Anti-HSP auto-antibodies enhance HSP-induced pro-inflammatory cytokine production in human monocytic cells via Toll-like receptors

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Abstract

Auto-antibodies against heat shock proteins (HSPs) are frequently found in the sera of patients with rheumatic and other autoimmune diseases. However, it is unclear whether these auto-antibodies play a role in the pathophysiology and etiology of these diseases. We found that a murine anti-HSP60 mAb enhanced the production of IL-8 and tumor necrosis factor-α induced by human HSP60 in the human monocytic cell lines THP-1 and U937, and human peripheral blood monocytes. Similar enhancement was observed with the combination of human HSP70 protein and a murine anti-HSP70 mAb. The enhancing effects were also observed for F(ab')₂ fragment, but not for monovalent Fab fragment. This suggests that the enhancement is due to cross-linking of HSP by the anti-HSP antibodies. The induction of IL-8 was dramatically suppressed by the transfection of a dominant-negative mutant of Toll-like receptor 4. We also found that sera from patients with rheumatic autoimmune diseases, which contained higher anti-HSP60 auto-antibody titers than sera from healthy donors, significantly enhanced the IL-8 production induced by human HSP60 in THP-1 cells. We propose that auto-antibodies against HSPs have the potential to play a pathogenic role in rheumatic autoimmune diseases by enhancing inflammatory reactions.

Introduction

Heat shock proteins (HSPs) are highly conserved proteins among prokaryotes and eukaryotes. The most important function of HSPs is molecular chaperones (1), which assist in the correct folding of proteins synthesized de novo or denatured by stresses such as heat shock. HSPs have therefore been regarded as intracellular molecules. However, recent studies strongly suggest that HSPs can also act as potent activators of the immune system outside of cells (2). In the innate immune system, HSPs act as immunostimulators like various microbial substances. Mammalian HSP60 and HSP70 are recognized by pattern recognition molecules, the Toll-like receptors (TLRs) (3–6), just as microbial substances are. When TLRs interact with microbial substances such as LPS, peptidoglycan, bacterial lipoproteins and viral RNA, they transduce intracellular signals to activate the transcription factors nuclear factor-κB (NF-κB) and interferon regulatory factor-3 (IRF-3) (7). The NF-κB up-regulates transcription of various immune mediators, such as pro-inflammatory cytokines (IL-8 and tumor necrosis factor-α (TNF-α)], antimicrobial peptides (defensin), cell adhesion molecules (ICAM-1 and E-selectin) and MHC. IRF-3 up-regulates transcription of interferon-β in cooperation with NF-κB. The TLR system is very important for the initial defensive response against both bacterial and viral infections. In the adaptive immune system, HSPs act as extracellular molecular chaperones and adjuvants (2, 3, 8). Cell-surface receptors for HSPs have been identified on antigen-presenting cells (APCs), for example, CD91 (low-density lipoprotein receptor-related protein/α₂-macroglobulin receptor) has been proposed to be a receptor for gp96, HSP70, HSP90 and calreticulin (9), and lectin-like oxidized low-density lipoprotein receptor-1 (Lox-1) and CD40 are suggested to be receptors for HSP70 (10, 11). Extracellular HSPs bind to various polypeptides including tumor antigens. The HSP-polypeptide complex binds to the HSP receptor and is internalized into APCs by endocytosis, and the polypeptides are efficiently recruited into the antigen presentation...
Auto-antibodies enhance cytokine induction by HSP

Auto-antibodies against HSPs are frequently found in sera from patients with rheumatic diseases and atherosclerosis (12–14). We recently reported that anti-HSP auto-antibodies are found in the cerebrospinal fluids from patients with immune-mediated neuropathies, such as Guillain–Barre syndrome (15), chronic inflammatory demyelinating polyneuropathy and multiple sclerosis (16). The pathogenic significance of such auto-antibodies is often discussed, but their role on the pathophysiology and etiology of the diseases has not yet been established. In this study, we show evidence that the anti-HSP auto-antibodies modulate the HSP-induced inflammatory reaction.

