The FASEB Journal article fj.08-128785. Published online April 3, 2009.

The FASEB Journal • Research Communication

Mitochondrial heat shock protein (HSP) 70 synergizes with HSP60 in transducing endothelial cell apoptosis induced by anti-HSP60 autoantibody

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ABSTRACT **Heat shock protein (HSP) 60, up-regulated by endothelial cells (ECs) to resist stress, is the target of a subgroup of apoptosis-inducing anti-EC autoantibodies (Abs) in human vasculitides. Given that HSP60 is not a transmembrane protein, the mechanism by which these auto-Abs induces apoptosis is unclear. EC membrane proteins were analyzed using bidimensional electrophoresis and Far Western blot, and the HSP60 receptor was identified by mass spectrometry. Heat stress-dependent synthesis of HSP60 and receptor was examined by semiquantitative RT-PCR, and expression was examined by flow cytometry and indirect immunofluorescence. Interaction was demonstrated by coimmunoprecipitations. Lipid rafts were purified to evaluate specific localization, and the apoptotic response was investigated by blocking monoclonal Ab. Mitochondrial HSP70 (mtHSP70) was identified as an HSP60 receptor. Stress was required for ECs to upregulate mRNA and express mtHSP70 on their surface. HSP60 and mtHSP70 colocalized and interacted within lipid rafts. They were associated with chemokine CC motif receptor 5 (CCR5), also induced at the mRNA and protein levels in stressed ECs. CCR5 was involved in the anti-HSP60-triggered apoptosis of ECs. These results provide new insights into the mechanism by which anti-EC auto-Abs from vasculitides induce apoptosis of ECs.—Alard, J.-E., Dueymes, M., Mageed, R. A., Saraux, A., Youinou, P., Jamin, C. Mitochondrial heat shock protein (HSP) 70 synergizes with HSP60 in transducing endothelial cell apoptosis induced by anti-HSP60 autoantibody. *FASEB J.* 23, 000–000 (2009)**

Key Words: antibodies · stress · endothelium · vasculitis · cell surface interaction · CCR5

Anti-endothelial cell (EC) Abs accompany all systemic autoimmune diseases associated with vasculitis. For example, they are detected in patients with systemic lupus erythematosus (1), polyarteritis nodosa (2), and systemic sclerosis (3). Furthermore, apoptosis (4, 5), Ab-dependent cell cytotoxicity (6, 7), and complement-dependent lysis (8, 9) have, thus, been ascribed to these auto-Abs. They promote the death of ECs and thereby participate in the pathophysiology of the disorders. One of the key issues relates to how EC death is achieved through their specificity (10). To clarify this situation the target antigens (Ags) have been interpreted as being either expressed in complex structural aggregates on ECs or as circulating Ags associated into their membranes.

One of the prominent members of the latter group of EC membrane-associated proteins is heat shock protein (HSP) 60. Specificity for this protein is frequently encountered in the screening of anti-EC Ab (AECA)-positive sera (11) and the related Abs shown to commit ECs into apoptosis (12). However, how HSP60 transduces apoptotic signals in ECs once engaged by anti-HSP60 AECAs remains an enigma. Given that HSP60 lacks transmembrane and intracellular domains, it is likely that this chaperon interacts with other structures to convey proapoptotic signals. The presumed associated molecule would be the one to endow the complex with the capacity to transmit proapoptotic signals. The identification of molecules that associate with HSP60 is complicated by the importance of the number of candidates that could be involved in this process. They have been distributed into two families of candidate receptors (13). The first consists of receptors involved in internalization of HSPs. Examples of these include CD91 and Lox-1. The second family comprises signaling receptors, such as Toll-like receptor (TLR) 2, TLR4, CD40, and chemokine CC motif receptor 5 (CCR5). Theoretically, any member of the two families of proteins could combine with others at structural or functional levels. For example, Lox-1 and TLR2 synergize in mounting the response to HSPs (14).

In view of the available evidence, two sets of mechanisms can be predicted. One relies on the binding of HSP60 to a single receptor that transduces the necessary signals directly. This process operates through a typical receptor-ligand interaction. As such, it would be

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doi: 10.1096/fj.08-128785

specific and limited by the concentrations of the interacting molecules (15). Interestingly, such a mechanism involving HSP60 was recently ascribed to the surface of ECs, although the receptor was not found (16). To identify it, several experiments were carried out. Some of them established that TLR2, TLR4, and CD14 were implicated in transducing the proinflammatory effects of HSP60 (17–19), suggesting that they could be common receptors for HSP60. Additional experiments in TLR-deficient mice, coupled with inhibition studies with monoclonal Abs, however, have shown that HSP60 binds to ECs irrespective of TLR2, TLR4, and CD14 expression (15, 16). The fact that the CD91 receptor is shared by several HSPs (20) also provides this candidate with credibility. However, CD91 is not involved in this process, because anti-CD91 Abs do not inhibit the binding of HSP60 to ECs (15).

The second set of mechanisms for how HSP could transduce proapoptotic signals corresponds with a cascade of events including numerous receptors. For example, once released from stressed ECs, HSP60 first increases the number of TLR4 molecules and then binds to them. The resulting intracellular signals (21) in turn up-regulate the expression of TLR2 (22), thus interacting with its ligand HSP70 (23), which is simultaneously shed by stressed ECs (24). TLR2 is then activated (25), and apoptosis of ECs is subsequently promoted (26).

