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Protective effects of hsp70 in inflammation

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Abstract. Inflammation results from the recruitement to a given tissue or organ and the activation of leucocytes, among which the monocytes-macrophages play a major role. These phagocytic cells produce high levels of reactive oxygen species (ROS) as well as cytokines. Whereas both ROS and cytokines have the potential to regulate the expression of heat shock (HS)/stress proteins (HSP), it appears that these proteins in turn have the ability to protect cells and tissues from the deleterious effects of inflammation. The mechanisms by which such protection occurs include prevention of ROS-induced DNA strand breaks and lipid peroxidation as well as protection from mitochondrial structure and function. In vivo, HS protects organs against a number of lesions associated with the increased production of ROS and/or cytokines. In an animal model for adult respiratory distress syndrome, an acute pulmonary inflammatory condition, HS completely prevented mortality. HSP (hsp70 in particular) may also exert protective effects in the immune system by contributing to the processing and presentation of bacterial and tumoral antigens. The analysis of the expression of hsp70 may prove of diagnostic and prognostic value in inflammatory conditions and therapeutical applications are being considered.
Key words. Heat shock proteins; inflammation; reactive oxygen species; nitric oxide; lipid peroxidation; tumor necrosis factor α; interleukin 1; adult respiratory distress syndrome.

Introduction

The first function to be attributed to heat shock proteins (HSP) was thermotolerance, i.e. protection from further exposure to heat shock (HS). One of the major reasons for the subsequent interest of biomedical research in HSP has been the hypothesis that HSP would provide protection not only against HS, but against a wide array of other stresses. Initially, this possibility was investigated with respect to types of stresses which themselves induced a HS response, such as arsenite. More recently, interest has been shown in other aspects of the protective effects of HSP, including their possible contribution to protective immunity. In this review, we will focus on the protective potential of HSP against the deleterious effects, on cells and organisms, of the toxic mediators of inflammation. We will concentrate on the following aspects of HSP-mediated protective effects:

1) protection from toxicity and cell death mediated by reactive oxygen species (ROS) (including NO),
2) protection from cytokines, in particular tumor necrosis factor alpha (TNF-α) and interleukin 1 (Il-1),
3) roles of hsp70 in immune protection, and
4) HSP induction in inflammatory disease and in vivo evidence for HSP-mediated protection in animal models.

Protection from ROS-mediated toxicity and cell death

ROS and inflammation

Inflammatory reactions result from the recruitment and activation of phagocytic cells (monocytes-macrophages [mϕ], neutrophils, eosinophils). These phagocytes are the host's first line of defense against a wide variety of pathogens[8]. Their ability to kill invading microorganisms depends to a large extent on the respiratory burst, a sequence of events whereby the activated phagocyte reduces molecular oxygen, giving rise to toxic species (fig. 1). The primary biochemical event of the respiratory burst is the univalent reduction of oxygen to superoxide (O_2^-) by the complex enzymatic system, NADPH oxidase[1,7]. Subsequent univalent reduction leads to the formation of other oxygen species including hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), the latter reaction being catalyzed by iron. Stimulated neutrophils also release myeloperoxidase (MPO), which catalyzes the conversion of H_2O_2 and Cl^- to the potent oxidant hypochlorous acid (HOCl) as well as singlet oxygen ($^1O_2$). ROS contribute to the amplification of the inflammatory reaction at least in part by providing chemotactic lipids from arachidonic acid metabolism[1,7].

In addition, mϕ, neutrophils and a growing list of other cells generate nitric oxide ($NO^{\bullet}$) when stimulated with a number of phagocytic stimuli such as mycobacterial-derived products, or with lipopolysaccharides (LPS),