Methods

Cell lines

The human monocytic cell lines THP-1 and U937 and the murine macrophage cell line RAW264.7 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were routinely cultured in RPMI-1640 containing 10% fetal bovine serum (FBS).

Preparation of monocyte/macrophage fraction from human peripheral blood mononuclear cells (PBMCs)

Monocyte/macrophage fraction was prepared from PBMCs according to the method of Wahl and Smith (17). Mononuclear cell fraction was prepared from heparinized blood of healthy adults by using Mono-Poly resolving medium (MP Biomedical, Irvine, CA, USA). PBMCs (5 x 10^6 cells) in 2 ml medium (RPMI-1640 containing 5% FBS and 50 μM 2-mercaptoethanol) were added to 24-well tissue culture plate and cultured for 6 h. The adherent cells were washed three times with PBS(–) and used as human peripheral monocyte/macrophage fraction. The cells were cultured in RPMI-1640 medium containing 20% FBS.

Reagents

The endotoxin concentration was determined with a colorimetric limulus amebocyte lysate (LAL) assay kit, Endospecy (Genzyme-T echne, Minneapolis, MN, USA) and HSP60 and HSP70 (Stressgen). Titers of anti-HSP60 auto-antibodies in human sera were determined using an ELISA as described previously (18).

Human sera

Human sera were donated from patients with rheumatoid arthritis (RA) (n = 23), systemic lupus erythematosus (SLE) (n = 20) and Sjögren’s syndrome (n = 9) and healthy adults (n = 23) (18). Diagnosis of RA (19), SLE (20) and Sjögren’s syndrome (21) was based on published criteria.

Stimulation of cells with HSP and anti-HSP antibodies

THP-1 and U937 were pre-treated with 1 x 10^{-8} M 1α, 25-dihydroxyvitamin D3 (active vitamin D3) for 24 h before stimulation. The pre-treated cells were treated with stimulants, such as HSP protein or a mixture of HSP protein and anti-HSP antibodies.

Transfection of dominant-negative forms of TLRs

Expression plasmids carrying dominant-negative versions of human TLR2, TLR4 and TLR5 (22) were kindly provided by Michael F. Smith (University of Virginia). The dominant-negative versions were in deletion of the carboxy-terminal portion of the molecules (TLR2 truncated at amino acid 670, TLR4 at amino acid 700 and TLR5 at amino acid 685). The cDNAs for the truncated proteins were inserted into pcDNA3.1-Neo (Invitrogen, Carlsbad, CA, USA). The plasmids were transfected into cells by the lipofection method using Superfect reagents (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Twenty-four hours after transfection, the transfected clones were selected by the treatment with 300 μg ml^{-1} G418 for 4 days and cloned by limiting dilution method. After the selection of transfected clones by G418, the cells were treated with active vitamin D3 and then the stimulants as above. At least, three clones were examined for confirmation that similar results were obtained. For control study of efficacy of dominant-negative TLRs (dnTLRs), LPS derived from Escherichia coli O111:B4 (Sigma–Aldrich, St Louis, MO, USA) and peptidoglycan derived from Staphylococcus aureus (Fluka, Steinheim, Switzerland) were used as stimulants for the examination of dnTLR4 and dnTLR2, respectively.

Statistical analysis

We analyzed statistical significance between two groups by the unpaired t-test with Statview software (SAS Institute Inc., Cary, NC, USA).