The present study was designed to identify receptors for HSP60 on the surface of stressed ECs and to analyze mechanisms by which related Abs induce apoptosis of ECs. Contrary to prediction, the process was accounted for neither by the single receptor mechanism nor by the aggregated receptor paradigm. In contrast, HSP60 interacted with mitochondrial HSP70, which itself bound to the CCR5 chemokine receptor. As a result, CCR5 activation is a prerequisite for the ability of anti-HSP60 Ab to induce ECs into apoptosis.

MATERIALS AND METHODS

Cell culture

Human umbilical vein ECs (HUVECs) are the most common source of human ECs, easily purified and used largely for *in vitro* studies. However, their usage for the detection of AECAs proved extremely difficult on a daily basis due to variation in cell numbers and cellular viability. Therefore, EAhy926 cells were substituted for HUVECs as a cell substrate (27). This EC line, which corresponds to the fusion of HUVECs with the permanent epithelial cell line A549-8, is appropriate to overcome the HUVEC-dependent variations. Although the epithelial cell partner may introduce bias in the data interpretation, results from *in vitro* experiments using EAhy926 cells may be supplemented with HUVECs. For these reasons, both HUVECs and EAhy926 were used herein.

The EAhy926 EC line was kindly provided by Dr. Cora-Jean S. Edgell (University of North Carolina, Chapel Hill, NC, USA). The cells were grown in DMEM supplemented with 10% FCS, 2 μM glutamine, 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and 50 mg/L gentamicin. They were cultured at 37°C in a humidified incubator with 5% CO_2. Stress was induced by a 7-h culture of the cells at 42.5°C.

HUVECs were prepared by digestion in 0.1% collagenase (Sigma-Aldrich Corp., St. Louis, MO, USA) and grown to confluence (28). The cells were passaged twice, harvested, and seeded in culture wells at 10^5 cells in 100 μl of DMEM supplemented with 10% FCS and 100 IU/ml of polymyxin B. HUVECs were subjected to stress by exposure to temperatures of 42.5°C for 5 h. All reagents used were lipopolysaccharide-free, as ascertained by a quantitative chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Emerainville, France).

Two-dimensional electrophoresis

Cells were scraped from culture flasks with trypsin, washed, and resuspended in a homogenization buffer (1 M sucrose, 100 mM Tris HCl, 100 mM EDTA, 50 mM $MgCl_2$, 1 μM leupeptin, 1 μM pepstatin, and 1 μM aprotinin) as described previously (28). After three freezing-thawing cycles in liquid nitrogen and three 15-s sonications, the nuclei were removed by centrifugation at 500 *g* for 10 min. Supernatants were collected and centrifuged at 10,000 *g* for 30 min to separate the organelle fraction, and the remaining supernatants were ultracentrifuged at 100,000 *g* for 30 min to separate the membrane-enriched protein fraction. This fraction was resuspended in solubilization buffer containing 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propenesulfonate and 1% Triton X-100. Solubilized proteins were obtained by centrifugation at 10,000 *g* for 45 min, and their concentration was determined using the Micro BCA protein assay kit (Pierce, Brebières, France) after acetone precipitation.

Protein extracts were resuspended in rehydration buffer containing 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 1% Triton X-100, 7 M urea, 2 M thiourea, 0.48% Bio-Lyte 3/10, and 1% tributylphosphine and loaded onto 10-cm linear pH 3–10 immobilized pH gradient strips (Bio-Rad, Marnes-la-Coquette, France) or 18-cm nonlinear pH 4–8 immobilized pH gradient strips for isoelectric focusing. After 16 h of passive rehydration, proteins were focused with a protean immunoelectrofocalization cell (Bio-Rad) for 20,000 and 160,000 V · h for the 10- and 18-cm strips, respectively. The strips were then incubated for 15 min at room temperature in a primary equilibration solution (0.375 M Tris, 6 M urea, 34.5% glycerol, 2% SDS, and 70 mM DTT) and for 20 min at room temperature with a second equilibration solution (in which DTT was replaced with 135 mM iodoacetamide). They were then loaded onto the top of a 10% polyacrylamide gel and subjected to electrophoresis for 4 h to resolve the proteins, based on their molecular mass.

Coimmunoprecipitation

In some experiments, cell surface proteins were cross-linked with 20 μg/ml bis(sulfo-*N*-succinimidyl) for 20 min at 4°C. After addition of 1 vol of 10 mM Tris, membrane-enriched fractions were obtained as described above using a modified solubilization buffer (50 mM Tris, 1% Triton X-100, and 150 mM NaCl). Protein solutions were precleared with protein G beads for 1 h at 4°C and precipitated for 1 h at 4°C with anti-HSP60 (VWR International, Strasbourg, France) or anti-HSP70 (Ozyme, Saint Quentin en Yvelines, France) Ab-coated protein G beads. The proteins were washed in modified solubilization buffer, and retained proteins were eluted with 0.1% Triton X-100 and 0.1 M triethylamine, pH 11.8, for 15 min. After acetone precipitation, proteins were resolubilized in the modified solubilization buffer and resolved by Western blotting.

