Detection of HSP60 on the membrane surface of stressed human endothelial cells by atomic force and confocal microscopy

Gerald Pfister[2,*], Cordula M. Stroh[1,*], Hannes Perschinka[2], Michaela Kind[3], Michael Knoflach[3], Peter Hinterdorfer[1,‡] and Georg Wick[3]

[1]Institute for Biophysics, University of Linz, A-4040 Linz, Austria
[2]Institute for Biomedical Aging Research, Austrian Academy of Sciences, and [3]Institute of Pathophysiology, University of Innsbruck, Medical School, A-6020 Innsbruck, Austria

*These authors contributed equally to this work
‡Author for correspondence (e-mail: peter.hinterdorfer@jku.at)

Accepted 26 January 2005
Journal of Cell Science 118, 1587-1594 Published by The Company of Biologists 2005
doi:10.1242/jcs.02292

Summary

The highly conserved and ubiquitous heat shock proteins (HSP) are essential for the cellular homeostasis and efficiently trigger cellular responses to stress conditions. Both microbial and human HSP act as dominant antigens in numerous infectious and autoimmune diseases such as atherosclerosis, inducing a strong immune-inflammatory response. In the present study, the surface localization of HSP60 on stressed and unstressed human umbilical venous endothelial cells (HUVECs) was investigated using sensitive high resolution microscopy methods and flow cytometry. Confocal laser scanning microscopy (CLSM) revealed an increase of HSP60 in the mitochondria and on the surface of heat-stressed living and fixed HUVECs compared to unstressed cells. Atomic force microscopy (AFM), which has developed as sensitive surface-probe technique in biology, confirmed the presence of HSP60 on the membrane of stressed cells at an even higher lateral resolution by detecting specific single molecule binding events between the monoclonal antibody AbII-13 tethered to AFM tips and HSP60 molecules on cells. The interaction force (force required to break a single AbII-13/HSP60 bond) was 59±2 pN, which correlated nicely to the 51±1 pN measured with isolated HSP60 attached to mica surfaces. Overall, we found clear evidence for the occurrence of HSP60 on the surface of stressed HUVECs in a very similar patchy distribution pattern in living and fixed cells. The relevance of our findings with respect to the role of HSP60 in atherogenesis is discussed.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/8/1587/DC1

Key words: HUVEC, HSP60, atomic force microscopy, confocal microscopy, atherosclerosis

Introduction

Heat shock proteins (HSP) are ubiquitous and structurally highly conserved molecules. They have important physiological functions in folding and intracellular transport of newly synthesized proteins and in preventing their aggregation and misfolding. HSP are also important in a number of diseases such as cancer, and several autoimmune diseases. There is increasing evidence that atherosclerosis is also an immune-mediated disease (Ross, 1999; Hansson, 2001) and that members of the HSP60 family may be the main antigen (Wick et al., 1995; Wick and Xu, 1999; Wick et al., 2001; Wick et al., 2004). In particular, microbial HSP60 constitutes a major antigen recognised by the immune system during bacterial infections (Kaufmann, 1990). Owing to the strong homology between microbial and human HSP60 an immune reaction against infectious microbes may lead to a cross-reactive autoimmune response against the native human HSP60, which is increasingly produced in arterial endothelial cells (ECs) stressed by classical atherosclerosis risk factors (Mayr et al., 1999; Wick and Xu, 1999; Wick et al., 2001; Wick et al., 2004). Hence, the atherogenic importance of the stress-induced production of HSP60 in ECs provides the rational basis for our analysis of HSP60 on the surface of ECs after stress exposition.

We have applied two highly sensitive methods, i.e. confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM) for the surface detection of HSP60 on both living and fixed stressed, and unstressed human ECs. In CLSM, illumination and detection are confined to a defined, restricted volume in the cell, which improves the resolution by a factor of up to 1.4 (White and Amos, 1987). Out-of-focus structures receive almost no illumination and therefore produce only small interfering signals. Moreover, using CLSM it is possible to image successive, single optical planes throughout a cell, which can later be digitally combined to produce a detailed three-dimensional reconstruction. These features made CLSM the optical method of choice to locate HSP60 on the surface of ECs in this study.

Compared to optical microscopy, AFM [first introduced by Binning et al. (Binning et al., 1986)] gives an even better spatial resolution (in the order of a few nanometers on biological samples), but needs more time to collect data. The additional potential of the AFM in detecting ultra-low surface forces

at high lateral resolution, allows the measurement of intermolecular forces between individual molecules. For this, one binding partner is attached to the surface of the object to be analysed while the moving tip of the AFM is functionalized with the other one and unbinding forces of individual receptor-ligand pairs can be quantified (Lee et al., 1994; Florin et al., 1994; Dammer et al., 1995; Hinterdorfer et al., 1996; Allen et al., 1997). A spacer molecule, used to bind the ligand to the AFM tip, enhances the mobility of the ligand, thereby increasing its chance to specifically bind to the cognitive receptor attached to the surface (Hinterdorfer et al., 1996; Hinterdorfer et al., 1998). In addition, it helps to discriminate between specific and nonspecific interactions. Since this method also works with receptors embedded in membranes of live and fixed cells (Lehenkari and Horton, 1999) we aimed to prove the presence of HSP60 on the cell surface of stressed ECs and to measure the interaction strength between HSP60 and a monoclonal antibody (AbII-13) directed against human HSP60, which was coupled to the AFM tip.

Materials and Methods

Cell culture

Human umbilical venous endothelial cells (HUVECs) were isolated as described previously (Amberger et al., 1997). Cells were passaged in 0.2% gelatine-coated polystyrene flasks (BD, France) in endothelial cell basal medium (CC-3121, BioWhittaker) with EGM SingleQuots supplements and growth factors (CC-4133, BioWhittaker) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells (10^5) were seeded on a 0.2% gelatine-coated coverslide and used for the experiments at >80% confluence as described previously (Henderson et al., 2003).

