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Minireview

Cell stress induced HSP are targets of regulatory T cells: A role for HSP inducing compounds as anti-inflammatory immuno-modulators?

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Abstract T cell responses to heat shock proteins (HSP) have disease suppressive activities through production of anti-inflammatory cytokines in patients and in models of inflammatory diseases. There is evidence that the anti-inflammatory activity of HSP-specific T cells depends on their recognition of endogenous HSP epitopes as expressed by stressed cells at sites of inflammation. Previously, we have demonstrated that such T cells can be induced by conserved sequences of microbial HSP. Now we propose that drug induced up-regulation of endogenous HSP can contribute to anti-inflammatory T cell regulation.

Keywords: Heat shock proteins; T cells; Immunologic tolerance; Cell stress; Adjuvant arthritis; Immuno-therapy

1. Cell stress leads to HSP up-regulation

Heat shock protein (HSP) expression can be induced in cells through various forms of stress, such as hyperthermia (fever), nutrient starvation, oxidative- and toxic stress and exposure to inflammatory cytokines [1,2]. On the basis of their molecular weights, HSP can be organized in HSP families, including the HSP100, HSP90, HSP70, HSP60, HSP40 and the small heat shock protein families [3]. Most likely because of their critical significance for cell survival under conditions of stress, the primary structures of many individual members of the HSP families remained highly conserved throughout evolution. For some HPS family members expression is mainly constitutive and for others the expression is highly inducible. Depending on the cell type and applied stress, protein expression of inducible HSP family members is increased. The inducible HSP70 family members are among the most prominent proteins up-regulated under stress. In addition, the expression of HSC70 is an example of constitutive HSP expression [1,4,5].

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Abbreviations: HSP, heat shock protein or proteins; HSF, heat shock factor; HSR, heat shock response; FR-WBH, fever-range whole body hyperthermia; GALT, gut associated lymphoid tissue; GGA, geranylgeranyl acetone; PG, proteoglycan; IL10, interleukin-10; EAE, experimental allergic encephalomyelitis; JIA, juvenile idiopathic arthritis

Upon stress, augmented expression of HSP is controlled by a family of heat shock transcription factors (HSFs) among which HSF1 is essential for induction of the so-called heat shock response (HSR). Under physiologic conditions HSF1 is maintained in its monomeric, inactive form by binding to molecular chaperones like HSP70 and HSP90. After stress, HSF1 is released and translocates to the nucleus. Subsequently, trimerization, binding to heat shock elements (HSE) and hyperphosphorylation finally result in transcription of HSPs [6,7]. Since altered HSP function has been associated with the development of several diseases, including immune dysfunctions [8], compound induced modulation of HSP expression became an emerging field of drug development and will be discussed briefly below. We will focus on compounds that can be administered orally, since the intestinal mucosa seems most relevant for induction of T cell immunoregulation. In addition, up-regulation of HSP by hyperthermia will be discussed.

The enhanced HSP induction through compounds which we discuss can obviously be of relevance for aging, as elderly individuals have a known reduced HSP inducibility [9,10]. In this way such artificial HSP enhancement could counter the age dependent increased risk of autoimmune diseases, which is a possible reflection of the growing dysbalances of the immune system during senescence (see Fig. 1).

2. Several drug related compounds are HSP expression enhancers

Activators of HSF can be categorized into HSP inducers and co-inducers. An inducer activates HSF in the absence of additional stress. A co-inducer partially activates components of the HSR, further stress signals are required for full activation of HSP transcription [11,12].

Compounds that inhibit HSP90 can act as HSP inducers. HSP90 inhibitors, like the benzoquinone ansamycins herbamycin A and geldanamycin, have been shown to bind to HSP90 ensuing in a disturbance in the binding of HSP90 to HSF. Then, released HSF is further activated leading to HSP expression [13,14].