Western and Far Western blotting

Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After 1 h of blocking with PBS containing 5% milk protein, the membranes were probed for 1 h with specific Abs diluted in PBS supplemented with 1% milk protein and 0.1% Tween 20 for Western blot analyses. Bound Abs, goat polyclonal anti-HSP60 (Abcam plc, Cambridge, UK), or rabbit polyclonal anti-HSP70 (Sigma-Aldrich Corp.), were amplified with biotinylated anti-Ig Ab (Jackson ImmunoResearch West Grove, PA, USA) and developed with horseradish peroxidase (HRP)-conjugated streptavidin (Amersham Biosciences, Orsay, France). For the Far Western blot experiments, PVDF membranes were first blocked with PBS containing 3% BSA for 2 h and then were incubated with PBS containing 1% BSA and 2 μg/ml recombinant HSP60 (Sigma). After another blocking step with PBS containing 5% milk protein, they were probed with anti-HSP60 mAb.

Detection of cellular expression

HUVECs were suspended at 2×10^6 cells/200 μl of ice-cold PBS, and incubated for 1 h at 4°C with primary anti-HSP60, anti-HSP70, or anti-CCR5 (BD Biosciences, Le Pont de Claix, France) mAbs. Then they were washed in ice-cold PBS and further incubated for 1 h at 4°C with FITC-conjugated secondary Abs. After three washes, the cells were suspended in 400 μl of PBS for flow cytometry analysis in an Epics XL flow cytometer (Beckman Coulter, Villepinte, France). Unstained cells and the FITC-conjugated developing reagent alone were used as controls to set the level of positivity.

For indirect immunofluorescence (IIF) staining, HUVECs were cultured onto 10-well slides until confluence. After application of stress, cells were washed three times with PBS, incubated for 30 min at 4°C with primary Abs, washed again, and incubated using FITC- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary Abs for 30 min at 4°C. Cells were fixed in PBS and 2% paraformaldehyde for 15 min, and slides were mounted in glycerol and examined with a TCS NT confocal microscope (Leica Microsystems, Wetzlar, Germany).

RNA isolation and RT-PCR amplification

Total RNA was extracted from resting and stressed HUVECs grown at confluence using RNable reagent (Eurobio, Les Ulis, France) and reserve-transcribed in 20 μl with Superscript II RNase H-reverse transcriptase (Invitrogen Life Technologies, Illkirch, France). For RT-PCR amplifications, reactions were performed with 1 μl of cDNA using the primer pairs listed in **Table 1** with *Taq* DNA polymerase (Invitrogen Life Technologies) as follows: denaturation at 94°C for 5 min, starting amplification with five touchdown cycles (94°C for 30 s, 61°C for 1 min, and 72°C for 1 min), amplification with a decreasing temperature for 30, 35, or 40 cycles (94°C for 30 s, 56°C for 1 min, and 72°C for 1 min), and final extension at 72°C for 10 min. RT-PCR products were analyzed on 2% agarose gels stained with SYBR Green for HSP70 and ethidium bromide for CCR5. The expected size for each specific product is indicated in Table 1.

Assessment of apoptosis

HUVECs were harvested from the second passage of the culture, suspended at 10^4 cells/100 μl of DMEM supplemented with 10% FCS serum and 100 IU/ml of polymyxin B. The cells were then preincubated or not with anti-CCR5 mAb. After a 48-h incubation with 8 μg/ml of anti-HSP60 mAb, accessibility of phosphatidylserine on the cell surface was evaluated to determine the frequency of cells in the early-stage of apoptosis. To this end, cells were stained with FITC-conjugated annexin V in association with propidium iodide (PI) to exclude dead cells according to the manufacturer's instructions (Beckman Coulter). Dead cells were recorded as positive for annexin V and PI, viable cells were recorded as negative for annexin V and PI, and apoptotic cells were considered as positive for annexin V and negative for PI. Percentages of annexin V-positive cells within the PI-negative population were then calculated after flow cytometry analysis. Hypoploidy of the cells was also evaluated to determine the proportion of cells in late-stage apoptosis. This was performed by staining the nuclei with 10 μg/ml of PI in 0.1 M sodium citrate supplemented with 0.1% Triton X-100 buffer and analysis by flow cytometry. Reduction in PI staining intensity compared with that for control cells was taken as a measure of hypoploidy.

Study of lipid rafts

The cholesterol-enriched microdomains of the cell membrane, referred to as lipid rafts (LRs), were isolated based on their insolubility in detergents and buoyant density on a sucrose gradient (29). After incubation with anti-HSP60 mAb, HUVECs were washed with TNE buffer (25 nM ice-cold Tris-HCl, pH 7.4; 150 mM NaCl; and 5 mM EDTA). The cell suspensions were then lysed for 30 min on ice in 1% Triton X-100 in TNE buffer with protease inhibitors. One milliliter of supernatant was mixed with 1 ml of 85% sucrose in TNE and transferred to the bottom of a centrifuge tube. This mixture was overlaid with 3 ml of 35% sucrose and 1.5 ml of 5% sucrose in TNE. The samples were centrifuged in a MLA-80 rotor at 180,000 *g* for 17 h at 4°C in a Beckman OptimaMax ultracentrifuge (Beckman Coulter). The insoluble fractions at the interface of 35 and 5% sucrose were collected, resolved by SDS-PAGE, transferred to a PVDF membrane, and blotted with biotinylated cholera toxin B (Sigma-Aldrich Corp.), anti-HSP60, anti-HSP70, or anti-CCR5 Abs developed with HRP-streptavidin, or HRP-goat anti-mouse Ab.

