Cytokine function of heat shock proteins

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HEAT SHOCK PROTEINS (HSPs) are the most phylogenetically conserved proteins present in all prokaryotes and eukaryotes (20, 27, 45). Traditionally, HSPs are regarded as intracellular molecules. With the availability of recombinant bacterial and human HSPs, there has been an intense interest in the extracellular function of HSPs in recent years. It has been shown that HSPs are potent activators of the innate immune system, capable of inducing proinflammatory cytokine production by the monocyte-macrophage system and the activation and maturation of dendritic cells (antigen-presenting cells) in a manner similar to the effects of lipopolysaccharide (LPS) and bacterial lipoprotein, e.g., via CD14/Toll-like receptor2 (TLR2) and CD14/TLR4 receptor complex-mediated signal transduction pathways. However, recent evidence suggests that the reported cytokine effects of HSPs may be due to the contaminating LPS and LPS-associated molecules. The reasons for previous failure to recognize the contaminant(s) as being responsible for the reported HSP cytokine effects include failure to use highly purified, low-LPS preparations of HSPs; failure to recognize the heat sensitivity of LPS; and failure to consider contaminant(s) other than LPS. Thus it is essential that efforts should be directed to conclusively determine whether the reported HSP cytokine effects are due to HSPs or to contaminant(s) present in the HSP preparations before further exploring the implication and therapeutic potential of the putative cytokine function of HSPs.

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Invited Review
served ATPase domain binds ADP and ATP tightly and hydrolyzes ATP, whereas the COOH-terminal domain is required for polypeptide binding (20, 27). The HSP90 family includes the cytosolic Hsp90 (α and β) and the ER form, gp96 (gp94). Glucose-regulated proteins (grp) such as grp78 and grp94/gp96 are molecular chaperones in the ER that are upregulated in response to glucose starvation and other stressful stimuli that disrupt protein folding in the ER (30, 70). For more detailed description of HSPs, we refer the reader to excellent reviews of the subject (20, 27, 33, 45, 55, 70, 87).

Bacterial HSPs, particularly Hsp60 and Hsp70, are highly immunogenic, capable of inducing antibody production and T-cell activation (92). The antibodies and T cells against bacterial Hsp60 and Hsp70 also recognize mammalian Hsp60 and Hsp70, respectively, due to cross-reactivity (36). These anti-Hsp60 and anti-Hsp70 antibodies and T cells induce tissues and cause inflammatory reactions. Thus Hsp60 and Hsp70 have been implicated in the pathogenesis of a number of autoimmune diseases and inflammatory conditions such as type 1 diabetes (1, 19), Crohn’s disease (77), atherosclerosis (56, 87), and juvenile chronic arthritis (60, 64).

HSPs also play important roles in antigen presentation, cross-presentation, and tumor immunity (43, 72, 73). HSPs of the cytosol, such as Hsp70 and Hsp90, and of the ER, such as gp96, bind antigenic peptides generated within the cells and are part of the endogenous pathway of antigen presentation by the major histocompatibility complex (MHC) class I molecules (32, 43, 74). Peptides that are chaperoned by HSPs when released extracellularly are taken up by antigen-presenting cells via α2-macroglobulin receptor (CD91)-mediated endocytosis, resulting in representation by the MHC molecules (9, 43, 75). Vaccination of mice with Hsp70, Hsp90, and gp96 isolated from murine tumor cells elicits immune response sufficient for tumor rejection and suppression of metastatic tumor progression (73). This tumor immunity results from tumor-derived peptides associated with the HSPs, rather than from the HSPs themselves (80).