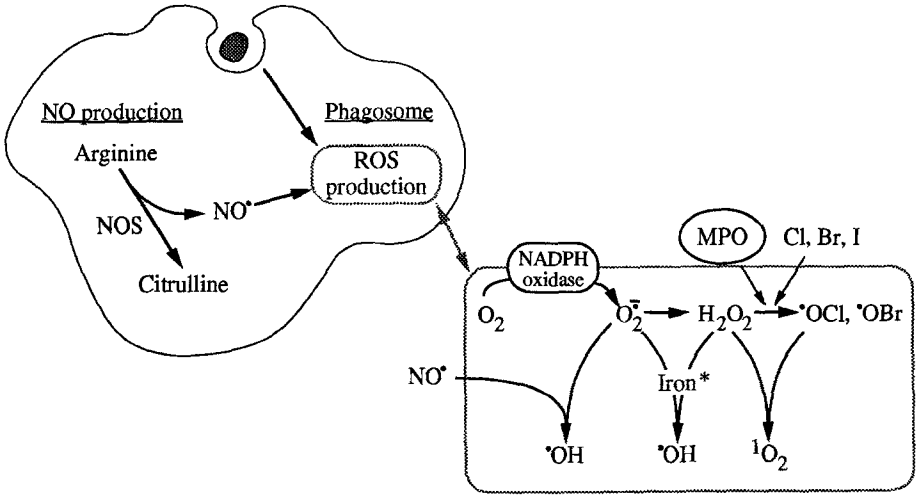

Figure 1. Production of ROS during phagocytosis.

cytokines or calcium ionophores. The nitrogen atom of NO• is derived from the N-guanidino terminal group of the amino acid, L-arginine, whereas the oxygen atom is provided by molecular oxygen (O_2)[31]. Molecular targets of NO• include heme proteins, non-heme iron-sulfur enzymes, DNA and ROS such as O_2^- (ref. 16). Depending on its nature, the target molecule can either be activated (e.g., the heme-protein soluble guanylate cyclase) or inhibited (e.g., the non-heme iron protein ferritin) as a result of reacting with NO•. Alternatively, NO• reacts with other ROS to yield compounds with even more powerful oxidant activity. For example, NO• and O_2^- interact with each other yielding •OH via the peroxynitrite anion $ONOO^-$, an unstable species at physiological pH, which is protonated to give peroxynitrous acid ONOOH which spontaneously decomposes to $NO_2^•$ and •OH (ref. 3). Peroxynitrite has strong prooxidant properties and contributes to free radical-dependent toxicity.

NO is synthesized from its precursors by the NO synthases (NOS). The complementary DNA for various isoforms of the NOS family have been cloned, and their amino acid primary structure sequenced. There are two major subgroups of NOS isoforms, constitutive and inducible. One of the inducible NOS isoforms probably mediates the cytotoxic activity of activated macrophages against numerous pathogens and intracellular microorganisms[33]. Another inducible NOS isoform, which accounts for pathological synthesis of large amounts of NO•, is thought to be the major cause of the refractory hypotension seen in human septic shock[36].

In infection and subsequent inflammation, ROS act primarily as a first line of defense against pathogens, and secondarily as a toxic factor leading to tissue damage and amplification of the inflammatory reaction. On the other hand, they are important second messengers in the effects of cytokines and in the metabolism of arachidonic acid (see below). Furthermore, ROS signal for HSP induction, thus conveying protection where lesions may occur.

Does HS/HSP protect cells from ROS, including NO•?

ROS induce HSP synthesis both in pathogens (prokaryotes) and in host cells (eukaryotes). There are also a number of pieces of evidence indicating that HSP may protect pathogens as well as eukaryotes from the toxic effects of ROS, thus serving similar protective function for both.

A role for HSP in virulence and resistance has been suggested in a number of bacteria, and in parasites, including *Leishmania braziliensis panamensis*[46]. Mutant Salmonella resistant to oxidative stress overexpress a number of HSP, whereas bacteria unable to overexpress HSP (normally induced during the infection of mϕ and the resulting oxidative stress) are able to survive within mϕ, but lose their virulence[5,6]. Log-phase, avirulent *L. donovani*; promastigotes are more sensitive to H_2O_2 than stationary, virulent, promastigotes[50]. Pre-exposure of avirulent promastigotes to HS induces a level of resistance to H_2O_2 similar to that found in stationary promastigotes. Both heat-shocked and stationary promastigotes display increased hsp70 transcription, and transcriptional activity is a prerequisite for HS-induced H_2O_2 resistance (prevention of this effect by actinomycin D).