Cell and protein preparation for CLSM and AFM

Cells for CLSM were grown in gelatine-coated Falcon polystyrene 60×15 mm tissue culture dishes (No. 3004, BD). Each experiment was performed in duplicates. Cells were heat-stressed for 30 minutes at 42°C, followed by a 6-hour recovery period at 37°C to allow for full synthesis of HSP60. For observation of live cells, stressed and unstressed cells were incubated at 37°C for 60 minutes with monoclonal antibodies against HSP60 [AbII-13; supernatant derived from human hybridoma cell line, clone II-13 raised against human recombinant HSP60 protein PKK13A lacking the first 30 amino acids (Singh and Gupta, 1992)], and a FITC-labelled rabbit anti-mouse IgG secondary antibody (No. 261, Dako, 37°C, 60 minutes). The monoclonal antibody II-13 is highly specific for human HSP60 and has been used to identify human HSP60 in several studies (Xu et al., 1994; Sigal et al., 2001). After washing with phosphate-buffered saline (PBS, pH 7.2), cells were immediately observed using CLSM at room temperature (RT). In parallel, cells were fixed using acetone/methanol (50/50; v/v) at –20°C for 2 minutes, or paraformaldehyde (PFA, 4% in PBS) at 4°C for 30 minutes, respectively.

Immunofluorescence staining was performed at RT. After blocking with 1% bovine serum albumin (BSA, Sigma) in PBS for 60 minutes cells were incubated with AbII-13 for 60 minutes. Cells were then washed with PBS and incubated with a FITC-labelled rabbit anti-mouse IgG secondary antibody (No. 261, Dako) for 60 minutes. DNA was labelled using the red fluorescent dye TOPRO-3 (Molecular Probes, USA), and cells were embedded in 90% buffered glycerol in PBS for confocal microscopy (μRadiance confocal scanning system; Bio-Rad, UK).

For AFM, HUVECs were grown onto coverslides and heat-stressed as described. After a 6-hour recovery period at 37°C, they were fixed with 2% glutaraldehyde. Incubation with glutaraldehyde is the most rapid fixation method and leaves the cell membrane impermeable and the binding capacity of the proteins intact. Control cells were treated the same way except that they were not heat stressed. For AFM on isolated HSP60, HSP60 was electrostatically adsorbed to freshly cleaved mica sheets for 20 minutes at 1-10 μg/ml HSP60 in 10 mmol/l Tris buffer (pH 7.5) containing 150 mmol/l NaCl and 2 mmol/l $NiCl_2$, and subsequently washed.

Flow cytometric assessment of HSP60 surface expression on HUVECs

Cells were grown and treated as described above. For flow cytometric analysis 5×10^5 cells were harvested from confluent monolayers and incubated at 37°C for 60 minutes with monoclonal antibodies against HSP60 (AbII-13), and a FITC-labelled rabbit anti-mouse IgG secondary antibody (No. 261, Dako; 37°C, 60 minutes). Cells were simultaneously labelled with propidium iodide, washed and analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The gate for HSP60-positive cells was set for cells with significantly higher fluorescence intensity than unstained control cells and cells that were only stained with the FITC secondary antibody, respectively (data not shown). All cells with significantly higher FITC fluorescence intensities than these controls, i.e. cells in the R2 gate, were considered as being HSP60 positive (Fig. 4b). Living cells with an intact cell membrane are impermeable to propidium iodide, whereas cells being necrotic lose their membrane integrity and become permeable, i.e. positive for propidium iodide. Hence, propidium iodide can be used as a measure of cell membrane integrity.

Scanning force microscopy/spectroscopy

We used a MAC mode PicoSPM magnetically driven dynamic force microscope (Molecular Imaging, Tempe, AZ). Topography images were recorded in the MACmode (Han et al., 1996; Han et al., 1997a; Han et al., 1997b; Raab et al., 1999). For the detection of antibody-antigen recognition, force-distance cycles were performed at RT using antibody-coated cantilevers (cantilevers from Veeco GmbH, Mannheim, Germany) with 0.02-0.1 N/m nominal spring constants in the conventional contact force spectroscopy mode at 50-200 nm sweep range and 1 Hz sweep rate. Block experiments were carried out with 43 μg/ml AbII-13 in PBS. Spring constants of cantilevers were determined using the thermal noise method (Hutter and Bechhoefer, 1993) and analysis of interaction forces was performed using Matlab (Math Works Inc., Natick, MA) as previously described (Baumgartner et al., 2000).

Conjugation of antibody and HSP60 to tips

Conjugation of antibody (AbII-13) directed against HSP60 to AFM-tips was done using a flexible PEG (polyethylene glycol)-crosslinker as described before (Haselgruebler et al., 1995; Hinterdorfer et al., 1996; Hinterdorfer et al., 1998). HSP60 was conjugated to PDP (2-(pyridyldithio)propionyl)-PEG tips via its intrinsic cysteins in buffer A (100 mmol/l NaCl, 50 mmol/l NaH_2PO_4, 1 mmol/l $EDTANa_2$, adjusted to pH 7.5 with NaOH) at a concentration of 18 μg/ml for 2 hours before they were extensively washed using buffer A.

Results

Occurrence of HSP60 on the surface of HUVECs

Freshly isolated HUVECs at passages below five were used for all experiments. The cells formed a uniform monolayer and no significant morphological changes, condensation or fragmentation of nuclear DNA was observed in unstressed and stressed cells (Fig. 1). We analysed both unstressed HUVECs kept in routine cell culture and stressed cells after their exposure to heat stress (42°C for 30 minutes followed by a 6-