**HSPs AND CYTOKINE FUNCTION**

The above-described molecular chaperone function and presentation of antigens depend on the peptide-binding properties of HSPs. Recent studies suggest that HSPs may also have potent cytokine-like function independent of peptide binding. HSPs such as Hsp60, Hsp70, and gp96 from a variety of sources, including purified preparations from bacterial (23, 65, 69) and mammalian (4, 10, 52, 68, 71, 83) sources as well as recombinant bacterial (15, 22, 37, 39, 53, 54, 66, 81, 86, 91) and human (5, 6, 13, 17, 18, 37, 38, 51, 81, 82) products, have been shown to be potent activators of the innate immune system (85). These HSP preparations have been shown to induce the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-12 and the release of nitric oxide (NO) and C-C chemokines by monocytes, macrophages, and dendritic cells. They also induce the maturation of dendritic cells as demonstrated by the upregulation of MHC class I and II molecules, CD86, CD40, etc. (10, 21, 52, 68, 71, 86). The Hsp60 and Hsp70 preparations purified from bacterial sources or from recombinant bacterial and human products are capable of inducing the above effects in concentrations ranging from <1 μg/ml to a few micrograms per milliliter, whereas Hsp70, Hsp90, and gp96 isolated from mouse liver require concentrations that are one to two orders of magnitude higher (e.g., 10–100 μg/ml). These HSP cytokine effects, compared with their molecular chaperone function, are unique in that they require no HSP-associated peptides, no ATP hydrolysis, no cofactors, and no protein complex assembly. A new term, “chaperokine,” has been coined for HSPs to indicate their dual functions as molecular chaperones and cytokines (5).

Furthermore, the observed HSP cytokine effects are mediated via the CD14/Toll-like receptor (both TLR2 and TLR4) complex signal transduction pathways leading to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), i.e., ERKs (p42 and p44 extracellular signal-regulated kinases), JNK (c-Jun NH2-terminal kinase), and p38 kinase (5, 6, 15, 18, 38, 51, 81–83). The CD14 and TLR receptor complexes are pattern recognition receptors involved in the innate immunity for the pathogen recognition and host defense (3, 47). CD14, the lipopolysaccharide (LPS) receptor, is a glycoprophatidyl inositol (GPI)-anchored membrane protein lacking transmembrane and intracellular domains (28, 76). TLRs are type I transmembrane proteins with an extracellular domain containing a leucine-rich repeat and a cytoplasmic domain analogous to that of the IL-1 receptor (IL-1R) family (47, 67). An adapter protein, MyD88 (myeloid differentiation protein 88), binds to the Toll/IL-1R homology (TIR) motif through its own TIR motif, whereas a death domain on its COOH terminus recruits IL-1R-associated kinase (IRAK) to the complex (48). IRAK is then autophosphorylated and released from the complex to bind TRAF6 (TNF receptor-associated factor 6), which can then activate either the NF-κB or the MAPKs (47, 49). Together with CD14 and an accessory protein MD2, TLR4 initiates signaling cascades in response to LPS, whereas TLR2 initiates the signal cascades in response to bacterial lipoprotein, Gram-positive bacteria, yeast, and spirochetes (2, 14, 34, 47, 67).

The reported activation of the innate immune system by HSPs, as described above, has been hailed as an important new function of HSPs with broad biological significance. The induction of proinflammatory cytokines by Hsp60 and Hsp70 may contribute to the pathogenesis of autoimmune diseases and chronic inflammation (55). Chlamydial Hsp60 frequently colocalizes with human Hsp60 in macrophages of atherosclerotic plaques (39). Induction of proinflammatory cytokine release from macrophages by chlamydial Hsp60 would provide a potential mechanism by which chlamydial infections may promote atherosclerosis and precipitate acute ischemic events (37, 39). Likewise, the activation and maturation of dendritic cells by gp96 may be responsible for the gp96-induced tumor immunity by inducing both the innate and adaptive immune responses (50). Thus it has been proposed that through their cytokine function, HSPs may serve as a “danger signal” to the innate immune system at the site of tissue injury (17, 85) and that HSPs could be the endogenous ligands for the TLR2 and TLR4 (51, 82). In fact, HSPs are considered to be the prototype of endogenous ligands for Toll-like receptors (12). There is considerable interest to further explore the implications and therapeutic potential of these HSP cytokine effects (7, 55, 88).
HSP AND CYTOKINE FUNCTION

HSPS VS. CONTAMINANTS

The reported HSP cytokine effects are similar to those of LPS and bacterial lipoprotein. Because the recombinant bacterial and human HSPs are produced by *Escherichia coli* expressing HSP cDNAs, the final preparations may be contaminated with bacterial products. Likewise, HSP preparations isolated from bacteria or murine tissues are also frequently contaminated with LPS. The fact that various HSPs all have similar effects and share the same CD14/TLR2 and CD14/TLR4 receptor complexes is of considerable concern.

