Stress proteins as targets for anti-inflammatory therapies

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Microbial heat shock proteins (HSPs) are ubiquitous and highly immunogenic. In healthy humans, B- and T-cells with specificity for self-HSP can be easily detected. In patients with chronic inflammatory diseases, raised levels of antibodies and T-cells with reactivity to self-HSP have been observed. Based on this and other evidence, this raised immune reactivity might be the result of stress-induced upregulation of self-HSP during inflammation and is possibly caused by tissue destruction. More importantly, immunization with conserved sequences of microbial-HSP increases resistance to the induction of autoimmune disease. Together, it appears that immune reactivity directed towards self-HSP can be part of a regulatory immune effector mechanism that contributes to maintenance of self-tolerance and has anti-inflammatory activity. Boosting of such anti-inflammatory effector mechanisms by artificial immunization offers attractive immunotherapeutic possibilities.

Heat shock proteins

Their function

Heat shock proteins (HSPs) have essential functions in all cellular organisms, and are classified by their MW and function (for reviews, please see Refs 1–4). Most of the known families of HSP have 'chaperone' functions. This means that HSPs engage in transient interactions with other cellular proteins to guide their assembly or transport within cells, to assist these proteins in their physiological function, or to protect and correct them from stress-induced damage. The mechanism of these aspects of HSPs is being widely studied and it is expected that this information will yield novel methods of manipulating essential cellular functions for the production of new therapies. For example, approaches are being examined that use HSPs to protect against ischemia- and reperfusion-induced damage of transplanted organs and for protection against the consequences of circulatory and haemorrhagic shock[5]. Furthermore, in the case of the GP96, HSP70 and HSP90, the protein- or peptide-binding efficacy of HSP appears to have been successfully exploited for the isolation of, and immunization with, tumour-specific antigens for cancer immunotherapies[6–8]. Despite HSPs being intracellular proteins, there is recurrent evidence for cell-surface expression of HSPs. Such expression on tumour cells might enable the specific targeting of tumour cells, while surface expression in bacteria[9] might enhance virulence by enabling bacteria to enter host cells that have an HSP receptor[10]. It is also suggested that there is an HSP receptor on dendritic cells, the crucial professional antigen-presenting cells (APCs) for initiating immune responses[11]. Thus, dendritic cells could take-up HSP-associated (chaperoned) antigen produced by stressed organisms, or even by complete microorganisms expressing HSP for presentation to the immune system[12].

Stress proteins are dominant immunogens

Cellular stress increases the production of HSP to a unique extent. Hence, cells under stress process large quantities of HSP epitopes, which are presented by their major histocompatibility complex (MHC) molecules. Receptors specific for these HSP epitopes on T-cells will detect this raised expression, while also remaining subject to the regulatory mechanisms of peripheral tolerance. Many other self-antigens can

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also be recognized by the immune system. However, the frequency of immune cells that have self-HSP receptors might be an underestimate, as repeated contact with microbial-HSP has increased the number of immune cells expressing microbial-HSP-specific receptors. In fact, for HSP60 and HSP70, immunization with microbial-HSP triggers not only microbial-HSP-specific T-cells, but also self-HSP crossreactive T-cells[13,14]. This aspect of immune behaviour is possibly connected to regulatory control of the inflammatory process, as exemplified in models of experimentally induced autoimmunity[15].

Stress proteins and autoimmune diseases

HSP60 in experimental arthritis

Early studies showed that, in a model of mycobacteria-induced adjuvant arthritis in rats, transfer of disease is possible using a single T-cell clone raised against whole *Mycobacterium tuberculosis* organisms[16]. Although initially, the antigen in the mycobacteria remained obscure, the T-cell clone cross-recognized an antigen associated with cartilage proteoglycans, indicating mimicry between a mycobacterial antigen and cartilage[17]. Subsequent searches for the antigen of the arthritis-producing T-cell on recombinant mycobacterial antigens showed that the HSP60 (or HSP65) of mycobacteria is the crucial antigen[18]. Furthermore, epitope mapping showed that the 180–188 amino acid sequence is the mimicry- producing epitope. However, this epitope is not conserved, suggesting that the self-antigen associated with cartilage is not self-HSP60, but another proteoglycan-associated molecule.