HUVECs were incubated for 30 min on ice with AlexaFluor 594-cholera toxin B (Molecular Probes, Illkirch, France) to target the LRs and with anti-HSP60 or anti-HSP70 Ab revealed by FITC-anti-mouse IgG to localize these HSPs with

TABLE 1. *Sequences of oligonucleotides used as primers for the amplification of cDNA in RT-PCR*

Primer	Oligonucleotide sequence (5′-3′)	Product length (bp)
mtHSP70 sense	GGAAGGACCGAGCTCTTCTCGC	137
mtHSP70 antisense	CAGGAGTAGGTGGTGCCCAGGTC	
CCR5 sense	ATTTCCCCTCCAAGGTATGG	360
CCR5 antisense	TGGTCTCCTTGCCCTAAATG	
18S RNA sense	GGCTACCACATCCAAGGAAGGCAG	109
18S RNA antisense	CCAATTACAGGGCCTCGAAAGAGTC	

respect to the LRs. The cells were then examined using a confocal microscope.

RESULTS

Screening of EAhy926 cells

We have previously shown that heat-stressed HUVECs expressed HSP60 and that anti-HSP60 Abs induced their apoptosis (12). Because EAhy926 cells are substituted for HUVECs as a cell substrate for the detection of AECAs on a daily basis, we wished to confirm the expression of HSP60 on the surface of these EC lines by flow cytometry and IIF. The experiments revealed that HSP60 was accessible on the surface (**Fig. 1*A***) without the need for stimulation, suggesting that these cells are inherently activated.

Because of the lack of leader peptide, HSP60 does not have the capacity to bind to the membrane but rather needs to be inserted into an as yet unknown receptor. To identify it, we chose the Far Western blot approach. EAhy926 cell membrane-enriched proteins were dispensed into three aliquots, and each was resolved on a bidimensional gel. The first was stained with Coomassie blue to validate the protein extraction procedure (Fig. 1*B*, left panel). The second was probed with anti-HSP60 mAb after the proteins were blotted onto a PVDF membrane to localize the endogenous HSP60 (Fig. 1*B,* center panel). From the third gel, proteins were transferred to another PVDF membrane, and incubated with recombinant HSP60 before being probed with anti-HSP60 mAb (Fig. 1*B,* right panel). This third gel unveiled one additional spot relative to the second. The 69-kDa molecular mass of the additional spot and its 5.5 isoelectric point were consistent with this protein representing mitochondrial HSP70 (mtHSP70) as described in the databases (also called mortalin, GRP75, or PBP74) (30). This additional spot was cut out from the Coomassie blue-stained gel, and its protein sequence was determined by mass spectroscopy. Its identity as mtHSP70 was demonstrated by this analysis (Fig. 1*C*) and further confirmed by flow cytometry and IIF analyses (Fig. 1*D*).

Stress-induced translocation of mtHSP70 to HUVEC membrane

Given that membrane HSP70 is restricted to neoplastic epithelial cells (31), its expression may be irrelevant to the endothelial nature partner of the EAhy926 hybridoma. The HUVECs were examined by flow cytometry and IIF to determine whether they inherently expressed membrane mtHSP70 or whether stress induced mtHSP70. The results showed that HUVECs did not inherently express mtHSP70 and that heat stress induced the expression of the protein (**Fig. 2*A***). Semiquantitative RT-PCR also indicated that stress up-regulated the transcription of the mtHSP70 mRNA (Fig. 2*B*). This result suggests that mtHSP70 expression is triggered at the transcriptional level, upstream of translocation of its protein product to the surface of normal ECs.