Ample examples exist in the literature demonstrating how contaminants can lead to misleading conclusions. For example, in 1998 with the use of the commercially available LPS preparation, it was first reported (90) that TLR2 mediated the LPS-induced activation of NF-κB and could be the long sought after LPS signal transducer. There followed a period of uncertainty regarding whether TLR2 or TLR4 was the LPS signal transducer (35, 59, 61). In 2000, it was then demonstrated that TLR2 could not mediate cellular response by repurified commercial preparations of LPS (31, 78). In 2002, two lipoproteins (Lip12 and Lip19) extracted from *E. coli* LCD25 LPS were identified to be the major components responsible for TLR2-mediated cell activation in the commercial LPS preparations (40). Thus failure to recognize the presence of lipoproteins in the commercially available LPS preparation led to the erroneous attribution of lipoprotein signal transducer, TLR2, as the LPS signal transducer (35, 90).

Investigators are cognizant of the possibility of contamination, particularly LPS, and have attempted to rule out the possibility of LPS contamination being responsible for the observed HSP cytokine effects. Most studies have used two criteria: first, LPS is resistant to heat inactivation (5, 17, 18, 23, 38, 65, 66, 86), and second, LPS effects are inhibitable by polymyxin B (5, 17, 18, 38, 65, 66, 86). Other less frequently used criteria include the effect of anti-HSP antibodies (15, 65) and other LPS inhibitors such as lipid IVa (5), LPS (10, 52), or lipid A (18) from *Rhodopseudomonas spheroides*. Because the observed HSP cytokine effects were heat sensitive, either not inhibitable or only partially inhibitable by polymyxin B, not inhibitable by other LPS inhibitors, and/or inhibitable by anti-HSP antibodies, it was concluded that the observed HSP cytokine effects could not have been due to LPS contamination. However, doubts about these criteria have been raised. Wallin et al. (85) noted that highly purified murine liver Hsp70 had no cytokine effects even at concentrations as high as 200–300 μg/ml. On the other hand, a LPS-contaminated preparation at Hsp70 concentrations as low as 50–100 ng/ml caused cytokine effects that were heat sensitive and were not inhibitable by polymyxin B.

Recent studies in which HSP preparations essentially free of LPS were used suggest that the previously reported cytokine function of HSPs may be due to the contaminants. Bausinger et al. (11) reported that LPS-free recombinant human Hsp70 (rhHsp70) did not induce the activation of dendritic cells. Gao and Tsan (24, 25) demonstrated that LPS was heat sensitive (Fig. 1) and that the ability of commercially available rhHsp70 to induce TNF-α production was entirely due to the contaminating LPS (24), whereas that of rhHSP60 was due to contamination by LPS as well as LPS-associated molecules (25). Reed et al. (63) reported that the activation of NF-κB and the production of NO by gp96 were due to LPS contamination. Importantly, all of these investigators demonstrated that these highly purified, essentially LPS-free HSPs retained their normal molecular chaperone functions or ATPase activity (11, 24, 25, 63). Thus failure of Hsp60, Hsp70, and gp96 to induce cytokine or NO production by macrophages or to activate antigen-presenting cells was not due to defective HSPs as a result of purification.

The fact that LPS is sensitive to heat inactivation (24, 25, 84) has not been widely appreciated. Most investigators are not aware that macrophages are extremely sensitive to LPS. LPS at a concentration of 0.1–0.2 ng/ml is sufficient to maximally induce TNF-α release from murine macrophages (24). However, in most studies LPS was used at concentrations ranging from 10 to 500 ng/ml to test for heat sensitivity (5, 17, 18, 23, 38, 65, 66, 86). At these concentrations, even if heat treatment inactivated 99% of the LPS used in the studies, there would still be sufficient residual LPS to induce TNF-α release, giving the impression that LPS was heat resistant. Thus, unless one uses an LPS concentration similar to the LPS concentration present in the HSP preparation, the result could be misleading.