Non-steroidal anti-inflammatory drugs (NSAIDS) can co-induce HSP expression. Sodium salicylate induces trimerization and DNA binding of HSF without induction of hyper-

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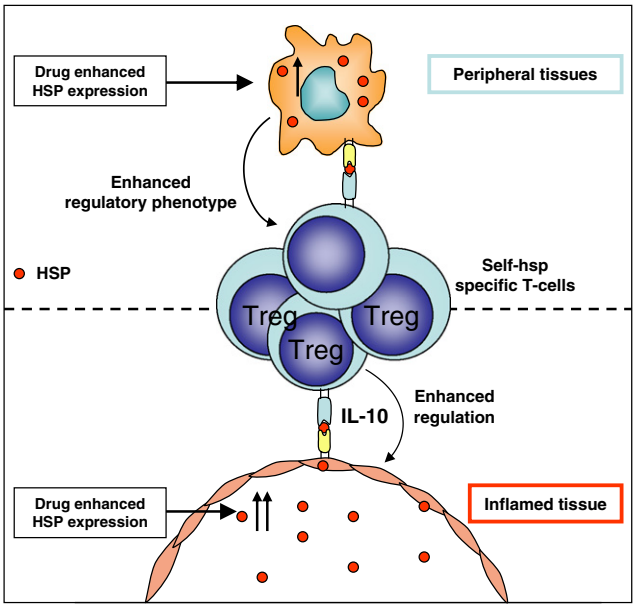

Fig. 1. HSP inducing and co-inducing compounds enhance the expression of HSP in tissue cells, including MHCII (major histocompatibility complex) positive cells which then present HSP epitopes to HSP-specific CD4+ T cells. A variety of mechanisms [39] (microbial HSP exposure in the tolerising gut mucosa; presentation by non-professional antigen presenting cell (APC) lacking co-stimulatory molecules; HSP acting as partial agonist; priming in the presence of stress cytokines such as IL10) are imposing a regulatory phenotype on these CD4+ T cells. These HSP-specific regulatory T cells can recognize induced HSP as presented by stressed cells at the site of inflammation and dampen inflammation by production of suppressive cytokines such as IL10. And also here the HSP inducing compounds can further enhance local HSP expression.

phosphorylation [15]. Indomethacin, in contrast to sodium salycilate, can induce both DNA binding and hyperphosporylation [16]. In the search for non-toxic HSP inducing compounds, ample attention was paid to herbal medicines. Several of them are known for their anti-inflammatory and anti-tumour effects. Celastrol, a member of the triterpene family of compounds, has been shown to activate HSF1 with kinetics similar to that of heat stress. Moreover, Celastrol can amplify heat shock induced HSP expression [17]. The herbal medicine constituents, glycyrrhizin and paeoniflorin, were reported to have an enhancing effect on HSP expression. Glycyrrhizin was acting as a co-inducer. In contrast, Paeoniflorin could induce HSP expression without supplementary stress [18].

Curcumin, the major compound of the seasoning tumeric, was described to increase HSP27, αB crystallin and HSP70 expression in combination with arsenite or heat stress. In contrast, treatment with curcumin alone did not have an effect on HSP expression. The elevated HSP expression was seen in both cultured cells and after in vivo administration, in rat tissues [19]. The hydroxyl amine derivates, arimoclomol and bimoclomol have been suggested to co-induce HSP expression. Arimoclomol induces Hsp70 and Hsp90 expression [20]. Bimoclomol has been reported, to have cytoprotective effects by up regulation of HSPs in several in vivo and in vitro models [21–23]. Anti-ulcer drugs like geranyl geranyl acetone (GGA), rebamipide and carbenoxolone can induce or co-induce HSPs. Among the anti-ulcer drugs GGA, an acrylic isoprenoid, is best described. Oral treatment with GGA protected from ischemia reperfusion induced liver injury via HSP up-regulation [24]. Depending on the cell type GGA can either act as an inducer or a co-inducer. Recently, GGA has been described to bind to HSP70 leading to dissociation of HSP70 from HSF after which free HSF could bind to DNA [25].

3. Hyperthermia induced HSP up-regulation

Hyperthermia pre-treatment can induce thermo-tolerance and has been shown to be protective against various types of injury. HSP up-regulation upon heat shock is extensively studied in multiple cell lines, primary cell cultures and in vivo models. HSP up-regulation and the kinetics thereof seem to depend very much on cell type and heat shock conditions used.