Results

HSP60 and HSP70 induce IL-8 and TNF-α in human monocytic cells

Numerous studies have found that HSP60 and HSP70 transduce pro-inflammatory signals via TLR4 and/or TLR2 (4–6). However, recent reports have suggested that endotoxin contamination, rather than the HSPs, was responsible for the stimulation (23, 24). LPS contamination in HSP preparations is a serious problem, and it is still controversial whether HSPs stimulate TLRs. We therefore carefully examined the ability of
HSP60 and HSP70 proteins to induce pro-inflammatory cytokines using a commercially available low endotoxin grade of HSP preparations. We also confirmed that the level of endotoxin contamination in the preparations was less than 5 EU mg\(^{-1}\) endotoxin, so that the final stimulation culture contained endotoxin derived from HSP proteins at a concentration of less than 0.05 EU ml\(^{-1}\). U937 and THP-1 cells, which were differentiated by active vitamin D\(_3\), showed up-regulated production of IL-8 and TNF-\(\alpha\) by stimulation with HSP60 and HSP70 (Fig. 1A, B and D). The enhancing activity of HSPs was weaker than that of a typical stimulant, LPS. We repeated the experiments in the presence of an endotoxin-neutralizing agent, polymyxin B (10 \(\mu\)g ml\(^{-1}\)), which did not affect the induction of IL-8 and TNF-\(\alpha\) by HSPs (data not shown). We also found that HSP60 and HSP70 significantly enhanced IL-8 production of human adherent PBMCs (monocyte/macrophage fraction) (Fig. 1C). The response to HSPs seems to vary among cell lines, although the reason is not known. In their experiments using the murine macrophage line RAW264.7, Gao and Tsan (23, 24) reported that neither HSP60 nor HSP70 induced pro-inflammatory cytokines and suggested that the induction observed by others was due to endotoxin contamination. We also observed that neither did RAW264.7 respond to HSP60 and HSP70 (Fig. 1E) nor did undifferentiated THP-1 and U937 (data not shown). Stimulation with active vitamin D\(_3\) was required for HSP to induce IL-8 production, as it was for a response to LPS. Our results clearly show that HSP60 and HSP70 can induce pro-inflammatory cytokines in differentiated human monocytic cell lines and peripheral blood monocytes/macrophages.

**Anti-HSP antibodies enhance HSP-induced pro-inflammatory cytokine production in monocytic cells**

We examined the effect of anti-HSP mAb on HSP-induced inflammatory cytokines. Commercially available anti-HSP60 and anti-HSP70 mAbs were checked for LPS contamination using a colorimetric LAL assay. Both preparations contained less than 5 EU mg\(^{-1}\) endotoxin, so that endotoxin contamination derived from the HSPs and anti-HSP mAbs in the final stimulation culture was less than 0.1 EU ml\(^{-1}\). These endotoxin levels were judged to be sufficiently low for this study. A mixture of HSP60 and a murine anti-HSP60 mAb induced dramatically higher amounts of IL-8 and TNF-\(\alpha\) than HSP60 alone in THP-1 and U937 cells treated with active vitamin D\(_3\) (Figs 2A and 3A). Furthermore, the anti-HSP60 mAb alone did not alter IL-8 production (data not shown, see control experiments in Fig. 5). Similarly, a murine anti-HSP70 mAb strongly enhanced HSP70-induced IL-8 and TNF-\(\alpha\) (Figs 2B and 3B). Anti-HSP70 mAb

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**Fig. 1.** HSP60- and HSP70-induced IL-8 (A, B and C) and TNF-\(\alpha\) (D and E) production in human monocytic cell lines THP-1 and U937 treated with active vitamin D\(_3\), human adherent PBMCs (monocyte/macrophage fraction) and murine macrophage cell line RAW264.7. Cells or vitamin D\(_3\)-treated cells (in case of THP-1 and U937) were treated with 1 and 10 \(\mu\)g ml\(^{-1}\) HSP60 or HSP70 or 0.1 and 1 \(\mu\)g ml\(^{-1}\) LPS. After 24 h culture, the concentration of IL-8 or TNF-\(\alpha\) in the culture supernatant was determined by ELISA. The experiments were carried out in triplicate. The results are expressed as means ± SD. Experiments of adherent PBMCs (C) were carried out using PBMCs derived from three healthy adult donors. Representative result is shown above.
alone did not show any enhancing effect. The enhancing effects of anti-HSP antibodies on HSP-induced IL-8 production were also observed for adherent PBMCs (Fig. 2C and D).