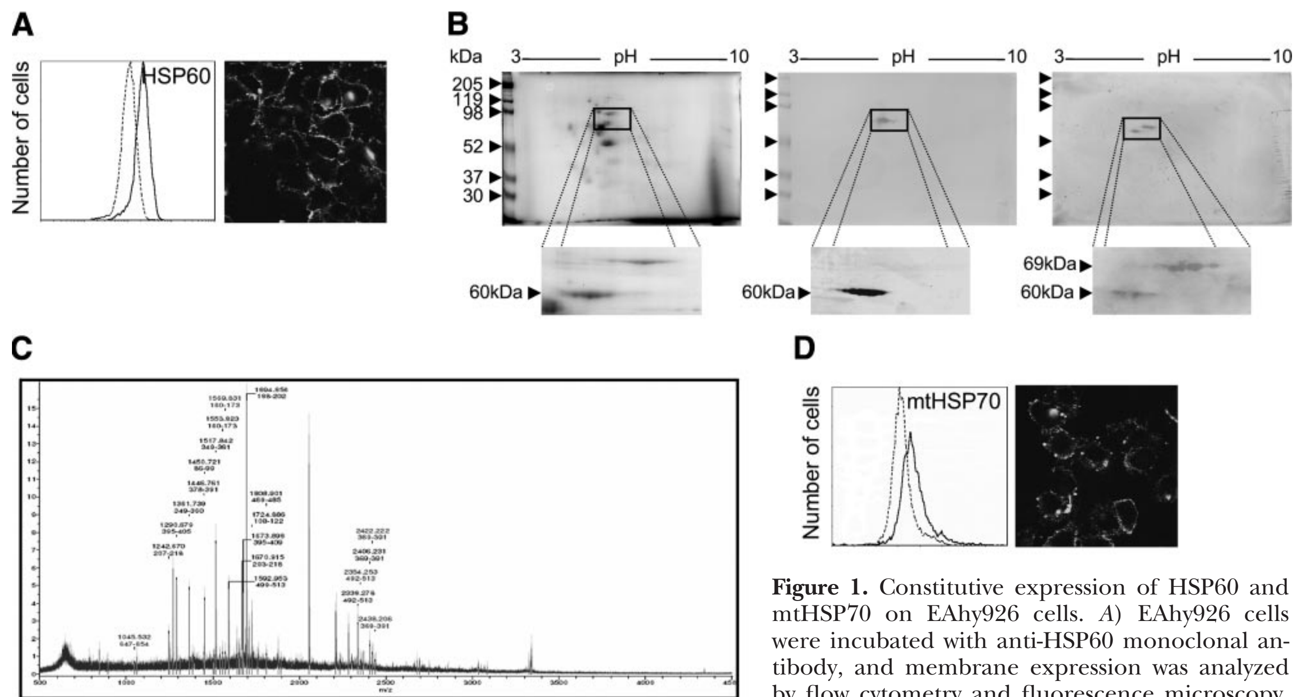
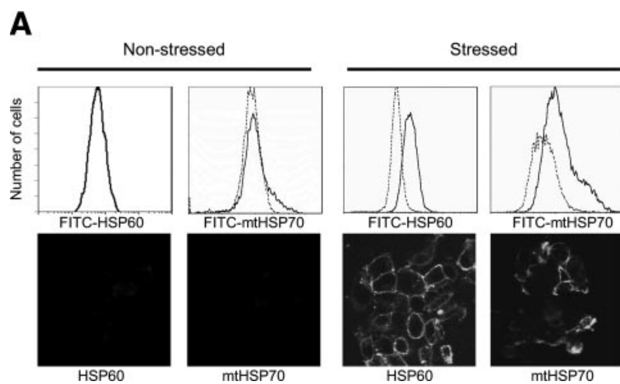


Figure 1. Constitutive expression of HSP60 and mtHSP70 on EAhy926 cells. *A*) EAhy926 cells were incubated with anti-HSP60 monoclonal antibody, and membrane expression was analyzed by flow cytometry and fluorescence microscopy. *B*) Three EAhy926 cell membrane-enriched protein aliquots were electrophoresed on bidimensional gels. Left panel: staining with Coomassie blue. Center panel: Western blot with monoclonal antibody against HSP60. Right panel: loading with HSP60 before Western blot analysis with anti-HSP60 monoclonal antibody. *C*) Mass spectroscopic spectrum of mtHSP70 recognized by HSP60. *D*) EAhy926 cells were treated with anti-HSP70 monoclonal antibody, and expression was analyzed by flow cytometry and fluorescence microscopy. Dotted histograms, background staining with secondary antibody; open histograms, staining with the primary antibody.



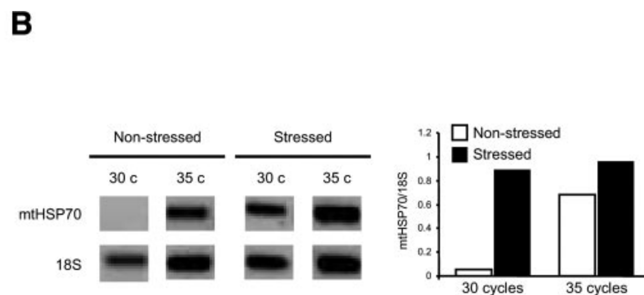


Figure 2. Stress-induced expression of HSP60 and mtHSP70 on HUVECs. HUVECs were cultured at 37°C or stressed by a 5-h heating at 42.5°C. *A*) Cells were incubated with monoclonal antibody to HSP60 or to mtHSP70, and expression was analyzed by flow cytometry and fluorescence microscopy. Dotted histograms, background staining with secondary antibodies; open histograms, staining with primary antibodies. *B*) cDNAs from resting and stress-activated cells were synthesized, and RT-PCR was performed for mtHSP70 and 18S mRNA for 30 and 35 cycles, respectively. Ratios of mtHSP70 to mRNA were calculated by densitometry.