Studies have shown that immunization of rats and mice with the recombinant mycobacterial HSP60 does not induce arthritis, but can protect against future arthritis induction[19]. Analysis of T-cell responses in these protected rats revealed that HSP60 immunization had induced T-cells responding to 180–188, but also to other epitopes. One such epitope was the highly conserved sequence 256–265, and T-cells responding to this epitope cross-recognized the mammalian (rat, mouse and human) HSP60 molecule[14]. Furthermore, passive transfer of a T-cell line responding to this epitope protected against active adjuvant arthritis induced by mycobacteria, as well as that induced by the arthritogenic synthetic oil, avridine. Similar protection was found in rats immunized with the synthetic 256–265 peptide sequence of mycobacterial HSP60. Hence, this appears to be a fundamental protective mechanism with a general influence on arthritic inflammation.

T-cell responses to HSP60 in rheumatoid arthritis patients

Analysis of T-cell responses in lymphocytes from the peripheral blood and synovial fluid of arthritis sufferers has shown the varying frequencies of T-cells specific for HSP60[20,21]. In juvenile rheumatoid arthritis (JRA), especially those with oligo-articular (four joints or less) JRA (OA-JRA), responses to recombinant human-HSP60 are prominent, whereas in poly-articular (five joints or more) JRA (PA-JRA) patients, such responses are usually absent[22,23]. Interestingly, by contrast with PA-JRA patients, OA-JRA patients are known to remit spontaneously. Thus, in children, the presence of proliferative T-cell responses to human-HSP60 can be used as an indicator of the spontaneously remitting form of the disease. Remission was also preceded by elevated HSP60 reactivity. Furthermore, T-cells responding to raised HSP60 levels demonstrated features of a regulatory response, such as increased CD30 expression and the production of inhibitory cytokines (e.g. interleukin 10, IL-10).

In adult rheumatoid arthritis (RA) patients, indirect evidence for a similar protective or regulatory activity of human-HSP60-responsive T-cells has been obtained. T-cells taken from adult RA patients and cultured in the presence of IL-4 responded when stimulated with human-HSP60, but not when stimulated with mycobacterial-HSP60, suggesting that human-HSP60-responding cells are also regulatory (Th2). Furthermore, in co-culture with RA synovial cells, which produce the pro-inflammatory mediator tumour necrosis factor α (TNFα), the HSP60-responding T-cells inhibited TNFα production, again compatible with the regulatory nature of HSP60-responding T-cells[24].

The mechanism of HSP60-induced protection in arthritis

The involvement of a self-reactive T-cell repertoire in protection against arthritis appears to be at odds with the perception that self-reactivity produced the adverse effects of autoimmunity. Several explanations have been suggested to explain why self-reactivity directed against self-HSP controls inflammatory responses[25] (Fig. 1).

Firstly, levels of bacterial-HSP are prominent near to the tolerizing gut mucosa, the site known to induce oral tolerance[26,27]. T-cells with specificity for conserved bacterial-HSP might continuously detect the presence of their epitopes in, or close to, the gut-associated lymphoid tissue (GALT), which might lead to the development of their regulatory phenotype. Hence, the presence of bacterial-HSP homologues from over-expression of mammalian-HSP in the inflamed joint (or other sites), will trigger the HSP-specific T cells to express their earlier-adopted regulatory phenotype. Inflammation will, therefore, be controlled through the production of suppressive cytokines (bystander suppression), such as IL-10 (Ref. 28).