In conventional models for in vivo hyperthermia, a short heat treatment, 15–20 min, is applied at relatively high temperature (42–43 °C). Heat preconditioning of rats at 42 °C for 15 min, attenuates acute ischemic renal injury. In the latter study, protein expression of HSP70 but not HSP27, HSP32 and HSP90 was significantly increased. Administration of quercitin, an HSP70 inhibitor, almost completely reversed protection [26]. Ostberg et al. propose that fever-range whole body hyperthermia (FR-WBH, 39.5–40 °C for 6 h) is a more accurate model for heat stress than the conventional models because it more closely mimics physiological conditions. In this model up-regulation of HSP70 and HSP110 was found in multiple mouse tissues [27]. Likewise, we could detect increased Hsp70 but not Hsp60 expression after 6 h in vivo FR-WBH in spleen cells of mice (unpublished results).

4. HSP inducing compounds can be anti-inflammatory by inhibiting NF-κB

Many compounds that (co-)induce the heat shock response (HSR) are also NF-κB inhibitors. In the non-inflammatory state NF-κB is kept inactive in its cytosolic form complexed to its inhibitor I-κBα. Firstly, proteasome inhibitors prevent degradation of I-κBα by the proteasome and thereby prevent activation of NF-κB and its translocation to the nucleus. At the same time proteasome inhibition increases the cytosolic content of incorrectly folded proteins, thereby inducing the HSR.

Secondly, many inducers of the HSR are known to block the NF-κB pathway directly not only by stabilizing I-κBα but also by inducing I-κBα gene expression [28]. Therefore, various compounds may act in an anti-inflammatory fashion directly in the cell by blocking the pro-inflammatory NF-κB cascade, but simultaneously through the induction of the HSR thereby evoking immune modulation by the provision of targets for HSP-specific regulatory T cells.

5. Endogenous cell stress induced HSP at the site of inflammation are targets of regulatory T cells

The role of heat shock proteins in experimental models of inflammatory diseases was first discovered in the model of adjuvant induced arthritis in rats [29]. Disease associated T cell lines and clones raised against heat-killed mycobacteria, the disease triggering antigen in this model, were found to

recognize a protein cloned from *M. bovis* BCG (Bacille Calmette Guerin) with a molecular weight of 65 kDa. This 65 kDa protein turned out to be the HSP60 of mycobacteria. Interestingly, immunization studies with this recombinant protein in the same rat arthritis model showed that HSP60 immunized animals developed a resistance against the induction of adjuvant arthritis.

Steve Anderton [30] was able to generate a series of T cell lines from rats after immunization with mycobacterial HSP60. These T cells were tested in adoptive transfer experiments and one of them was transferring resistance. This particular T cell line was found to recognize a highly conserved sequence of HSP60 and as expected, this T cell did cross-recognize the mammalian homologue of the mycobacterial protein: rat HSP60. The other T cells that did not transfer resistance were also mapped and none of them cross-recognized mammalian HSP60.

The same was also found for mycobacterial HSP70 [31]. Immunization with mycobacterial HSP70 protected against adjuvant arthritis in rats, and also protected in a model induced with a synthetic oily compound called avridine. Also here mapping studies revealed that only T cells recognizing highly conserved sequences were transferring protection and were cross-responding to the mammalian homologous proteins. Various studies using different routes of exposure to peptides representing conserved HSP60 and HSP70 T cell epitopes, have shown immuno-regulatory effects of these conserved sequences in a multitude of disease models.

From these studies we must conclude that immune exposure to microbial HSP was capable of inducing a regulatory T cell response and that such regulation depended on a T cell response that included a repertoire of (endogenous) self-HSP-specific T cells.