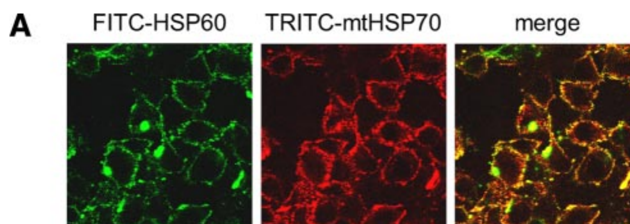
HSP60 interacts with mtHSP70

Our observations raised the possibility that mtHSP70 could be the receptor for HSP60. Confocal microscopy allowed us to assess colocalization of HSP60 and mtHSP70, and, indeed, HSP60 colocalized with mtHSP70 on the membrane of EAhy926 cells (**Fig. 3*A***). We then performed reverse coprecipitation experiments of the two HSPs (Fig. 3*B*). Immunoprecipitation with mAbs to each protein coprecipitated the other protein, and the blot showed the molecular mass of the two resulting complexes to be 150 kDa. Of note, there was only one band after precipitation with anti-HSP60 suggesting that HSP60 interacts only with mtHSP70, whereas several bands appeared after precipitation with anti-HSP70, suggesting that mtHSP70 interacts with HSP60 and other as yet unidentified proteins.

HSP60-induced apoptosis involves mtHSP70

Colocalization of HSP60 and mtHSP70 on the EC surface in the LR signaling platforms would facilitate the transduction of HSP60-induced apoptosis messages. The results of our IIF experiments confirmed that the two HSPs were associated with LRs (**Fig. 4*A***). This finding suggests that intracellular signaling may be triggered by HSP60 recognition on stimulation of the associated mtHSP70. Although mtHSP70 is itself not a transmembrane molecule, microbial HSP70 can bind to CCR5 (32) and through this link promote apoptosis of HUVECs (33). We, therefore, asked the question as to whether the anti-HSP60-induced apoptotic message was transduced by association of mtHSP70 with CCR5. Because we were unable to detect any CCR5 staining by confocal microscopy, protein-enriched fractions of LRs were analyzed by Western blotting (Fig. 4*B*). The results confirmed that CCR5 was present in the LR fractions. Of note was the fact that expression of CCR5 on the surface of ECs was also dependent on stress. Although virtually absent in resting HUVECs, the transcript for CCR5 was up-regulated and protein was detectable after stress (Fig. 4*C*).

The role of CCR5 in the transduction of apoptotic signals was then evaluated. Apoptosis triggered by anti-HSP60 mAb binding was assessed after stress activation of HUVECs (Fig. 4*D*). The role of CCR5 was confirmed by experiments showing that the early stage of apoptosis (Fig. 4*D,* left) and the late stage of apoptosis (Fig.



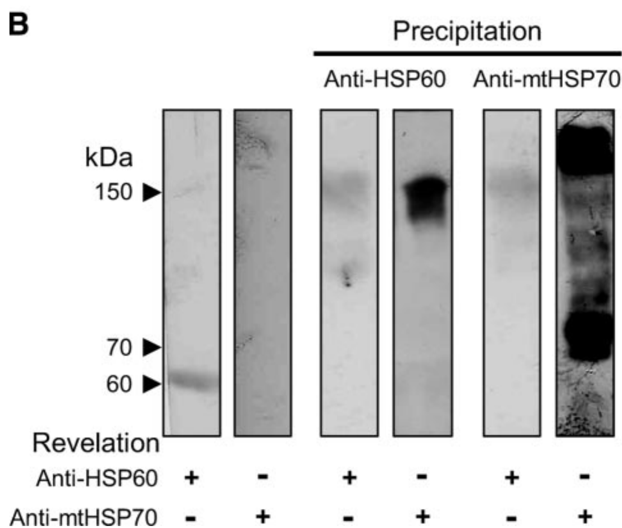


Figure 3. Interaction of HSP60 with mtHSP70. *A*) EAhy926 cells were incubated with goat polyclonal anti-HSP60 antibody and anti-HSP70 monoclonal antibody, developed with FITC-conjugated anti-goat immunoglobulin and TRITC-conjugated anti-mouse immunoglobulin, respectively, and analyzed by confocal microscopy. *B*) HSP60 and mtHSP70 were coimmunoprecipitated from membrane-enriched protein extracts of EAhy926 cells with anti-HSP60 and anti-HSP70 monoclonal antibodies, respectively. Proteins were then Western blotted using goat polyclonal anti-HSP60 or rabbit polyclonal anti-HSP70 antibodies. Recombinant HSP60 served as a positive control (first two lanes).