It is probable that the HSP–antibody complex simultaneously interacts with an HSP receptor, such as TLR, and an Fc receptor on the cells. So we also examined the effect of a monovalent Fab fragment and a divalent F(ab')2 fragment lacking Fc region of the anti-HSP mAbs on HSP-induced IL-8 production. Fab fragments derived from the anti-HSP60 mAb and anti-HSP70 mAb did not significantly enhance HSP induction of IL-8 production (Fig. 4). In contrast, F(ab')2 fragments showed enhancing activity, which was not significantly different from intact IgG. The results indicate that the enhancing effect of anti-HSP mAbs on HSP induction of pro-inflammatory cytokines is due to cross-linking of HSP by specific mAbs, and is irrelevant to Fc region of antibodies.

HSPs transduce pro-inflammatory signals via TLR4

We next examined which HSP receptors on human monocytic cells contribute to pro-inflammatory cytokine induction by HSPs transduce pro-inflammatory signals via TLR4
transfected THP-1 cells with expression plasmids encoding dominant-negative mutants of TLR2, TLR4 and TLR5. The transfected clones were selected by G418 for 4 days. At least three clones derived from each transfection were examined. Dominant-negative effects of dnTLR4 and dnTLR2 in the transfected THP-1 cells were confirmed by the response to specific stimulants, LPS and peptidoglycan, respectively (Fig. 5C and D). Only cells expressing the dnTLR4 showed significant suppression of IL-8 induction by HSP60 and HSP70 (Fig. 5A and B). Furthermore, the enhancing activity of anti-HSP mAbs on the HSP-induced IL-8 production was also dramatically suppressed by transfection of the dnTLR4, but not the dnTLR2 and dnTLR5 (Fig. 5). HSP70/anti-HSP70 mAb-induced IL-8 was slightly suppressed in cells expressing the dnTLR2, but the suppression was not significant. The results suggest that both HSP60 and HSP70 mainly transduce pro-inflammatory signal via TLR4.

Sera from patients with rheumatic autoimmune diseases contain anti-HSP60 auto-antibodies and enhance HSP60-induced IL-8 production in monocyctic cell lines

There have been many investigations into the presence of auto-antibodies against HSP, especially HSP60, in sera from patients with autoimmune diseases, such as rheumatic diseases (12, 13). We hypothesized that the auto-antibodies against HSP60 found in patients’ sera would enhance the HSP60-induced production of IL-8 as the murine mAbs did. We examined HSP60-induced IL-8 production in the presence of sera containing HSP60 antibodies. The anti-HSP60 IgG titers in sera used in this study were shown in Fig. 7(A). Significant higher levels of anti-HSP60 antibodies were observed in sera from patients with RA, SLE and Sjögren’s syndrome than sera from healthy controls, although correlation between anti-HSP60 titers and disease activity was not observed in the studied patients (18, 25).

First, we confirmed whether the sera were appropriate to use in this study. Levels of serum HSP60 were less than 200 ng ml$^{-1}$, so no more than 40 ng ml$^{-1}$ HSP60 were contained in the final mixture used in this experiment. Furthermore, the sera contained less than 0.1 EU ml$^{-1}$ endotoxin as determined by LAL assay, so the final stimulation culture contained endotoxin derived from sera at a concentration of less than 0.02 EU ml$^{-1}$. We conclude that the effect of HSP60 and endotoxin contamination in the sera was not considered in this experiment. THP-1 culture supernatants in presence of patients’ sera (‘none’ in Fig. 6) contained higher levels of IL-8 than those in presence of control sera. The levels of IL-8 were around the detection limit of the ELISA system (20–30 pg ml$^{-1}$), but significantly different. The higher levels of IL-8 should originate from endogenous IL-8 in patients’ sera. Higher levels of IL-8 are found in sera from patients with RA and SLE related to disease status (26, 27).

We added the patients’ and normal sera to THP-1 cell culture in presence or absence of HSP60 (Fig. 6). The degrees of IL-8 induction by HSP60 in the presence of sera from rheumatic autoimmune disease patients were higher than those in the presence of sera from healthy adults. Significantly up-regulated IL-8 production induced by HSP60 was observed in the presence of sera derived from patients with RA, SLE and Sjögren’s syndrome compared with sera from healthy adults. The sera did not significantly alter IL-8 induction by other IL-8 inducers, such as LPS (data not shown). Significant correlation ($r^2 = 0.539$, $P < 0.0001$) between the IL-8 induction activity of sera and the...
anti-HSP60 antibody titer (Fig. 7C) was observed. These results suggest that anti-HSP60 auto-antibodies in sera from patients with rheumatic autoimmune diseases may potentiate the HSP60-induced inflammatory reaction.

