and even babies, before the appearance of any atherosclerotic changes.

Most CD3⁺ cells in the mononuclear infiltrates of early atherosclerotic lesions express the $\alpha\beta$ T-cell receptor (TCR), but an unexpectedly high proportion (>10%) is $\gamma\delta$ TCR⁺, representing a significant enrichment of this cell type compared with the peripheral blood of the same individuals⁴². It should be kept in mind that $\gamma\delta$ TCR⁺ cells show a preferential tendency to react with HSPs (Refs 43–45).

Conclusion

Figure 2 summarizes our concept of an autoimmune, inflammatory pathogenesis of the earliest stages of atherosclerosis. Microorganisms (bacteria, viruses

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