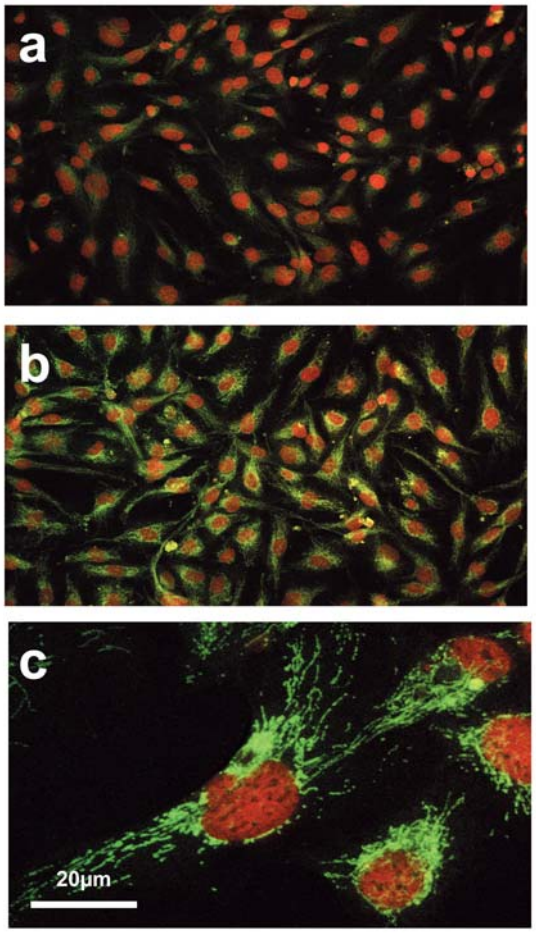

Fig. 1. Production of HSP60 in the mitochondria of HUVECs fixed with ice-cold acetone/methanol. The nuclei appear red after staining with the fluorescent DNA dye TOPRO-3 and excitation at 543 nm. HSP60 appears green after labelling with a FITC-conjugated monoclonal anti-HSP60 antibody and excitation at 488 nm. (a) Unstressed control cells. (b) Cells exposed to heat stress (42°C, 30 minutes). (c) Detailed view of mitochondrial localization of HSP60 in heat stressed cells.

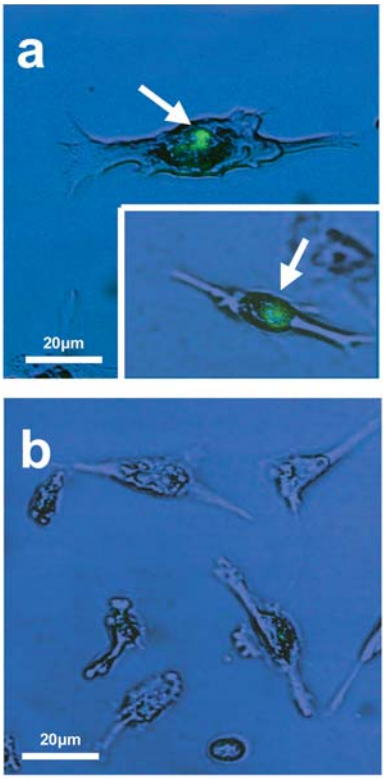

Fig. 2. CLSM images of HSP60 on the surface of living HUVECs after incubation with the FITC-conjugated monoclonal anti-HSP60 antibody II-13. All images are focused on the top of the cells. (a) Heat-stressed (42°C, 30 minutes) cell showing HSP60 on the cell surface (green regions marked by arrows). The insert shows another heat stressed cell expressing HSP60 on the cell surface. (b) Unstressed control cells with almost no surface expression of HSP60.

hour recovery period) and observed almost no production of HSP60 in unstressed cells, as evident from the low level of fluorescence (Fig. 1a). In contrast, the green colour in Fig. 1b revealed that there was a significant increase in HSP60 production in heat-stressed cells, which was predominantly mitochondrial (Fig. 1c). In addition to the intracellular presence, a significant portion of living stressed cells (10.9±3.6%) had exposed HSP60 on their surface, whereas unstressed controls showed no surface expression of HSP60 (0.3±0.05%, Figs 2-4). The ratio of cells showing HSP60 on their surface after heat stress was equivalent in cells analysed by confocal microscopy (10.9±3.6%; Fig. 2) and flow cytometry (9.5%). Hence, in response to stress there was a significant increase in the number of cells expressing HSP60 on their surface, from 1.1% in unstressed to 9.5% in stressed cells (Fig. 4b). The threshold value for cells we considered to express HSP60 on their surface was set by calibrating the FITC fluorescence of both unstained cells and cells only labelled with the secondary FITC antibody. Counterstaining with propidium iodide clearly proved the integrity of the cell membrane in both stressed and unstressed HUVECs (Fig. 4c).

Force detection of AbII-13 interaction with HSP60 on cells

The high sensitivity of the AFM and the soft cantilever used makes it possible to detect pN forces. It has been shown that single molecule receptor-ligand forces are typically in the range of 50-250 pN (Florin et al., 1994; Lee et al., 1994; Hinterdorfer et al., 1996). The principle of single molecule recognition force detection using AFM is depicted in Fig. 5a. In this so-called force-distance mode, the deflection angle of the cantilever is measured as a function of the vertical position of the cantilever. The AFM tips are conjugated with ligands (here antibodies) via distensible tethers at a very dilute surface concentration, so that only one of them has access to the receptors, which are bound to probe surfaces.

First, the tip approaches the surface (Fig. 5a; dotted trace). Far away from the surface (1) no bending of the cantilever is visible and the deflection and, thus, the force is almost zero. In the contact regime (2), the cantilever is deflected upwards because of the repulsive tip-sample interaction during contact. The measured deflection, Δz, is directly proportional to the

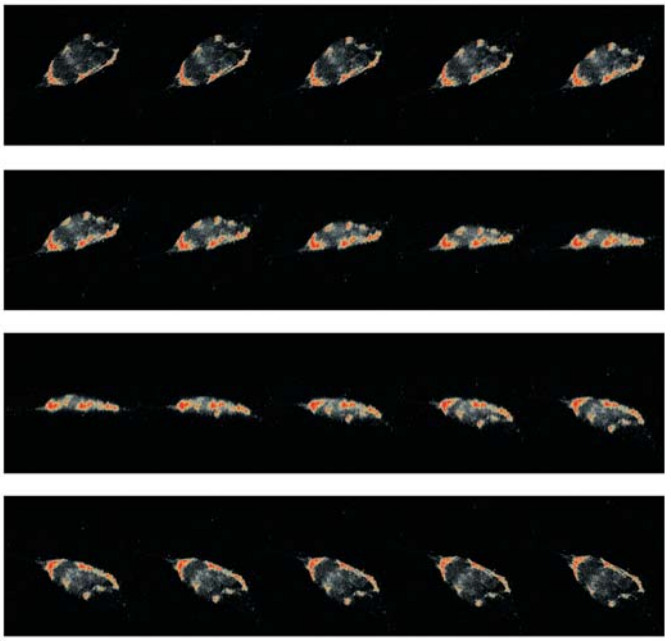

Fig. 3. Image series of a three dimensional reconstruction of 25 single zx-profiles of a living heat-stressed HUVEC. Surface HSP60 is presented as a thermo-false-colour image. The image series is from a video (see Movie 1 in supplementary material).

interaction force f, $f=k \times \Delta z$ (Hook's law), where k is the spring constant of the cantilever. The succeeding retraction of the cantilever (retrace, solid line) first results in relaxation of the repulsive force in the contact region (2 to 3). In case the antibody on the tip has bound to a receptor on the cell surface, an attractive force develops (downward deflection) and the crosslinker, via which the antibody is attached to the tip, will be stretched (3) (Kienberger et al., 2000). Finally, the antibody will detach from the antigen on the surface (3 to 4) at a distinct critical force, termed unbinding force, f_u (see arrow), and the cantilever jumps back to zero deflection (4).