Discussion

Anti-HSP auto-antibodies are frequently found in sera from patients with various diseases, such as rheumatic autoimmune diseases, atherosclerosis and cardiovascular diseases (12–14), but the role of anti-HSP auto-antibodies in the pathophysiology and etiology of these diseases is still obscure. One issue in question is whether HSPs are resident in intracellular compartments as molecular chaperones, because recent studies have suggested that HSPs are present extracellularly and on the cell surface. For example, Schett et al. (28) reported that anti-HSP60 antibodies have a cytotoxicity to vascular endothelial cells, which express HSP60 on the cell surface, via a complement-dependent mechanism. Extracellular HSPs have also been identified; for example, levels of serum HSP60 are associated with early human cardiovascular disease and with the presence of physiological stress in women (29, 30), and levels of serum HSP70 are related to the disease severity of chronic heart failure, myocardial infarction, atherosclerosis, and peripheral and renal vascular disease (31–34). Recent studies have suggested that extracellular HSPs act like inflammatory cytokines or various microbial substances called pathogen-associated molecular patterns, which are recognized by TLRs. Furthermore, they act as an adjuvant, which helps in the phagocytosis with generating auto-antibodies enhance cytokine induction by HSPs.
antigenic polypeptides in the APCs and antigen presentation of the peptides (2, 3, 8). In this study, we showed that sera from patients with rheumatic autoimmune diseases contain significantly higher titers of anti-HSP60 auto-antibodies and increased levels of pro-inflammatory cytokines induced by HSP60. We did not find significantly higher levels of circulating HSP60 in sera from patients with rheumatic autoimmune diseases compared with sera from healthy controls. However, extracellular HSPs may be generated by the destruction of tissues, such as joints in RA, in inflammatory sites during the progression of rheumatic diseases. High levels of IL-8 were found in synovial fluids but not in sera of RA patients (35). Anti-HSP auto-antibodies have been detected widely in various diseases as described in Introduction. So the enhancement of inflammation, which was generated by soluble HSP and anti-HSP auto-antibodies seems to contribute to an exacerbation factor of inflammatory reactions, such as increased production of inflammatory cytokines and chemokines, rather than to the generation of characteristic symptoms of each disease.

In the present study, we found that anti-HSP mAbs and autoimmune disease patients’ sera containing higher titer of anti-HSP antibodies enhance the production of pro-inflammatory cytokines induced by HSPs in human monocytes. However, we have not observed statistically significant correlation between disease activity and anti-HSP auto-antibody titers in a particular disease group. Further studies with larger populations are necessary to elucidate this point. In agreement with numerous reports (3–6), the HSPs transduced the inflammatory cell signal via TLR4, mainly. From the results of experiments using anti-HSP mAbs and their Fab and F(ab')2 fragments, the enhancing activity of the anti-HSP antibodies appears to be due to cross-linking of the stimulant, HSPs. And there is little contribution of interaction between Fc region of antibodies and Fc receptors on the monocyte. In conclusion, we propose a new pathogenic role for anti-HSP auto-antibodies in enhancing inflammatory reactions induced by extracellular HSP proteins.

Acknowledgements
We thank Michael F. Smith Jr for providing the expression plasmids encoding dominant-negative mutants of TLRs.

Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<td>dnTLR</td>
<td>dominant-negative TLR</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>HSP</td>
<td>heat shock protein</td>
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<td>IRF</td>
<td>interferon regulatory factor</td>
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<td>LAL</td>
<td>limulus amebocyte lysate</td>
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<td>NF-kB</td>
<td>nuclear factor-kB</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNF-α</td>
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References


Auto-antibodies enhance cytokine induction by HSP