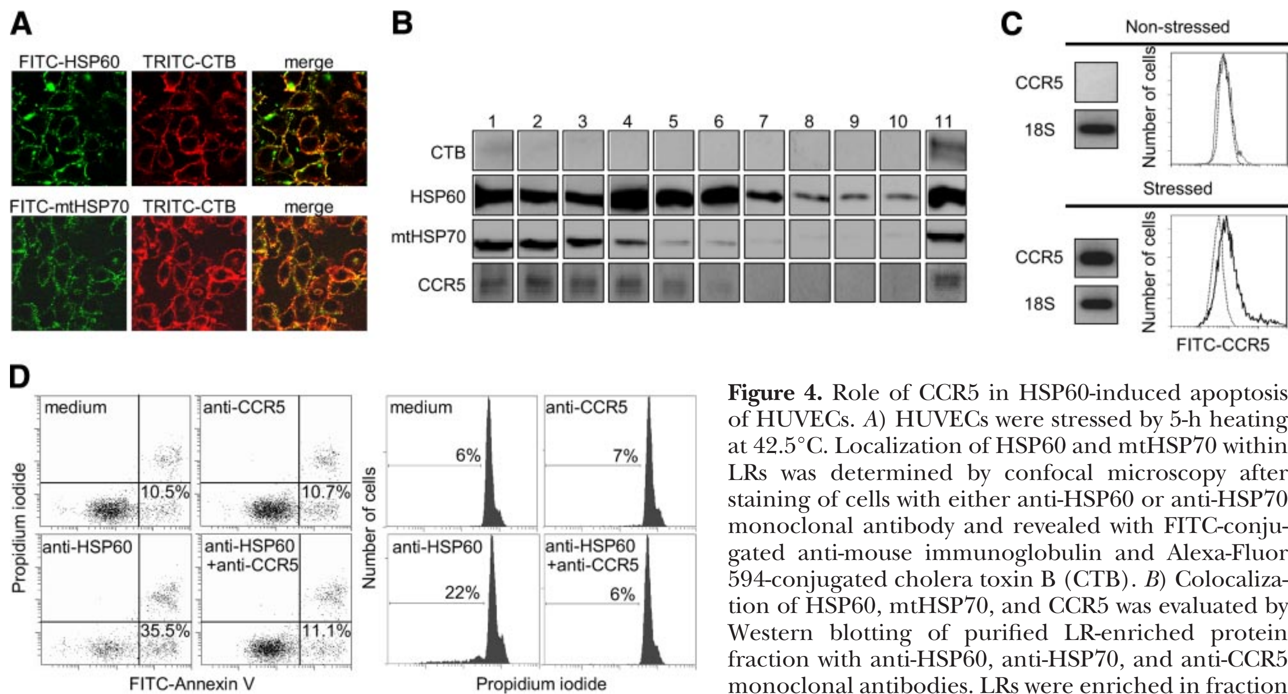


Figure 4. Role of CCR5 in HSP60-induced apoptosis of HUVECs. *A*) HUVECs were stressed by 5-h heating at 42.5°C. Localization of HSP60 and mtHSP70 within LRs was determined by confocal microscopy after staining of cells with either anti-HSP60 or anti-HSP70 monoclonal antibody and revealed with FITC-conjugated anti-mouse immunoglobulin and Alexa-Fluor 594-conjugated cholera toxin B (CTB). *B*) Colocalization of HSP60, mtHSP70, and CCR5 was evaluated by Western blotting of purified LR-enriched protein fraction with anti-HSP60, anti-HSP70, and anti-CCR5 monoclonal antibodies. LRs were enriched in fraction 11, as depicted by staining with CTB. *C*) cDNAs from resting and stressed cells were synthesized, and RT-PCR for CCR5 mRNA was performed at 40 cycles. Expression of CCR5 protein on the surface of resting and stressed HUVECs was analyzed by flow cytometry after incubation of cells with anti-CCR5 monoclonal antibody and development with FITC-conjugated anti-mouse immunoglobulin. Dotted histograms, background staining with secondary antibody alone; open histograms, staining with anti-CCR5 monoclonal antibody. *D*) Stressed HUVECs were incubated in medium alone or with anti-HSP60 monoclonal antibody, blocking anti-CCR5 monoclonal antibody, or anti-HSP60 in association with anti-CCR5. After 48 h, early stage of apoptosis was evaluated by staining of HUVECs with FITC-conjugated annexin V in combination with propidium iodide and analysis by flow cytometry (left panels). Percentages of annexin V-positive cells within propidium iodide-negative population are indicated. Late stage of apoptosis was also evaluated by staining of HUVEC nuclei with propidium iodide and analysis by flow cytometry (right panels). Percentages of hypoploid cells are shown.

4*D,* right) were blocked by incubating the cells with anti-CCR5 Ab (Fig. 4*D*).

All in all, these experiments indicate that HSP60 and mtHSP70 interact to initiate anti-HSP60-mediated apoptosis of stressed HUVECs with simultaneous up-regulation of the transmembrane CCR5 on the membrane required to transduce a message from the LRs.

DISCUSSION

Our experiments were designed to resolve the mechanisms by which anti-HSP60 Abs found in patients with autoimmune diseases could cause pathological conditions. Although the proapoptotic effects of Abs on HSP60 were established previously (12), the signaling cascade that ultimately transduced the signal was unknown. In this study we show that HSP60 interacts specifically with mtHSP70 on the surface of stressed but not resting ECs and confirm that this interaction leads to apoptosis through the CCR5.