Secondly, at the molecular level, the small differences in the amino acid sequence between microbial- and self-

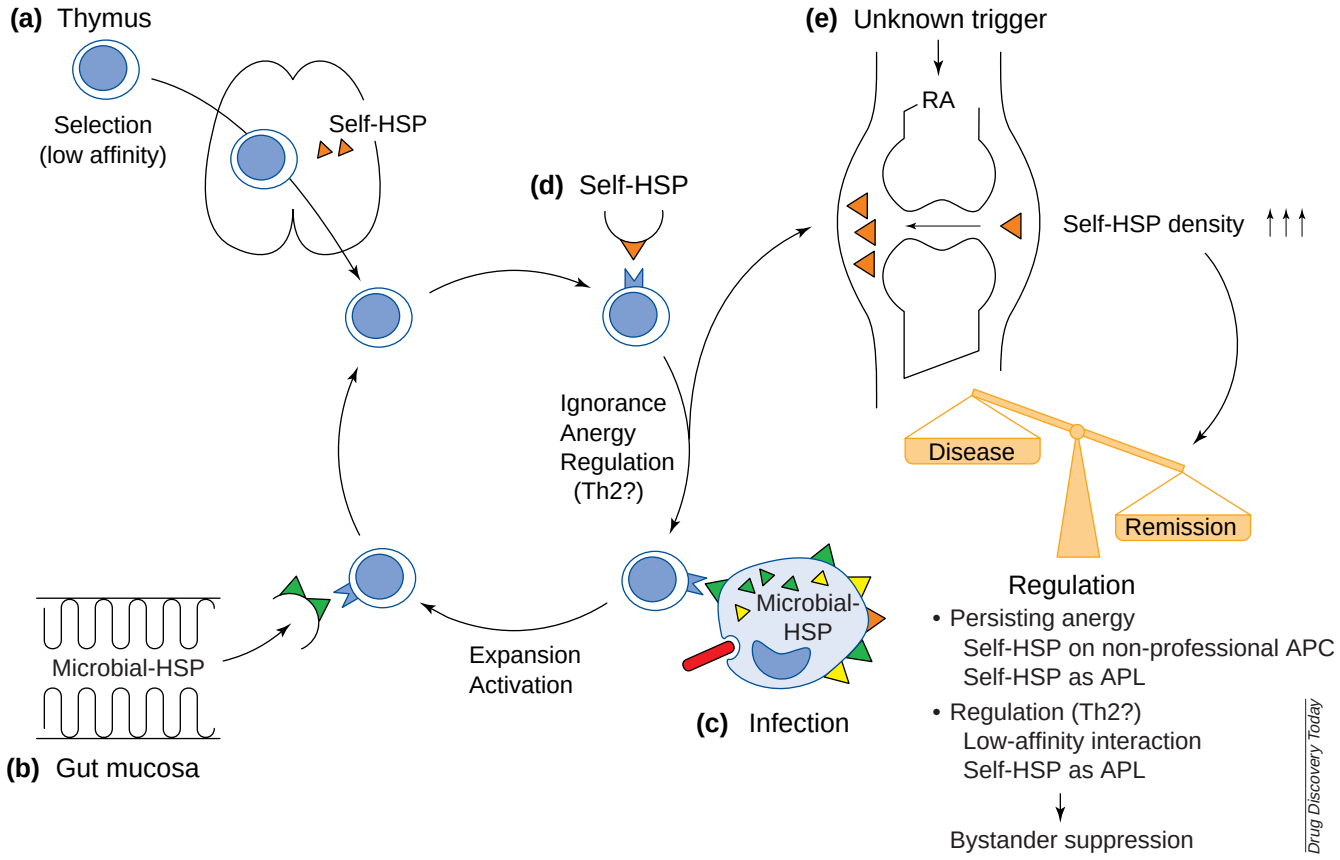

Figure 1. *Mechanisms through which heat shock proteins (HSPs) regulate inflammation. The expansion and activation of T-cells specific for HSP, for example by HSP immunization, will contribute to disease remission in arthritis and other inflammatory conditions. (a) Thymic selection leads to a T-cell repertoire of HSP-reactive T-cells. The mechanisms that contribute to the maintenance and safe containment of such self-reactive T-cells are schematically represented. Conserved microbial-HSP epitopes, as present in the periphery of the immune system in (b) the tolerizing gut mucosal environment or (c) transiently during infection, will activate self-HSP-reactive T-cells or will lead to their proliferation. (d) Low-level expression of self-HSP epitopes on (non-professional) antigen presenting cells will lead to ignorance, anergy or a regulatory phenotype in self-HSP-reactive T-cells. These latter mechanisms, activated by stress-induced overexpressed self-HSP molecules, are thought to activate regulatory control of inflammation in autoimmunity such as (e) in the joints of humans with rheumatoid arthritis. Conserved HSP epitopes are represented by coloured triangles: yellow indicates self (host origin)-HSP epitopes; blue indicates microbial-HSP (Figure taken from Ref. 22).*

HSP could be crucial. According to the current understanding of thymic selection, negative selection ensures the elimination of T-cells with high-affinity receptors for self-HSP. Positive thymic selection (the thymic areas involved in positive selection express HSP60)[29] produces a repertoire of T-cells with receptors that have a low affinity for self-HSP (self-HSP60 in this instance). T-cells that detect conserved microbial-HSP epitopes during infection, bacterial vaccination or in the (gut) environment will proliferate selectively. Naturally, such selective pressure will favour T-cells with a relatively high affinity for the microbial homologues. Hence, the resulting T-cell repertoire will have a high affinity for microbial epitopes, but low affinity for self-HSP epitopes. Studies using altered peptide ligands (APL – 'altered' meaning small modifications in the amino acid sequence) suggested that peptides that trigger T-cells with high affinity promote a more vigorous pro-inflammatory (Th1) T-cell response, which is advantageous in