We used AFM force measurements to verify the presence of HSP60 on cell surfaces. Fig. 5b shows a typical force-distance cycle for a single molecule antibody-antigen recognition event with an AbII-13 containing tip on the surface of a stressed cell. In the example shown, AbII-13/HSP60 dissociation occurs at a distance of 77 nm with a force of 90 pN. The specific binding event vanishes when free AbII-13 antibody is present in solution because the binding sites on the cell are occupied (block experiment). Thus, the antibody on the tip cannot bind to the HSP60 on the cell surface and retrace looks like trace (Fig. 5b, inset).

Unbinding force and binding activity of the AbII-13/HSP60 interaction

Force-distance cycles were recorded on five different cells (HUVECs) using tips to which an AbII-13 was coupled. For each cell, several positions on the cell surface were studied resulting in about 2000 force-distance cycles in total. After calibrating the spring constant of each cantilever used, empirical probability density functions (pdf) from the detected unbinding forces f_u were constructed (Fig. 5c). The maximum of the distribution (f_u=59 pN) reflects the most probable force upon which a single AbII-13/HSP60 bond dissociates under the force ramp used. This value falls in the range of previously measured receptor/ligand unbinding forces.

An overall binding probability, which is the probability of finding an unbinding event in a force-distance cycle or not, of 15.4% was obtained under the conditions described above (Fig. 5d). Blocking experiments, performed by injection of free antibody in solution reduced this probability to 5.8% (Fig. 5d). No binding at all was found when a non-coated tip was used instead of an AbII-13-coated tip on stressed cells. These results strongly support the specificity of the binding events observed. Thus, HSP60 must be present on the surface of stressed HUVECs.

Interactions of AbII-13-coated tips were also studied with non-stressed cells. About 1500 force-distance cycles were recorded on three different cells at various lateral positions and analysed, resulting in a binding probability of 3.9% (Fig. 5d). Since the binding activity on unstressed cells is dramatically decreased compared to the binding activity on stressed cells, HSP60 can only be present in trace amounts on unstressed HUVECs.

We also studied the binding of AbII-13 on AFM tips to isolated HSP60 (Fig. 6a) adsorbed onto mica. Six different data sets with a total of about 5500 force-distance cycles were recorded. The resulting probability density function of one of them is shown in Fig. 6b and yielded a most probable unbinding force of f_u=51 pN under the force ramp used. Since the unbinding force value of AbII-13 on isolated HSP60 almost exactly matches the value obtained on stressed HUVECs, additional evidence for the presence of HSP60 on the cell surface was found. The specificity of AbII-13 binding to isolated HSP60 is demonstrated in Fig. 6c. The binding probability was significantly reduced upon blocking with free AbII-13 in solution.

Discussion

HSP are ubiquitous proteins with a high degree of amino acid sequence homology across different species. The increased production of HSP is a central and protective cellular response to environmental and metabolic stress in all procaryotic and eucaryotic organisms (Morimoto, 1993; Hartl, 1996). Members of the HSP60 family belong to one of the most conserved protein families in evolution (Karlin and Brocchieri, 2000), with over 70% local sequence homology between procaryotic (microbial) and eucaryotic (human) HSP60. This high homology leads to the formation of shared B- and T-cell epitopes, which are cross-reactive between procaryotic and eucaryotic HSP60 (Elias et al., 1990; Zügel and Kaufmann, 1999). Since microbial HSP60s are major antigens in most infectious diseases, the crossreactivity has been discussed as a trigger in several autoimmune disorders such as rheumatoid arthritis and systemic sclerosis.

It has been shown that bacterial infections causally contribute to the pathogenesis of atherosclerosis (Mayr et al., 1999; Kiechl et al., 2001), and we have put forward the concept that the missing link between bacterial infections and atherosclerosis could be the crossreaction between microbial and human HSP60 in the vascular endothelium (Wick et al., 2001; Wick et al., 2004). In human cells, HSP60 is an

important mitochondrial protein (Bukau and Horwich, 1998), which is also found in various extramitochondrial locations (reviewed by Soltys and Gupta, 1999). These locations include foci on the endoplasmic reticulum (Soltys et al., 1996), unidentified vesicles and cytoplasmic granules (Soltys and Gupta, 1996) and the cell surface (Xu et al., 1994; Soltys and Gupta, 1997; Barazi et al., 2002).

With respect to the development of atherosclerosis, the expression of HSP60 on the surface of vascular ECs is an extremely interesting feature. Obviously, these cells are the first in direct contact with the immune system via circulating immune cells and antibodies. Hence, HSP60 on the surface of vascular ECs could function as a danger signal during and after an exposure to cellular stress factors, such as hypertension, free radicals, toxins, cytokines or fever. However, only Xu et al. (Xu et al., 1994) reported the existence of HSP60 on the surface of stressed vascular ECs. Schett et al. (Schett et al., 1995) found high levels of hsp60 mRNA expression in stressed ECs and concluded that human serum anti-hsp65 antibodies act as autoantibodies reacting with hsp60 on the surface of stressed endothelial cells, thus being able to mediate endothelial cytotoxicity. Other records of surface-associated HSP60 include wild-type Chinese hamster ovary cells (Soltys and Gupta, 1996), mouse liver and spleen cells (Belles et al., 1999), mouse macrophages (Habich et al., 2002), some human T-cell lines (Soltys and Gupta, 1997; Khan et al., 1998) and human breast and lung carcinoma cells (Barazi et al., 2002). Evidence for the existence of HSP60 on the surface of human vascular ECs has so far not been presented.