Our own work has concentrated on the protective effects of HSP against ROS-induced toxicity in host cells such as mϕ. In the human premonocytic line U937 we established that preexposure to HS partially, but significantly, protects these cells from subsequent exposure to hydrogen peroxide (H_2O_2)[40]. We also found that preexposure to HS prevented the inhibition of oxidative phosphorylation induced by the ATPase inhibitor oligomycin in the mutant line DS7 (a strictly aerobic glycolytic mutant of the Chinese hamster lung fibroblast line 023)[38].

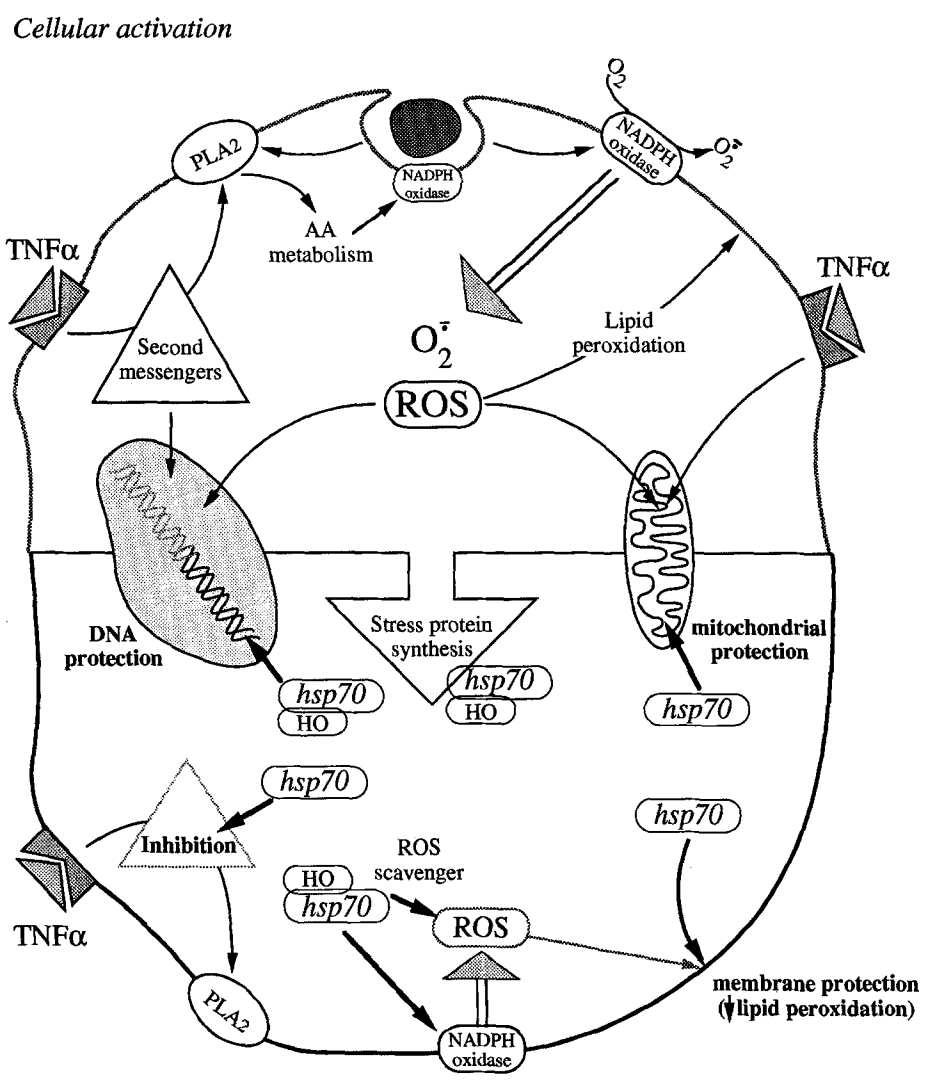

Figure 2. ROS, TNF-α and hsp70 in mϕ activation, oxidative damage and protection. Large amounts of ROS produced during phagocytosis via activation of the respiratory burst enzyme NADPH oxidase induce lipid peroxidation (membrane damage) and DNA damage, and alter mitochondrial respiration. Hsp70 may exert protection at the same levels (membranes, DNA, mitochondria). HO, often upregulated along with hsp70, may contribute to DNA protection and acts as a radical scavenger. Heat shock inhibits NADPH oxidase activity and HSP render the enzyme thermotolerant. TNF-α is also produced during phagocytosis, activates PLA_2 and further enhances the activity of NADPH oxidase. Hsp70 protects cells from the cytotoxic effects of TNF-α and inhibits the second messengers involved in its effects.