The existence of self-HSP reactive T cells has been demonstrated in many different studies, even in human umbilical cord lymphocytes [32] and in mice transgenic for HSP60 [33]. Apparently, thymus selection allows such a repertoire to develop. Microbial HSP are dominantly immunogenic and frequencies of HSP-specific T cells can be very high following microbial exposure. It makes sense to suppose that this T cell stems from a repertoire that has been selected in the thymus by self (endogenous) HSP peptides. Low affinity interactions for such self-HSP must have stimulated positive selection and must have allowed the self-HSP reactive T cells to survive negative selection. In the peripheral immune system, when the cells have left the central lymphoid organs, microbial HSP can be the full agonists for these T cells compatible with the immuno-dominant character of these HSP. At the same time, when self-HSP is expressed in the periphery, under conditions of cell stress, the self HSP can act as a partial agonist producing a regulated or actively regulatory response in these T cells .

In addition, at the site of inflammation HSP will be up-regulated in all (stressed) cells of which the majority will be tissue cells lacking co-stimulatory molecules. In the absence of co-stimulatory molecules T cells will adopt a state of anergy or regulation [34]. Through these mechanisms HSP-specific T cells can adopt a regulatory phenotype upon antigen recognition in the periphery of the immune system. And this may explain the tolerant state of these cells and that they can be present at high frequencies without causing damage. It is possible that this tendency of these cells to stay in a tolerant or regulatory state is further promoted by mucosal tolerance in the gut associated lymphoid system (GALT) for abundantly present microbial HSP from the gut flora. The tolerance for this collection of HSP in the gut microbiota will be dominated by tolerance for the conserved sequences, as these are shared among the variety of bacterial species present. Thus, there may be a natural focus on the conserved sequences of HSP to drive a repertoire of regulatory T cells.

In various studies HSP reactive T cells have been seen to produce the immuno-regulatory cytokine interleukin-10 (IL10) [35–37]. These cells are most likely regulatory T cells, induced in the periphery and reflecting mechanisms known to contribute to development of peripheral tolerance.

Some studies have also suggested that HSP are antigens seen by natural T regs [38]. Along the same line, studies in children with juvenile idiopathic arthritis (JIA) and adults with rheumatoid arthritis (RA) have indicated the potential of HSP to trigger in T cells the presence of FoxP3 which is a marker for natural T regs.

Thus, given the fact that regulatory T cells induced by microbial HSP have been seen to cross-recognize endogenous HSP through their specificity for conserved HSP sequences, it seems that up-regulated endogenous HSP in stressed cells is the target and the possible initiator of the local regulatory activity of these T cells.

How levels of endogenous expression of HSP at sites of inflammation or elsewhere in the body would influence that capacity of endogenous HSP reactive T cells to maintain or display regulatory activity is unknown.

Experimental manipulation of endogenous HSP expression, during disease or during maturation of the immune system may well lead to a further understanding of the possible contribution of cellular HSP expression to T cell mediated immune regulation.

Some drug compounds are known to have effects on endogenous HSP levels as discussed above. In the following section we will discuss some effects of such compounds in disease models with T cell involvement.

6. Administration of HSP up-regulators and effects on T cells in chronic inflammatory diseases

Earlier we described that T cells can respond to endogenous HSP and that this can lead to an anti-inflammatory phenotype. In other words, HSP up-regulated in inflamed tissues can be targets for HSP-specific regulatory T cells [39]. A relative deficiency of this protective activity in chronic inflammatory diseases can be due to a reduction of the HSP reactive T cell population itself or to a relatively low expression of HSP in the inflamed tissue where HSP are required as targets for triggering the regulatory T cells locally. Elsewhere we have reviewed how administration of (immunization with) HSP can reinforce the regulatory HSP-specific T cells [39]. Here we will present examples from the literature in which (co-)inducers of HSP may have enhanced the expression of HSP to serve as better targets for the HSP-specific regulatory T cells capable of inhibiting T cell mediated chronic inflammatory diseases. Additionally, enhanced expression in non-inflamed tissues also may have reinforced the induction of HSP-specific regulatory T cells.

6.1. Geranylgeranylacetone (GGA)

GGA, a specific HSP (co-)inducer, is given to patients for anti-ulcer therapy, its mode of action being local gastric parietal cell protection. In the experimental model of autoimmune uveoretinitis (EAU) in mice GGA treatment reduced disease which was associated with suppressed T cell responses against the disease inducing antigen, IRBP peptides 1–20 [40]. GGA was also found to be protective in trinitrobenzene sulfonic acid-induced (TNBS) colitis [41], a T cell mediated model for colitis and in dextran sulfate sodium-induced (DSS) colitis in mice [42].