infection. By contrast, peptides modified in the T-cell receptor contact positions, thereby reducing affinity, can skew the resulting T-cell response towards a regulatory (Th2) or inhibited response[30–33]. Hence, the low-affinity self-HSP epitope, which is overexpressed at the inflammation site, would skew the responding T-cell towards this regulatory and, thus, anti-inflammatory mode.

Thirdly, the ubiquitous (for HSP60, the low-level) constitutive HSP expression guarantees that T-cells will detect self-HSP epitopes on non-professional APCs and non-activated professional APCs that lack the co-stimulatory molecules necessary to induce a T-cell response. Recognition in the absence of proper co-stimulation puts T-cells into a state of anergy (total inhibition of function). Similarly, chronic antigenic stimulation *in vivo* produces an anergic regulatory phenotype in $CD4^+$ T-cells[34]. These and other recent observations indicate that these anergic T-cells are actually regulators that inhibit proliferation of other T-cells in co-culture when exposed to their antigen on professional APCs (Refs 35–37). Therefore, anergic self-HSP-reactive T-cells might exert a local suppressive effect when HSP60 is overexpressed by APCs in the inflamed area. Alternatively, through induction of cross-tolerance, professional APCs could present peripherally derived HSP in the secondary lymphoid organs to induce T-cell anergy there[38].

HSP70 and other conserved proteins in experimental arthritis

Other HSPs also have a protective effect in arthritis models. For example, HSP10, the smaller chaperone associated with HSP60, has a protective effect in rats[39]. Furthermore, the protection observed with mycobacterial-HSP70, depended on the recognition of a highly conserved HSP70 sequence[13,40]. As with HSP60, the T-cells responsive to this conserved epitope cross-reacted with the self (rat)-HSP70. Interestingly, on stimulation, these T-cells produced IL-10, supporting the suggestion that this HSP epitope also induced a regulatory phenotype in the responding T-cells. As might be expected from the induction of IL-10, the nasal administration of the same epitope protected against induction of both mycobacteria- and avridine-induced arthritis in rats.

In a separate series of experiments, other conserved and immunogenic bacterial proteins with mammalian homologues present in the recipients did not inhibit the development of arthritis, emphasizing the unique potential of HSP in this respect.