Extending these studies to the investigation of the targets and the mechanisms relevant to HSP-induced cellular protection, we analyzed in human mϕ the effects of HS on:

1) lipid peroxidation,
2) mitochondrial function and structure and
3) DNA, and
4) the toxic effects of NO (although for the latter the data available have been essentially generated by other groups in other cells).

Figure 2 schematically presents mϕ activation and hsp70-mediated protection.

Lipid peroxidation

Lipid peroxidation can be examined as a downstream measurement of damage to cellular constituents induced by diffusible peroxides. Polyunsaturated fatty acids that contain multiple carbon double bonds are subject to oxidative attack. Peroxidation is initiated by hydrogen abstraction by $^{\cdot}OH$ and potentiated by other ROS, but not by the lipid insoluble O_2^- (ref. 13). A conjugated diene results that reacts with O_2 to form a peroxy radical $ROO^{\cdot}$, which abstracts $H^{\cdot}$ from another fatty acid to establish an autocatalytic free radical chain

reaction. Using a method previously described[42], we determined lipid peroxidation in the human premonocytic line U937. We found that the level of thiobarbituric reactive substances (TBARs) induced by H_2O_2 was significantly reduced by preexposure to HS (table 1). This protective effect was also observed in human monocytes.

Lipid peroxidation leads to membrane damage and subsequent alterations in calcium homeostasis, which are essential components of cell death (whether occurring by necrosis or apoptosis). Levels of intracellular calcium play a key role in ROS-mediated cell toxicity and death. However, protection induced by HS occurs distal to the entry of calcium into the cells[23,40] and thermotolerance is induced in the virtual absence of calcium[40]. The protective effects of HS from lipid peroxidation are thus likely to occur previously to calcium entry.

HS protects human cells from lipid peroxidation.

H_2O_2(mM)	% Protection (n = 4)
5	18.7 ± 8.3
10	24.7 ± 8.0

Lipid peroxidation (MDA [malondialdehydes] in μmol/g of proteins) was measured in U937 cells (a human premonocytic line) exposed to H_2O_2 as described (Richard et al.[42]). We determined % protection mediated by preexposure to HS (44 °C, 30 min).

% protection was calculated as follows:

$$\% \text{ protection} = \frac{\left(\begin{matrix}\text{lipid peroxidation in} \\ H_2O_2\text{-treated cells}\end{matrix}\right) - \left(\begin{matrix}\text{lipid peroxidation in } H_2O_2\text{-treated} \\ \text{cells preexposed to HS}\end{matrix}\right)}{\text{lipid peroxidation in } H_2O_2\text{-treated cells}}$$

Mitochondria

The possibility that mitochondria represent an important target for the protective effects of HS against ROS relies on a number of observations to be reviewed elsewhere (Polla et al. in preparation). We are currently investigating this possibility using a newly-described cytofluorimetric method for analysing mitochondrial membrane potential and the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide JC-1 (ref. 10), in U937 cells preexposed or not to HS then exposed to H_2O_2. Preliminary results indicate that mitochondria may be a major target for the protective effects of HS (no alterations in mitochondrial membrane potential in pre-heated cells) (Polla et al., in preparation). These observations are consistent with the protective effects of HS (hsp70) against TNF-α (see below). Indeed, the first effect which may be observed after incubation of cells with this cytokine is an increase in mitochondrial production of O_2^- along with an alteration in membrane potential, which is not observed in TNF-α-resistant cells (our unpubl. data).