In our study, we clearly showed that stressed human vascular ECs do present HSP60 on their surface. Three highly sensitive methods (CLSM, AFM and flow cytometry) were employed and provided similar and reproducible results. All applied methods allowed for the detection of HSP60 on the surface of HUVECs and both flow cytometric and confocal microscopic analysis showed that in roughly 10% of heat stressed HUVECs HSP60 was expressed on the cell surface, while this was the case in only about 1% of unstressed control cells. With CLSM we could detect HSP60 on both living and fixed cells. A non-uniform, patchy distribution of HSP60 indicates its arrangement in clusters, which might well be associated to lipid rafts and specific HSP-receptors. We found a very similar distribution pattern on the surface of living and fixed cells, which demonstrates that the fixation did not alter the distribution of HSP60 on the cell surface or destroy its binding properties. Thus, we speculate that the surface-HSP60 is actively bound by a receptor-ligand mechanism.

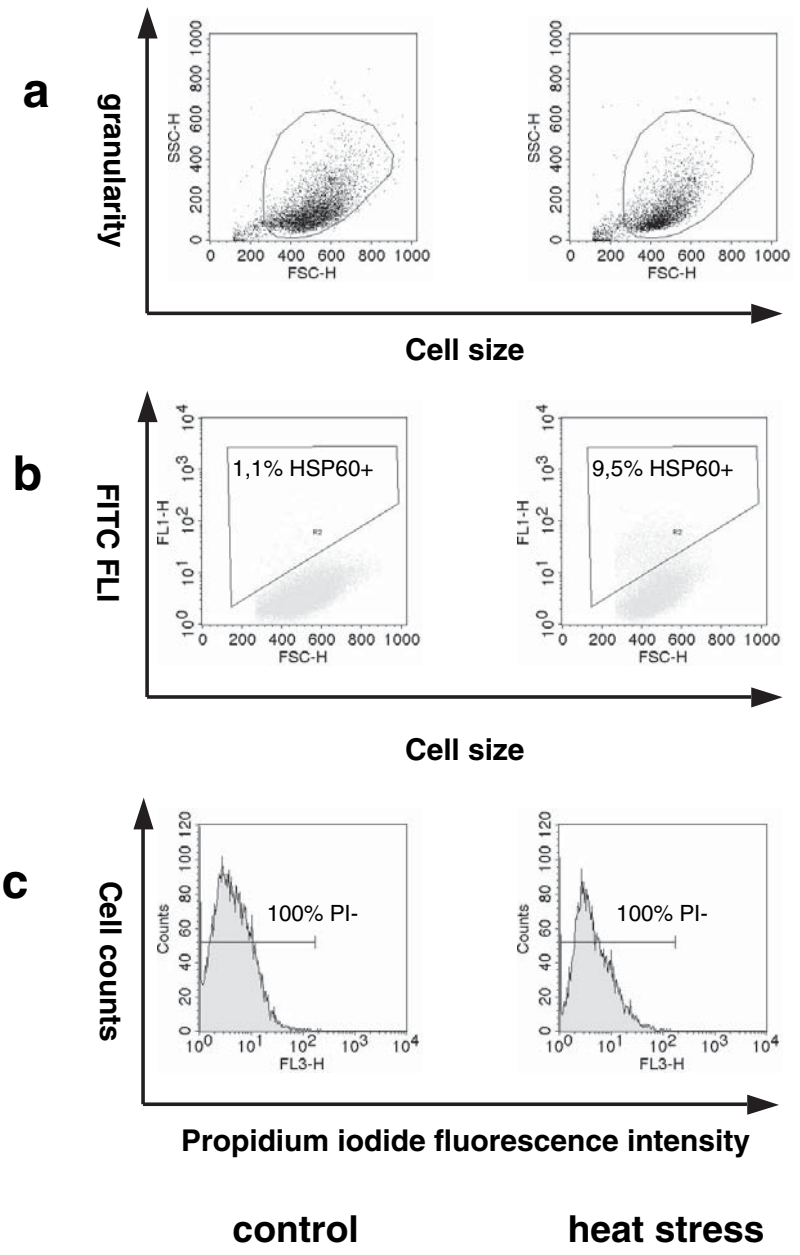

Fig. 4. Flow cytometric analysis of unstressed (left panels) and heat-stressed (right panels) living HUVECs. (a) Dot plots of forward scatter (cell size) against side scatter (surface granularity) showing similar size and granularity in control and heat-stressed cells. (b) Dot plots of forward scatter (cell size) against FITC fluorescence intensity (FITC FLI) showing a significant increase of cells with HSP60 surface expression from 1.1% in the control cells to 9.5% in heat-stressed cells. Cells have been incubated with the FITC labelled monoclonal antibody II-13 specific for human HSP60. (c) Histogram plots of cell counts against propidium iodide intensity showing full cell membrane integrity in control cells and heat-stressed cells. The full membrane integrity is indicated by the equal high percentage (100%) of propidium iodide negative cells in both unstressed and stressed HUVECs.

These findings are in agreement with other investigations of specific receptors for HSP60. Vabulas et al. (Vabulas et al., 2001) elucidated the role of Toll-like receptors 2 (TLR2) and TLR4 in activating the Toll/interleukin-1 receptor signalling pathway in innate immune cells via both endocytosed human and chlamydial HSP60. TLR4 has also been identified as a putative receptor for HSP60 in mouse macrophages by Ohashi et al. (Ohashi et al., 2000). Bulut and co-workers (Bulut et al.,