Confocal microscopic observations indicate that HSP60 and mtHSP70 colocalize on the surface of ECs and associate with LRs. Furthermore, coimmunoprecipitation studies revealed that HSP60 physically interacts with mtHSP70. These data suggest that, in association with LRs, HSP60 and mtHSP70 may participate in the transduction of intracellular signals. Several reports have shown that HSP70 is located in LRs (34–36) but, depending on the cell type, binds to different partners. Thus, when accessible on the external surface, phosphatidylserine can bind HSP70 (37), but glycosphingolipid Gb3 also enables anchorage of HSP70 in the LRs of tumor cells (36). However, we found no binding of annexin V to the surface of stressed cells, and Gb3 is weakly present in the LRs of either resting or activated HUVECs (38, 39). It is therefore unlikely that either phosphatidylserine or Gb3 serves as the interacting partner for the HSP60-mtHSP70 complex on ECs. In contrast, we found that CCR5, which is associated with LRs on carcinoma and T cells (40, 41), is expressed on the surface of stressed ECs and is associated with HSP60 and mtHSP70 in the LRs. Furthermore, we have shown that anti-CCR5 blocking Ab inhibited the anti-HSP60-induced apoptosis. CCR5 is thus a credible candidate for the binding of mtHSP70 and its associated molecule HSP60.

A recent study has demonstrated that bacterial HSP70 interacts with CCR5 (32) and with CD40 (42) on the surface of HUVECs and dendritic cells (DCs), respectively, which are professional antigen-presenting cells (APCs). Human HSP70 also interacts with CD40 but in a configuration different from that of bacterial HSP70. Thus, whereas bacterial HSP70 binds to CD40 *via* its COOH-terminal substrate-binding domain, human HSP70 binds *via* its NH_2-terminal nucleotide-binding domain, which may enable presentation of Ag peptide

(43). On monocytes, HSP70 can also use CD14, which is a glycophosphatidylinositol-anchored molecule devoid of an intracellular domain to promote the NF-κB-dependent proinflammatory properties of the cells (44). Human HSP70-induced NF-κB activity depends not only on CD14 but also on the myeloid differentiation protein 88 through use of TLR2, TLR4, or the heterodimer of both. Thus, proinflammatory cytokines are induced through engaging TLR2 and TLR4 but not CD14 alone (25). This finding suggests that HSP70 serves not only as a carrier for Ag peptides but also as a danger signal to the innate and adaptive immune systems. Interaction of HSP70 with both TLR2 and TLR4 in a CD14-dependent manner results in a synergistic enhancement of NF-κB activation and, consequently, elevated IL-6 production (45).

Lox-1 is another receptor for HSP70 on the surface of APCs. However, although ECs can function as occasional APCs and express Lox-1 (46), this role did not appear to be involved in the binding of HSP70 to the surface of HUVECs (47). Because resting HUVECs do not express TLRs, CD40, or Lox-1, another potential receptor for HSP70 should be present on their surface, with or without stress. Our results demonstrated that human HSP70 binds to CCR5 on the surface of stressed ECs, but whether this binding involves the same domain as that for bacterial HSP70 remains to be determined. However, some clues given by the current study suggest that this situation is unlikely. For example, human HSP70 coupled with HSP60 induces ECs into apoptosis, whereas bacterial HSP70 induces immature DCs to secrete IL-12 (32). Alternatively, this effect might be cell-dependent and, thus, accounted for by cell-specific receptors. On macrophages, HSP70 binds to CD91 and favors activation (20) but could also bind to CD40 and promote inflammation (43). On DCs, HSP70 binds to CD40 and Lox-1 and induces the production of TGF-β and IL-1 (48) and could also induce IL-12 production by after binding to CCR5 (32). On NK cells, HSP70 binds to CD94 and promotes cytotoxicity (49). Our data suggest that HSP70 binds specifically to CCR5 on the surface of HUVECs and thereby encourages an apoptotic response. This suggestion is consistent with the observation that HUVECs bind HSP70 independently of Lox-1, CD91, CD40, TLR2, and TLR4 (49). Although resting HUVECs express low levels of CCR5 (50), stress up-regulates its density, leading to activation of apoptotic pathways (33) probably *via* LR-dependent intracellular signalings.

With regard to HSP60-induced responses, note that stress-activated protein kinases JNK1/2 and p38, ERK1 and 2 kinases, and NF-κB are used in murine macrophages and in human embryonic epithelial cells transfected with cDNA for TLRs (17). HSP-induced signaling proteins for cytokine production have been shown to be distinct in macrophages, DCs (51), and activated ECs (52). These findings indicate that, depending on the cell type and the stimulating agent, receptors for HSP60 vary from cell to cell and that different responses can be obtained in a given cell type. Thus, stress specifically allows induction of apoptosis in ECs after the binding of anti-HSP60 Abs to HSP60, their target Ag, when it is associated with mtHSP70.

In conclusion, we have identified a novel function for HSP60. This HSP is known to be able to induce proinflammatory effects of macrophages that might influence the development of inflammatory and autoimmune conditions. We have demonstrated herein that, after stress, HSP60 interacts with mtHSP70 on the surface of ECs, thereby participating in the apoptotic response of ECs to anti-HSP60 auto-Abs (12). Further investigation is required to design new therapeutic strategies for the treatment of cardiovascular symptoms in patients with systemic autoimmune diseases associated with vasculitis. FJ

This work was supported by a grant from the Conseil Régional de Bretagne. We gratefully thank the maternity staff at Clinique Pasteur and Clinique de Kéraudren for their help with umbilical cord collecting over the years. We also appreciate the secretarial assistance of Simone Forest and Cindy Séné.

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Received for publication December 26, 2008.
Accepted for publication March 19, 2009.