Although LPS may be the most frequent contaminant, non-LPS contaminant(s) capable of inducing proinflammatory cytokines may also contribute to the reported cytokine effects of HSPs. Gao and Tsan (25) showed that 50% of the TNF-α-inducing activity of the commercially available rhHsp60 (no. NSP-540; StressGen Biotechnologies, Victoria, BC, Canada) was due to non-LPS contaminant(s) that was heat sensitive but not inhibitable by polymyxin B. The presence of non-LPS contaminant(s) could partially explain previous reports that the observed cytokine effects of HSPs were either not inhibitable...

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**Fig. 1.** Effect of heat inactivation on endotoxin activity and tumor necrosis factor (TNF)-α-inducing activity of lipopolysaccharide (LPS). A stock solution of LPS at 4 ng/ml was heated in a boiling water bath for 1 h. *A:* endotoxin activities of the non-heated LPS and heated LPS were determined using the Limulus amebocyte lysate assay. *B:* murine macrophages were treated with LPS or heated LPS at the indicated concentrations for 4 h. TNF-α concentrations in media were then determined. Values represent means ± SD of 3 experiments. *P < 0.05 vs. non-heated LPS. [Reprinted from Gao and Tsan (25) with permission.]
or only partially inhibitable by polymyxin B (5, 17, 18, 38, 65, 66, 85, 86).

It is thus important to determine conclusively whether HSPs can activate the innate immune system before further investigating the implication and therapeutic potential of the reported cytokine effects of HSPs. Only highly purified HSP preparations that are essentially free of LPS contamination should be used. Such low LPS preparations for rhHsp60 and rhHsp70 are commercially available. However, it is important to measure the endotoxin activity of each preparation by using the Limulus amebocyte lysate assay beforehand to ensure that the preparation is essentially free of LPS contamination. If the Hsp60 or Hsp70 preparation is contaminated with LPS, then LPS and LPS-associated proteins can be efficiently removed with the use of commercially available polymyxin B agarose gel without affecting the chaperone functions of HSPs (11, 24, 25). Because gp96 binds LPS tightly, the novel purification procedures as reported by Reed et al. (63) should be used.

AREAS OF UNCERTAINTY

Although recent evidence suggests that the cytokine effects that have been reported in the last 10 years may be due to the effects of contaminants such as LPS and LPS-associated molecules (11, 24, 25, 63), a number of uncertainties exist. It is not clear why anti-Hsp60 antibodies could inhibit Hsp60-induced proinflammatory cytokine production by macrophages (15, 65). It is possible that HSPs bind LPS and that binding of anti-HSP antibodies to HSPs interferes with the interaction of HSP-bound LPS with the CD14/TLR4 receptor complex, thus inhibiting the effect of LPS. There is supporting evidence that Hsp70, Hsp90, and gp96 bind LPS (16, 63, 79).

If the reported cytokine effects are conclusively shown to be due to contaminants, not due to HSPs themselves, then one important question will be whether HSPs have any effect on the innate immune system. Habich et al. (26) have described a macrophage receptor for Hsp60 that is specific and saturable, suggesting that Hsp60 may have some effect on macrophages that is different from the reported cytokine effects mediated through the CD14/TLR4 receptor complexes. Reed et al. (63) reported that a highly purified, low-LPS preparation of gp96 was able to elicit a marked increase in ERK phosphorylation but not the activation of p38 and JNK, an effect distinct from that of LPS. Thus HSPs may activate macrophage signal transduction independent of LPS.

In conclusion, extensive investigation in the past 10 years has suggested that HSPs may be potent activators of the innate immune system. It has been shown that Hsp60, Hsp70, Hsp90, and gp96 are capable of inducing the production of proinflammatory cytokines by the monocyte-macrophage system, as well as the activation and maturation of antigen-presenting cells in a manner similar to the effects of LPS and bacterial lipoprotein, e.g., via CD14/TLR2 and CD14/TLR4 receptor complex-mediated signal transduction pathways. However, recent evidence suggests that the reported cytokine effects of HSPs may be due to LPS and LPS-associated molecules. Thus it is essential that efforts should be directed to conclusively determine whether the reported HSP cytokine effects are due to HSPs or to contaminant(s) present in the HSP preparations before exploring further the implication and therapeutic potential of the putative cytokine function of HSPs.

REFERENCES


Invited Review

HSP AND CYTOKINE FUNCTION

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