6.2. Curcumin

Curcumin is a biologically active component of turmeric, inhibits NF-κB and is known to be a co-inducer of stress proteins [19]. Recently, a turmeric extract containing curcumin, given orally was shown to inhibit joint inflammation and periarticular joint destruction in a dose-dependent manner in the T cell mediated, experimental model of streptococcal cell wall-induced arthritis [43]. Also, an anti-arthritic effect of curcumin has been reported in one small clinical study of rheumatoid arthritis (RA) and in other studies of arthritis in animals [44,45]. In other patient studies oral curcumin was found to be a safe medication for maintaining remission in quiescent ulcerative colitis [46] and it induced improvements in other forms of inflammatory bowel disease [47].

Recently, it has been suggested that curcumin's reported beneficial effects in arthritis and colitis, but also in other T cell mediated chronic inflammatory disorders like allergy, asthma, atherosclerosis and diabetes, might be due in part to its ability to modulate the immune system, and that these findings warrant further consideration of curcumin as a therapy for immune disorders [48].

6.3. Geldanamycin

Geldanamycin by its direct binding to HSP90 activates the HSR response and induces HSP, and at the same time, inhibits NF-κB by inducing selective degradation (through autophagy) of the IkappaB kinase (IKK). IKK normally phosphorylates I-κB, which then detaches from cytosolic NF-κB enabling translocation to the nucleus of the latter [49].

Administration of 17-allylamino-17-demethoxygeldanamycin (17-AAG), a less toxic derivative of the naturally occurring geldanamycin, in mice immunized with myelin oligodendrocyte glycoprotein peptide to induce experimental autoimmune encephalomyelitis (EAE), prevented disease when given at an early time, and reduced clinical symptoms when given during ongoing disease. T cells from treated mice showed a reduced response to immunogen re-stimulation [50].

6.4. Hyperthermia

In EAE whole body hyperthermia (WBH) has been described to strongly reduce the incidence and severity of EAE [51]. T-cell activation, assessed by the production of interferon gamma (IFN-γ) in response to the EAE inducing immunogen myeloid oligodendrocyte antigen (35–55), was also decreased by the HSR.

Thus, in several disease models endogenous HSP induction has been seen to lead to suppression of disease. Although mechanisms involved could be manifold, in some cases evidence for immuno-modulatory effects was already obtained.

7. HSP induction and the possible contribution of mucosal tolerance to immuno-regulation

Most HSP inducers are administered orally. Therefore, we have to consider the effects of these drugs in inducing local HSP up-regulation in the mucosal tissues.

The mucosal immune system, as present for example in the gastrointestinal tract and airways, guards the major entry sites of the body against foreign antigens, and is thereto especially equipped with the ability to adjust the outcome of the immune response depending on the type of antigen encountered. The specific down regulation of unwanted and unnecessary systemic immune responses to innocuous antigens upon mucosal antigen encounter is called mucosal tolerance and has been described for both the intestinal and the respiratory mucosa [52,53]. This systemic immunological hypo-responsiveness is characterized by a reduced T and/or B cell response to the antigen firstly applied at the mucosal surface. A major mechanism contributing to mucosal tolerance is the active induction of antigen specific regulatory T cells, characterized by the secretion of regulatory cytokines such as IL-10 [53,54].

Mucosal application of bacterial HSPs has been shown to be a potent means of regulatory T cell induction in several models of chronic inflammatory diseases such as atherosclerosis, arthritis and diabetes [31,55]. Since up-regulation of self-HSPs is part of the inflammatory response in these diseases, the induction of self-HSP-cross-reactive T cells upon mucosal application of the highly homologous bacterial HSP might seem an attractive explanation for the development of the mucosally induced regulatory T cells with specificity for otherwise very immunogenic and potentially pro-inflammatory bacterial proteins. Indeed, in several studies of the protective mechanism of HSP, self-HSP cross-reactive T cells were found to produce regulatory cytokines, such as IL-10 and exhibit regulatory activity (Wieten et al. in prep, [31,56]). In addition, mycobacterial HSP70 mediated protection in the experimental mouse model of proteoglycan induced arthritis not only induced IL10 producing HSP-specific T cells, but also proteoglycan-specific T cells that produced IL-10 upon antigen specific stimulation (Wieten et al. unpublished results). This indicated that bacterial-HSP application induced a regulatory phenotype in both HSP specific and disease associated proteoglycan (PG)-specific T cells. These data imply that HSPs might be a therapeutic target for the induction of mucosal tolerance in cases where the auto-antigen is unknown and that microbial HSP can induce antigen specific regulatory T cells.