HSP reactivity in other autoimmune diseases

Antibodies and T-cell responses to HSP also occur in many other inflammatory disorders including autoimmune diseases. Although this reactivity is also found in healthy individuals, in inflammatory disorders, the level of reactivity is much higher (for reviews, see Refs 41–43). This increased HSP reactivity is probably due to the HSP overproduction caused by inflammation. Furthermore, in chronic persistent diseases, this raised immunity probably reflects an inadequate response that failed to control disease development. Until now, there have been few suggestions for what determines such a response to be insufficient or inadequate in humans.

Risk of inducing disease by HSP immunization

Despite intensive attempts, no induced arthritis has been noted in arthritis models following HSP immunization. The mycobacterial-HSP60 protein alone, which includes the 180–188 mimicry epitope that activates T-cells and has an arthritis-producing capacity, cannot induce the disease. Conserved regulatory epitopes, such as the 256–265 epitope[14], can take over and downregulate the potentially dangerous 180–188 reactivity. In addition, disease induction might require the adjuvant effect of other immune stimulatory compounds of whole mycobacteria, creating a pro-inflammatory environment (cytokines such as IFNγ, TNFα) that promotes a Th1-type response.

Monkeys have been immunized using HSP70 and HSP60, with no adverse effects[44]. Similarly, in children after immunization against pertussis, responses to HSP that was present in the vaccine developed without any adverse effects[45]. One exception is the induction of uveoretinitis in rats, using a bovine retina-derived HSP60[46], where immunization required using an emulsion containing complete Freund's adjuvant (mycobacteria). The homologous Yersinia-HSP60, shown to cross-react at the level of the T-cell, did not induce uveoretinitis. By contrast, immunization with a conserved human-HSP60 peptide, which differs from the rat sequence by one amino acid, produced induction of uveitis. Unexpectedly, the peptide also induced the disease after intranasal administration[47].

Possible drugs and therapies

Observations that exposure to HSP molecules or epitopes contributes to disease resistance (e.g. arthritis, diabetes) support earlier observations in various model systems (including diabetes and arthritis) that exposure to microbial flora reduces disease incidence. In rats, a decreased disease resistance was found in animals kept in a sterile environment, whereas re-introduction of bacterial flora restored resistance to induced arthritis[48]. Moreover, oral administration of HSP60 to rats reduces induction of arthritis[49].

Of interest in this respect is a recently introduced slow-acting anti-rheumatic drug called Subreum (Laboratoires OM, Geneva, Switzerland), extracted from selected *E. coli* strains. In RA patients, Subreum is administered orally and has disease-suppressive activity[50]. This drug contains HSP60 and higher quantities of HSP70 and, in rats, has been shown to trigger HSP-reactive T-cells and reduce

adjuvant arthritis when administered orally[51]. Obviously, the mode of action of such a complex compound is difficult to assess. However, it is possible that reactivity to *E. coli*-derived HSPs does contribute to its therapeutic effect. To fully understand this mechanism, the responding T-cells in treated patients need to be tracked, and the immunological phenotype of such responding cells characterized regarding their disease suppressive potential.

The first clinical trial in RA using these compounds was analyzed using a T-cell capture assay. Patients were treated orally with a peptide (amino acid sequence QKRAA) derived from DnaJ, which is a smaller HSP-component of the HSP70 complex, and responding T-cells were 'captured' from the peripheral blood using liposomal MHC–QKRAA complexes. No adverse effects were found with the oral treatment, while QKRAA specific T-cells were skewed towards the Th2 (supposedly regulatory) phenotype[52].

To develop such peptides into therapeutically active drugs for arthritis and other chronic disorders, techniques for manipulating a chronically disregulated inflammatory condition will be required. However, knowing the immunologically dynamic nature of most of these processes, it is likely that the proper immunotherapeutic intervention will, at least, hamper further disease progression. This in itself would be an attractive goal, especially given the relatively non-toxic nature of such peptide (or protein) therapies.

It should then be possible to select various candidate sequences for clinical research, tailoring to every possible genetic (MHC) context.

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