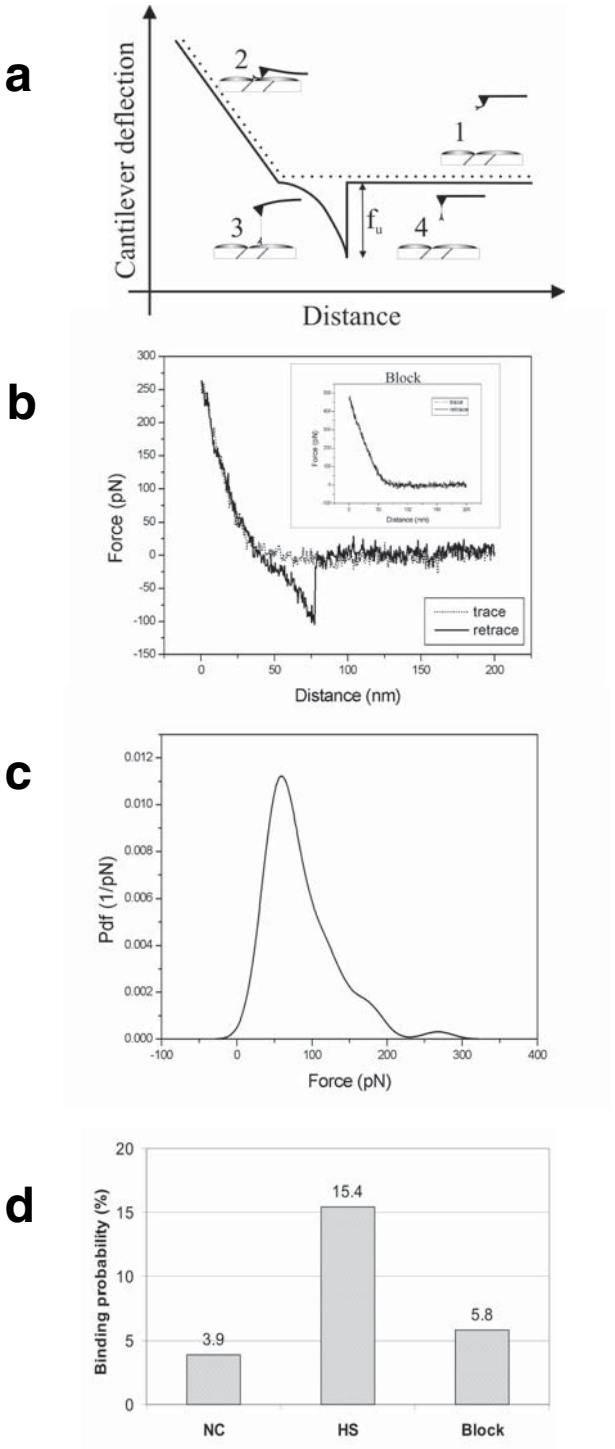

Fig. 5. Force spectroscopy of AbII-13-coated tip on HUVECs. (a) Schematic representation of a force-distance cycle carried out to measure specific molecular forces. (b) The force-distance cycle on HUVECs with an AbII-13-coated AFM tip shows specific interaction in the retrace (jump at 75 nm). The specific interaction is blocked using free AbII-13 in solution (inset). (c) The probability density function (pdf) is constructed from an ensemble of forces, gives the distribution of unbinding forces f_u. (d) Binding probabilities of AbII-13-coated tips on non-stressed cells (NC), heat-shocked cells (HS) and on heat shocked cells blocked with free AbII-13 in solution (Block), proving the specificity of the measured molecular interactions.

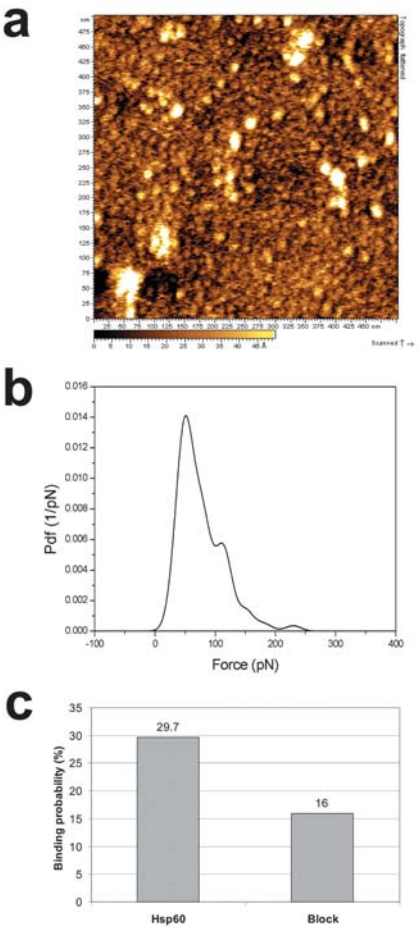

Fig. 6. Force spectroscopy of AbII-13-coated tip on a protein layer of isolated HSP60. HSP60 was electrostatically adsorbed onto mica. (a) Topography image of HSP60 on mica. Single proteins of about 3 nm in height and small aggregates cover the surface of the 500×500 nm large area almost completely. The false colour bar at the bottom of the image (0 to 5 nm, from dark to bright) indicates the heights of the objects. (b) Probability density function (pdf) of specific molecular forces giving the distribution of unbinding forces f_u. (c) Binding probabilities of AbII-13-coated tips on isolated HSP60 without (left) and with free AbII-13 in solution (Block), proving the specificity of the measured molecular intersections.

2002) demonstrated chlamydial HSP60 activation of mouse macrophages and ECs through TLR4 and MD2 and discussed the relevance of this process for the initiation of inflammation during atherogenesis. By examining the expression of certain adhesion molecules and the nuclear transcription factor NF-κB, Kol et al. (Kol et al., 2000) found the CD14 receptor to be responsible for HSP60-induced activation of the innate immune response in human vascular endothelium, smooth muscle cells and macrophages. Additionally, TLR4 and CD14 receptor-independent receptors for HSP60 and HSP70 have been identified (Triantafilou et al., 2001; Lipsker et al., 2002).

According to our concept of atherogenesis (reviewed by Wick et al., 2004), we assume that the HSP60 we found on stressed ECs is autologous and may act as a danger signal in vivo, triggering inflammatory diseases such as atherosclerosis. This is in accordance with the observation that in unstressed HUVECs we could detect HSP60 exclusively in mitochondria

(Fig. 1a) whereas we observed a cytoplasmatic and surface expression of HSP60 in stressed HUVEC (Figs 1-3). That observation strongly supports our theory, that stressed ECs produce HSP60, which is then translocated from the mitochondria to the cytoplasm and finally to the cell surface. However, whether the surface HSP60 we detected was produced by the respective EC or was coming from surrounding cells is still an open question and has to be clarified in further studies.