Various mechanisms have been described to be important in the suppressive function of regulatory T cells. In the case of HSP-specific regulatory T cells it is attractive to speculate that these cells play an important role at the site of inflammation where self-HSP is abundantly present. Mucosal HSP-specific regulatory T cells can dampen the ongoing inflammatory response by local production of IL-10 at the site of inflammation thereby down-regulating pro-inflammatory cells and cytokines. In addition, mucosal regulatory T cells have been shown to use infectious tolerance as a mechanism to expand their regulatory function and convert naïve T cells to differentiate into regulatory T cells [57]. Such a mechanism might explain the observed IL-10 production by arthritogenic T cells in the proteoglycan induced arthritis model and might expand the regulation to different cells or antigens involved in the

inflammatory response. Moreover, these mechanisms might also play a role during the dampening of the inflammatory response under normal conditions thereby preventing excessive inflammation and subsequent organ damage.

For the reasons given above, the exposure to (microbial) HSP at the mucosal surface of the gut, may be a major factor in endowing the repertoire of HSP-specific T cells with regulatory potential. A local action of our HSP inducing compounds at the gut mucosa, in up-regulating endogenous self-HSP in the mucosa may therefore prepare the mucosa for attracting the attention of HSP-specific T cells and for creating a local environment optimal for setting these T cells into a regulatory mode.

8. Clinical development of HSP mediated immune intervention in chronic inflammatory diseases

Studies in JIA have shown that T cell responses to human HSP60 are predominantly found in patients with remitting forms of this disease (oligo-articular). In addition remission was shown to be preceded by raised proliferative responses to human HSP60. In a recent study Kamphuis et al. [37] have shown that such patients do respond to conserved HSP60 sequences and that the T cells of such patients do produce high levels of IL10.

First human trials have confirmed the immuno-modulatory capacity of HSP-derived peptides in chronic autoimmunity. In type 1 diabetes a mammalian HSP60 peptide – diaPep277 – comprizing a known T cell epitope in mice and humans has been used to immunize subcutaneously. This procedure was seen to temporarily arrest inflammation and beta-cell destruction in the pancreas resulting in a decreased insulin dependency. In the peptide specific T cells the inflammatory cytokine phenotype was seen to shift into the development of a regulatory (IL10 and IL4) cytokine phenotype [37,58,59].

The therapeutic potential of mucosal delivery of HSP peptides was indicated by a study in RA, where a DnaJ (HSP70 complex) peptide was given orally. Also here a shift towards a regulatory cytokine profile was noted in the peptide specific T cells. In a recent follow-up phase II trial a favorable clinical response was seen in the peptide treated group especially when the peptide was given in combination with hydroxychoroquine, a second line anti-rheumatic drug known to improve antigen presentation (Albani et al., personal comm.). Although in their infancy, based on first results, these HSP directed antigen specific approaches are promising and may lead to future effective therapies for the control of a wide spectrum of inflammatory diseases. If so, an important issue to address will be whether or not such interventions can lead to permanent remissions of disease. It may well be possible, that the result of such interventions will be determined, amongst others, by the continued exposure of the immune system to the relevant antigens, HSP in this case. As long as we are not aware of the exact factors that have led to the induction of disease or that have prepared the ground for disease producing immune deregulation, we must entertain the possibility that promotion of stress inducibility of endogenous HSP will be essential for the (permanent) establishment of disease suppressing immune regulation. The use of oral HSP up-regulating compounds for this purpose is then an attractive and further to be explored possibility.

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