DNA

During exposure to stress, HSP (hsp70 in particular) enter the nucleus, and are exported back to the cytoplasm during recovery from stress. HS, along with HSP, also induces DNA fragmentation and apoptosis[45], whereas thermotolerant cells (in which HSP expression is increased) are more resistant to apoptosis[32]. These and other observations led us to address the issue of whether DNA could represent a major target for the protective effects of HSP (protection from radical-induced genotoxicity). Using the method described by Birnboim et al.[4], we found that the frequency of alkali labile sites induced by the DNA-damaging agent bleomycin in the human premonocytic line U937 was significantly reduced by preexposure to HS[39] (Polla et al., in preparation). This protective effect was also observed when using agarose gels to visualise DNA: preexposure of the same cells to HS prevented both internucleosomal fragmentation as induced by HS itself (apoptosis) and the appearance of DNA smears (random DNA strand breaks, alkali-labile sites) as induced by H_2O_2 or bleomycin (Polla et al., in preparation).

Along these lines, we found that when we exposed the cells to various stresses (such as erythrophagocytosis or cadmium) inducing not only the classical HSP but also the oxidation-specific stress protein heme oxygenase (HO), we were unable to induce either DNA fragmentation or alkali labile sites and strand breaks[34]. These results suggest that HO (and/or ferritin) may play a major role in stress-induced protection against DNA damage. In order to test this hypothesis, HO should be constitutively overexpressed in cells or tissues and the prevention of the induction of radical-mediated genotoxicity should then be examined.

HS-mediated protection from NO·

Little is known at the present time about the specific protection by HS/HSP against the toxic effects of NO·. Data available (see below) suggest that HS inhibits the activity of the inducible NOS, as it has been demonstrated for another ROS-generating enzyme, NADPH oxidase[27].

Protection from cytokines

There are numerous ways in which ROS are involved in inflammation, one of them being the link between ROS and cytokines. Cytokines such as Il-1 and TNF-α contribute to acute and chronic inflammation by activating phospholipase A_2(PLA_2), thus leading to the generation of lipid mediators of inflammation and further ROS, by modulating the production of extracellular matrix proteins and by activating a number of other cells. Binding of TNF-α to its receptor results in a rapid rise in intracellular (mitochondrial) ROS, whilst IL-1 may

exert similar effects under certain conditions; both TNF-α and IL-1 may prime the respiratory burst enzyme NADPH oxidase for increased O_2^- production. Links between cytokines and HSP other than those discussed below are reviewed in detail by T. Hall (this issue).

TNF-α

TNF-α is a multifunctional cytokine with a crucial role in immune and inflammatory reactions. By analogy with ROS, TNF-α exerts both beneficial effects by contributing to anti-infectious defenses and killing tumor cells and deleterious effects by leading to tissue damage and organ wasting.

The protective role of HSP against TNF-α-mediated cytotoxicity has been particulary studied in tumor cells but could be extended to other systems. Jäättelä et al. demonstrated that a short pretreatment of mouse fibrosarcoma cells (WEHI) at 39–42 °C decreased TNF-α-mediated lysis by approximately 50% (ref. 18). The effect was maximal when TNF-α was added 1 hour after HS, and then gradually declined, being almost undetectable after 2 days. This effect was found roughly to coincide with the kinetics of HSP induction in the tested cells. The same author then directly investigated the involvement of HSP themselves in cell resistance to TNF-α-mediated cytotoxicity. The human hsp70 and hsp27 genes were stably transfected into the highly TNF-sensitive WEHI-S tumor line in both sense and antisense orientations. Only cells overexpressing hsp70 were protected from the lytic effects of TNF-α, overexpression of hsp27 having no effect[19,20].

An interesting correlation was observed between TNF-α-sensitivity, TNF-α-induced arachidonic acid metabolism and hsp70 synthesis[21]. Indeed, an inhibition of arachidonic acid metabolism was observed in tumor cells overexpressing hsp70 (but not hsp27)[21]. Whatever the precise mechanism of hsp70-associated TNF-α-resistance, it was found to interfere with the TNF-α-induced signal transduction pathway at a step after receptor binding but before activation of PLA_2. In vivo, most of TNF-α is produced by activated mϕ. Thus, hsp70 induced in these cells by TNF-α may provide the same cells with a protective mechanism against the potential autotoxicity of TNF-α[12,24]. The possibility that the protection against TNF-α is mediated, at least in part, by an inhibition in the production of NO· or of its effects, also deserves consideration.