In addition to the CLSM and flow cytometry analysis data, we were able to unequivocally prove the presence of HSP60 on the surface of stressed human ECs using AFM. AFM has become a classical surface analysis method, capable of creating topography reliefs and detecting interaction forces with nm lateral resolution. A detailed view of the location of HSP60 on the outer surface of the HUVECs was obtained by tethering a cognitive ligand (monoclonal antibody AbII-13) to an AFM tip and sensing its interaction with stressed and unstressed cells. This configuration allowed for single molecule interaction force detection between the tip-coupled AbII-13 and potentially surface-presented HSP60. It was shown that AbII-13 showed significantly more binding activity to the surface of stressed HUVECs than to unstressed cells and that this binding activity was the result from a specific molecular recognition between AbII-13 on the AFM tip and HSP60 on the cell surface. Analysis of binding activity and unbinding force clearly revealed that HSP60 is exposed on the surface of HUVECs, however, only when previously stressed. First experiments with HSP60 coupled to the AFM tip showed interactions only with unstressed cells but not with stressed ones. This is another valid indication that HSP60 interacts with the cell membrane via a specific receptor. Our comprehensive data from CLSM and AFM provide a detailed mechanical picture of HSP60 expression and cell membrane surface binding.

This work was supported by the Austrian Research Fund (FWF, project 14741 to G.W., projects P12802/14549 to P.H.), the Merkur Insurance Company, Graz, Austria, and a donation by Julius Wallnöfer, Innsbruck, Austria. The human hybridoma cell line producing the monoclonal antibody II-13 was a generous gift from R. S. Gupta, McMaster University Hamilton, Ontario, Canada.

References

Allen, S., Chen, J., Davies, M. C., Dawkes, A. C., Edwards, J. C., Roberts, C. J., Sefton, J., Tendler, S. J. B., and Williams, P. M. (1997). Detection of antigen-antibody binding events with the atomic force microscope. *Biochemistry* **36**, 7457-7463.

Amberger, A., Maczek, C. and Jürgens, G. (1997). Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins. *Cell Stress Chaperones* **2**, 94-103.

Barazi, H. O., Zhou, L., Templeton, N. S., Krutsch, H. C. and Roberts, D. D. (2002). Identification of heat shock protein 60 as a molecular mediator of $\alpha 3\beta 1$ integrin activation. *Cancer Res.* **62**, 1541-1548.

Baumgartner, W., Hinterdorfer, P. and Schindler, H. (2000). Data analysis of interaction forces measured with the atomic force microscope. *Ultramicroscopy* **82**, 85-95.

Belles, C., Kuhl, A., Nosheny, R. and Carding, S. R. (1999). Plasma membrane expression of heat shock protein 60 in vivo in response to infection. *Infect. Immun.* **67**, 4191-4200.

Binning, G., Quate, C. F. and Gerber, C. (1986). Atomic force microscope. *Phys. Rev. Lett.* **56**, 930-933.

Bukau, B. and Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell* **92**, 351-366.

Bulut, Y., Faure, E., Thomas, L., Karahashi, H., Michelsen, K. S., Equils, O., Morrison, S. G., Morrison, R. P. and Arditi, M. (2002). Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J. Immunol.* **168**, 1435-1440.

Dammer, U., Popescu, O., Wagner, P., Anselmetti, D., Guentherodt, H.-J. and Misevic, G. N. (1995). Binding strength between cell adhesion proteoglycanes measured by atomic force microscopy. *Science* **267**, 1173-1175.

Elias, D., Markovits, D., Reshef, T., van der Zee, R. and Cohen, I. R. (1990). Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein. *Proc. Natl. Acad. Sci. USA* **87**, 1576-1580.

Florin, E. L., Moy, V. T. and Gaub, H. E. (1994). Adhesion forces between individual ligand-receptor pairs. *Science* **264**, 415-417.

Habich, C., Baumgart, K., Kolb, H. and Burkart, V. (2002). The receptor for heat shock protein 60 on macrophages is saturable, specific, and distinct from receptors for other heat shock proteins. *J. Immunol.* **168**, 569-576.

Han, W., Lindsay, S. M. and Jing, T. (1996). A magnetically driven oscillating probe microscope for operation in liquid. *Appl. Phys. Lett.* **69**, 1-3.

Han, W., Dlakic, M., Zhu, Y. J., Lindsay, S. M. and Harrington, R. E. (1997a). Strained DNA is kinked by low concentrations of Zn^{2+}. *Proc. Natl. Acad. Sci. USA* **69**, 10565-10570.

Han, W., Lindsay, S. M., Dlakic, M. and Harrington, R. E. (1997b). Kinked DNA. *Nature* **386**, 563.

Hansson, G. K. (2001). Immune mechanisms in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **21**, 1876-1890.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature* **381**, 571-579.

Haselgruebler, T., Amerstorfer, A., Schindler, H. and Gruber, H. J. (1995). Synthesis and application of a new poly(ethylene glycol) derivative for the crosslinking of amines with thiols. *Bioconjugate Chem.* **6**, 242-248.

Henderson, B. R., Pfister, G., Boeck, G., Kind, M., and Wick, G. (2003). Expression levels of heat shock protein 60 in human endothelial cells in vitro are unaffected by exposure to 50 Hz magnetic fields. *Cell Stress Chaperones* **8**, 172-182.

Hinterdorfer, P., Baumgartner, W., Gruber, H. J., Schilcher, K. and Schindler, H. (1996). Detection and localization of individual antibody-antigen recognition events by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* **93**, 3477-3481.

Hinterdorfer, P., Schilcher, K., Baumgartner, W., Gruber, H. J. and Schindler, H. (1998). A mechanistic study of the dissociation of individual antibody-antigen pairs by atomic force microscopy. *Nanobiology* **4**, 39-50.

Hutter, J. and Bechhoefer, J. (1993). Calibration of atomic-force microscope tips. *Rev. Sci. Inst.* **64**, 1868-1873.

Karlin, S. and Brocchieri, L. (2000). Heat shock protein 60 sequence comparisons: duplications, lateral transfer, and mitochondrial evolution. *Proc. Natl. Acad. Sci. USA* **97**, 11348-11353.

Kaufmann, S. H. (1990). heat shock proteins and the immune response. *Immunol. Today* **11**, 129-136.

