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

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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.

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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 HS60 on the cell surface of stressed ECs and to measure the interaction strength between HS60 and a monoclonal antibody (AbII-13) directed against human HS60, 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 as described previously (Amberger et al., 1997). Cells were passaged cell basal medium (CC-3121, BioWhittaker) with EGM SingleQuots as described previously (Amberger et al., 1997). Cells were passaged to achieve >80% confluence before experiments. Freshly isolated HUVECs at passages below five were used for all experiments. The cells were kept in routine cell culture and stressed cells after their exposure to heat stress (42°C for 30 minutes followed by a 6-hour recovery period at 37°C) were used for all experiments. The cells were fixed in 4% paraformaldehyde in PBS for 20 minutes at 4°C and embedded in 90% buffered glycerol in 10 mmol/l Tris buffer (pH 7.5) containing 150 mmol/l NaCl and 2 mmol/l NiCl2, and subsequently washed.

Flow cytometric assessment of HS60 surface expression on HUVECs

Cells were grown and treated as described above. For flow cytometric analysis 5×10⁵ cells were harvested from confluent monolayers and incubated at 37°C for 60 minutes with monoclonal antibodies against HS60 (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 HS60-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 HS60 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 HS60 to tips

Conjugation of antibody (AbII-13) directed against HS60 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). HS60 was conjugated to PDP (2-(pyridyldithio)propionyl)-PEG tips via its intrinsic cysteins in buffer A (100 mmol/l NaCl, 50 mmol/l NaH2PO4, 1 mmol/l EDTANa2, 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 HS60 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 cells after their exposure to heat stress (42°C for 30 minutes followed by a 6-
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. 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 heat-stressed cells labelled with the 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.
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 unbinding force $f_u$ 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.

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).

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...
HSP60 on membranes of stressed HUVECs

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.

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. 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.
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...
HSP60 on membranes of stressed HUVECs