IL-1

The protective effects of HS/hsp70 against IL-1 are particularly relevant to diabetes. Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disorder ultimately resulting in the destruction of the pancreatic β cells, which causes insulin deficiency. Amongst immune cells, mϕ are the first to invade pancreatic islets during the development of the disease. Mϕ-derived IL-1 (which is usually not cytotoxic) becomes toxic for the β cells probably via a mechanism involving ROS[9]. Recent evidence indicates that inducible NOS activity, through excessive production of NO by mϕ, is an essential component for the destruction of pancreatic islet[26].

In contrast to many other cells in which IL-1 does not induce a stress response, we found that exposure of β cells to IL-1 induces both hsp70 and heme oxygenase (HO)[15]. Since β cells are characterized by particularly low levels of endogenous antioxidant enzymes (scavengers), we suggested that hsp70 induced by IL-1 may compensate this defect by exerting a novel antioxidant mechanism[15]. In agreement with this hypothesis, Margulis et al. demonstrated that purified hsp70 introduced into β cell via liposomes renders these cells fully resistant to subsequent exposure to Il-1[28]. On the other hand, HS treatment reduced the lysis of rat islet cells induced by the NO-donor sodium nitroprusside, and prevented intracellular NAD^+ depletion (Bellman et al., pers. commun.). However, this protective effect did not seem to involve de novo protein biosynthesis (not affected by cycloheximide).

Immune protection

Many types of chronic inflammation are initially induced by antigen-specific activation of the immune system, and it has become apparent that HSP are immunodominant antigens of a variety of infectious agents[49]. Upon phagocytosis of these microorganisms, HSP may be up-regulated in the pathogen as well as in the host, and then antigenicially presented by the phagocytosing cell. Parastic diseases such as malaria, schistosomiasis and leishmaniasis as well as the bacterial disease leprosy and tuberculosis are all associated with both T- and B-cell responses to hsp70[49](discussed in more detail by Maresca, this issue).

The HSP induced in both host and invader may represent a common target, enabling organisms to respond to a variety of diverse infections. However, this induction is also a potential danger, which may be molecular mimicry and autoimmune disorders. For example, patients suffering from systemic lupus erythematosus (SLE) have been shown to possess autoantibodies to members of the hsp70 family[30]. The role of these autoantibodies remains speculative, but they could contribute to disease activity.

Some of the protective functions of hsp70 may depend on its normal cellular role, i.e. the molecular chaperoning of denatured or improperly folded proteins and their translocation across membranes. Several members of the hsp70 family (including the constitutively expressed hsp70 and the glucose-regulated protein grp78) have been described as participating in different steps of antigen processing and presentation[47]. DeNagel and

Pierce suggest that HSP could participate in both the assembly of MHC class II-peptide complexes and in the subsequent presentation of antigen[11]. We demonstrated that members of the hsp70 family optimize antigen processing and presentation and thus contribute to an efficacious immune response[29]. The more efficient the initial immune response to an infectious agent, the less risk there may be that this response will evolve into chronic inflammation; thus, upregulation of hsp70 and the concomitant increases in immune response may provide protection against chronic inflammation.

It has also been established that HSP (hsp70) play important roles in tumor immunity, both by presenting tumour-specific peptides and by controlling the activity of products of oncogenes involved in carcinogenesis. The former aspect of the protective effects of hsp70 in cancer is discussed in detail by Srivastava (this issue). The latter may be illustrated by the interactions of hsp70 with p53 (for review, see ref. 12a).

Mutations in the *p53* gene are among the most common genetic alterations in many forms of cancer[17]. Usually these mutations induce a conformational change, which contributes to the stability of the mutant protein p53, which may be found in a tight complex with hsp70[37]. This conformational change can alter the growth-suppressive function of p53. Using both wild-type and mutant p53 with a temperature-sensitive phenotype, Hainaut and Milner demonstrated that hsp70 complexes only mutant p53, not wild-type p53, in manner that requires the 28 carboxyl-terminal amino acids of p53[14]. Moreover, p53-hsp70 complexes can occur after post-translational switching from the wild-type to the mutant phenotype, indicating that complex formation can result from a change in the tertiary structure of pre-existing p53. While this switch is rapid and ATP-independent, the reverse switch from the mutant to the wild-type phenotype is slower, requires ATP hydrolysis, and involves hsp70. Hsp70, by regulating the folding of the mutant (promoter) from the wild-type (suppressor) p53 phenotype might be an important effector in the cellular pathways controlling normal cell growth.