Khan, I. U., Wallin, R., Gupta, R. S. and Kammer, G. M. (1998). Protein kinase A-catalyzed phosphorylation of heat shock protein 60 chaperone regulates its attachment to histone 2B in the T lymphocyte plasma membrane. *Proc. Natl. Acad. Sci. USA* **95**, 10425-10430.

Kiechl, S., Egger, G., Mayr, M., Wiedermann, C. J., Bonora, E., Oberhollenzer, F., Muggeo, M., Xu, Q., Wick, G., Poewe, W. and Willeit, J. (2001). Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study. *Circulation* **103**, 1064-1070.

Kienberger, F., Pastushenko, V. Ph., Kada, G., Gruber, H. J., Riener, C., Schindler, H. and Hinterdorfer, P. (2000). Static and dynamical properties of single poly(ethylene glycol) molecules investigated by force spectroscopy. *Single Mol.* **1**, 123-128.

Kol, A., Lichtmann, A. H., Finberg, R. W., Libby, P. and Kurt-Jones, E. A. (2000). Cutting Edge: Heat shock protein (HSP) 60 activates the innate immune response : CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J. Immunol.* **164**, 13-17.

Lee, G. U., Kidwell, D. A. and Colton, R. J. (1994). Sensing discrete streptavidin-biotin interaction with the atomic force microscope. *Langmuir* **10**, 354-357.

Lehenkari, P. P. and Horton, M. A. (1999). Single integrin molecule

adhesion forces in intact cells measured by atomic force microscopy. *Biochem. Biophys. Res. Commun.* **259**, 645-650.

Lipsker, D., Ziylan, U., Spehner, D., Proamer, F., Bausinger, H., Jeannin, P., Salamero, J., Bohbot, A., Cazenave, J. P., Drillien, R. et al. (2002). Heat shock proteins 70 and 60 share common receptors which are expressed on human monocyte-derived but not epidermal dendritic cells. *Eur. J. Immunol.* **32**, 322-332.

Mayr, M., Metzler, B., Kiechl, S., Willeit, J., Schett, G., Xu, Q. and Wick, G. (1999). Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of Escherichia coli and Chlamydia pneumoniae: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis. *Circulation* **99**, 1560-1566.

Morimoto, R. I. (1993). Cells in stress: transcriptional activation of heat shock genes. *Science* **259**, 1409-1410.

Ohashi, K., Burkart, V., Flohe, S. and Kolb, H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J. Immunol.* **164**, 558-561.

Raab, A., Han, W., Badt, D., Smith-Gill, S. J., Lindsay, S. M., Schindler, H. and Hinterdorfer, P. (1999). Antibody recognition imaging by force microscopy. *Nature Biotechnology* **17**, 902-905.

Ross, R. (1999). Atherosclerosis-an inflammatory disease. *N. Engl. J. Med.* **340**, 115-126.

Schett, G., Xu, Q., Amberger, A., van der Zee, R., Recheis, H., Willeit, J. and Wick, G. (1995). Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. *J. Clin Invest.* **96**, 2569-2577.

Sigal, L. H., Williams, S., Soltys, B. and Gupta, R. (2001). H9724, a monoclonal antibody to Borrelia burgdorferi's flagellin, binds to heat shock protein 60 (HSP60) within live neuroblastoma cells: a potential role for HSP60 in peptide hormone signaling and in an autoimmune pathogenesis of the neuropathy of Lyme disease. *Cell. Mol. Neurobiol.* **21**, 477-495.

Singh, B. and Gupta, R. S. (1992). Expression of human 60-kD heat shock protein (HSP60 or P1) in Escherichia coli and the development and characterization of corresponding monoclonal antibodies. *DNA Cell Biol.* **11**, 489-496.

Soltys, B. J. and Gupta, R. S. (1996). Immunoelectron microscopic localization of the 60-kDa heat shock chaperonin protein (Hsp60) in mammalian cells. *Exp. Cell Res.* **222**, 16-27.

Soltys, B. J. and Gupta, R. S. (1997). Cell surface localization of the 60 kDa heat shock chaperonin protein (60) in mammalian cells. *Cell Biol. Int.* **21**, 315-320.

Soltys, B. J. and Gupta, R. S. (1999). Mitochondrial-matrix proteins at unexpected locations: are they exported? *Trends Biochem. Sci.* **24**, 174-177.

Soltys, B. J., Falah, M. and Gupta, R. S. (1996). Identification of endoplasmic reticulum in the primitive eucaryote Giardia lamblia using cryoelectron microscopy and antibody to Bip. *J. Cell Sci.* **109**, 1909-1917.

Triantafilou, K., Triantafilou, M. and Dedrick, R. L. (2001). A CD14-independent LPS receptor cluster. *Nature Immunol*. **2**, 338-345.

Vabulas, R. M., Ahmad-Nejad, P., da Costa, C., Miethke, T., Kirschning, C. J., Häcker, H. and Wagner, H. (2001). Endocytosed HSP60s us Toll-like receptor 2 (TLR2) and TLR4 to activate the Toll/interleukin-1 receptor signalling pathway in innate immune cells. *J. Biol. Chem.* **276**, 31332-31339.

White, J. G. and Amos, W. B. (1987). Confocal microscopy comes of age. *Nature* **328**, 183-184.

Wick, G., Schett, G., Amberger, A., Kleindienst, R. and Xu, Q. (1995). Is atherosclerosis an immunologically mediated disease? *Immunol. Today* **16**, 27-33.

Wick, G. and Xu, Q. (1999). Atherosclerosis – An autoimmune disease. *Exp. Gerontol.* **34**, 559-566.

Wick, G., Perschinka, H. and Millonig, G. (2001). Atherosclerosis as an autoimmune disease: an update. *Trends Immunol.* **22**, 665-669.

Wick, G., Knoflach, M. and Xu, Q. (2004). Autoimmune and inflammatory mechanisms in atherosclerosis. *Annu. Rev. Immunol.* **22**, 361-403.

Xu, Q., Schett, G., Seitz, C. S. Hu, Y., Gupta, R. S. and Wick, G. (1994). Surface staining and cytotoxic activity of heat-shock protein 60 antibody in stressed aortic endothelial cells. *Circ. Res.* **75**, 1078-1085.

Zügel, U. and Kaufmann, S. H. (1999). Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clin. Microbiol. Rev.* **12**, 19-39.