Recent work exploring the uses of HSP as vaccines has produced some exciting discoveries. A study by Barrios et al. showed that vaccination using hsp70 as a carrier molecule for peptides derived from the circumsporozoite proteins of *P. falciparum* (the causative agent of malaria), elicited high titres of IgG. This resulted in protection for at least a year, which was T-cell dependent, as it was not seen in athymic mice[2]. Richter et al. investigated the high level of resistance to *S. Mansoni* in mice vaccinated with irradiated cercariae, and found that protection was related to the production of antibodies, a large percentage of which recognized hsp70[43,44]. These findings may provide clues as to the role of hsp70 in the immune system, and how its functions may be used to design future vaccines. These issues are discussed in more detail by G. del Giudice (this issue).

In vivo induction of HSP and protection

Up to now, pulmonary inflammation has been the subject of most of the interest devoted to HSP and inflammation in vivo. Alveolar macrophages (AM) from patients with a number of inflammatory lung diseases produce excess amounts of ROS. Based on the observation that exogenous ROS induce HSP, we hypothesized that endogenous production of ROS during disease may induce HSP in AM as an autoprotective mechanism[24]. We therefore investigated the ex vivo HSP synthesis by human AM recovered during bronchoalveolar lavage performed for diagnostic purposes in pulmonary inflammation of undefined etiology. In a series of 17 patients, we observed high levels of spontaneous HSP synthesis by the AM from 2 of them[41]. The alveolar inflammation of these two patients was characterized by large number of alveolar eosinophils. Eosinophilic inflammation is the hallmark of a number of specific diseases, including asthma and some parasitic infections. We and others are currently investigating the relationship between eosinophils and HSP induction in disease.

One model disease to consider with respect to the in vivo protective effects of HSP is adult respiratory distress syndrome (ARDS). ARDS is an acute pulmonary inflammation which is initiated by a number of diverse triggers and is characterized by a massive generation of ROS within the lung, along with activation of PLA2 and production of TNF-α. Despite major efforts to develop new therapeutic approaches, ARDS remains lethal for about 50% of the patients. The implications of HSP in this disease have been recently reviewed[22]. Circulating inflammatory cells of some patients suffering from ARDS express increased levels of hsp70 (ref. 25). On the other hand, in a rodent model for ARDS, developed by Slutsky's group, in which intratracheal administration of PLA2 leads to a reproducible mortality of 30% (ref. 48), preexposure of the animals to HS, with massive induction of hsp70 within the lung, was associated with a remarkable decrease in pulmonary inflammation and the complete prevention of lethality[48]. The enhanced survival induced in septic animals by experimental hyperthermia probably results from a decreased expression of the inducible NOS (Villar et al., pers. commun.), an effect which is observed in pre-heated AM, as compared to control AM, upon exposure to endotoxin. However, it has not yet been established in this model whether the protection that hyperthermia exerts against potential deleterious effects of $NO^{\bullet}$ actually involves HSP synthesis. The use of transgenic animals overexpressing hsp70 may contribute to addressing this important issue.

Atherosclerosis is another disease in whose pathogeny the increased production of ROS and TNF-α by inflammatory mϕ plays a key role. There is evidence, in atherosclerosis as in ARDS, both for an increased expression of hsp70 and for a protective effect of hsp70 against disease-associated lesions[35]. The number of inflammatory diseases in which these observations may hold true will probably be growing in the next few years.

In conclusion, we propose that HSP (hsp70 in particular) should prove to be an important new diagnostic marker for inflammatory diseases in the near future. Once the precise biochemical mechanisms for the protective effects of hsp70 are understood, therapeutic applications in inflammatory conditions associated with increased production of ROS and TNF-α should rapidly follow. The beneficial effects of fever, which have been known for many years, may well be related to increased HSP expression.

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