Contribution of heat shock proteins to cell protection from complement-mediated lysis

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Abstract

The possible participation of hsc70 and hsp70 in cellular protection from complement damage was studied. Human erythroleukemia K562 cells were pretreated with reagents affecting hsc70 or hsp70, and cell sensitivity to lysis by antibody and human complement was examined. Treatment with deoxyspergualin, an hsc70 inhibitor, sensitized K562 cells to complement lysis, whereas treatment with ethanol, butanol or hemin, inducers of hsc70 synthesis, protected the cells from complement-mediated lysis. Incubation of K562 at either 42°C or with the amino acid analogue L-azetidine-2-carboxylic acid induced synthesis of hsp70, but not of hsc70. The latter treatment also conferred elevated resistance to complement lysis on K562 cells. Pretreatment of K562 cells with sub-lethal doses of complement desensitizes them to lethal complement doses. No effect of sublytic complement on synthesis of hsc70 and hsp70 was found. However, the results demonstrated that complement stress causes translocation of hsc70 from the cytoplasm to the K562 cell surface. Two monoclonal and two polyclonal antibodies identified hsc70 on the surface of intact, viable complement-stressed cells, while antibodies directed to hsp70 did not bind to these cells. Altogether, the results suggest that the heat shock proteins hsc70 and hsp70 play a role in cell defense against complement.

Introduction

Cells express a variable degree of resistance to the lytic action of the complement membrane attack complex (MAC). The MAC is a molecular complex composed of the five terminal complement proteins C5–C9 that form a transmembrane cylindrical structure (1). By a mechanism not clearly understood, this complex leads to a necrotic-type cell death. Under certain conditions, it may also induce apoptosis (2,3). In addition, cells treated with sublytic MAC doses undergo a rapid, transient phenotypic transition of desensitization to complement-mediated lysis, that was named complement-induced protection (4). The cellular variability in sensitivity to complement-mediated lysis has been attributed to the action of several protective mechanisms (reviewed in 5). Cells bear on their surface several membrane proteins that block the complement cascade at different critical stages. The level of expression of such regulatory proteins is directly correlated with resistance to lysis by complement (5,6). On the other hand, various cellular activities are also essential to promote cell resistance to MAC. Thus, cells can rapidly shed or ingest the complement complexes in a Ca^{2+}-dependent process (7,8). Protein phosphorylation processes, involving protein kinase C (9) and ERK (10) and de novo protein synthesis (4), support cellular recovery mechanisms that determine the outcome of the MAC attack. Insights into the cellular damage repair mechanisms are just beginning to accumulate.

Heat shock and thermotolerance have been studied for over a decade and the leading finding was that pre-exposure to sublytic heat shock transiently desensitizes cells to elevated lethal temperatures (11–13). Heat shock triggers synthesis of several transcription factors, followed by synthesis of heat shock proteins, including the hsp70 multi-gene family. Some members of the hsp70 family, such as hsc70, are constitutively expressed and respond only weakly to heat. Under normal conditions, the hsc70 serves as a molecular chaperone escorting proteins to their various intracellular compartments and assisting their translocation across membranes (14–16). In addition, hsc70 plays a major role in endocytosis, uncoating clathrin-coated vesicles (17,18). Under stress conditions, the hsp70s participate in repair of damaged proteins and membranes, and dissociation of protein aggregates. Heat
stress causes an immediate but transient translocation of hsp70 from the cytosol into the nucleus (19,20). Similarly, oxidative stress was shown to induce nuclear translocation of hsc70 (21).

Members of the hsp70 family have been also implicated in cell protection from other toxic compounds, such as hydrogen peroxide (21–23) and from metabolic stress (20,24). Transgenic mice expressing a high level of human hsp70 had elevated resistance to ischemic myocardial injury (25,26). Introduction of human hsp70 into WEHI-S cells conferred resistance to toxicity by tumor necrosis factor (27). hsp70 was whereas SPA-816 binds only to hsc70.

Introduction of human hsp70 into WEHI-S cells conferred hsp70 and hsc70, MA3-009 and SPA-811 bind only to hsp70, whereas SPA-816 binds only to hsc70.

Thermotolerance and the complement-induced protection phenomenon share some resemblance. The two shock responses depend on de novo synthesis of proteins, develop within minutes and decay after several hours. Hence, the possible involvement of heat shock proteins in cell protection from complement shock was investigated. As shown here, both hsc70 and hsp70 can probably protect cells from the lytic effects of complement. Interestingly, sublytic complement activation was found to induce translocation of hsc70 from the cytosol to the exoplasmic side of the plasma membrane of K562 cells.

Methods

Buffers and reagents

Experiments were carried out in Dulbecco’s PBS, pH 7.4, containing 1 mM CaCl₂ and 1 mM MgCl₂ (PBS). Cells were washed with PBS containing 10% fetal bovine serum (Gibco, Grand Island, NY) (PBS/FBS). Chemicals employed in this study were all of analytical grade. Molecular weight standards for SDS–PAGE were obtained from BioRad (Richmond, CA). Deoxyxyspergualin (DSG) was obtained from Dr S. G. Nadler, Bristol-Myers Squibb (Princeton, NJ). Hemin and L-azetidine-2-carboxylic acid were purchased from Sigma (St Louis, MO). The ViaProbe viability probe, a composition of 7-aminotoxino-mycin D, was from PharMingen (San Diego, CA). QIIFIT mouse mAb calibration kit for flow cytometry was purchased from Dako (Glostrup, Denmark). Bovine brain hsc70/hsp70 was purified as described before and stored at 4°C in ammonium sulfate (29).

Sera and antibodies

Normal human serum (NHS) was used as a source of complement. It was freshly prepared from healthy donors and divided into aliquots. Heat inactivation (HI) of complement in NHS was performed for 30 min at 56°C. All sera were kept frozen at −70°C until used. Polyclonal antibodies directed to K562 were prepared in rabbits or mice by s.c. or intramuscular injections respectively of intact cells in PBS. The antibody titer was determined in a cytotoxicity assay using the Trypan blue exclusion method.

hsc70-specific rabbit antisera was prepared by s.c. injections of hsc70(623–643) synthetic peptide (sequence not present in hsp70) conjugated to keyhole limpet hemocyanin (KLH; Sigma). The rabbit was first immunized with the antigen in complete Freund’s adjuvant (Difco, Detroit, MI) followed by two boosts in incomplete Freund’s adjuvant (Difco). Pre-bleeds were used as normal rabbit serum (NRbS) controls. This antiserum reacted by Western blotting with purified hsc70 and not with hsp70 (not shown). The mouse mAb MA3-007, MA3-006 and MA3-009 were purchased from Affinity Bioreagents (Golden, CO), isotype-matched mouse IgG1 MOPC-31c was from Sigma, and the rabbit antisera SPA-816 and SPA-811 were from StressGen Biotechnologies (Victoria, BC, Canada). According to the manufacturers’ Technical Specifications, MA3-007 and MA3-006 are specific to both hsp70 and hsc70, MA3-009 and SPA-811 bind only to hsp70, whereas SPA-816 binds only to hsc70.

Cell culture

K562 human erythroleukemia cells were kept in culture in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS (Gibco), 1% glutamine and an antibiotic mixture of penicillin (100 U/ml)/streptomycin (0.4 mg/ml) (Biolab, Jerusalem, Israel).

Lysis of K562 cells by antibody and complement

K562 cells (0.5×10⁶) were incubated for 30 min on ice with 100 µl of rabbit or mouse anti-K562 antiserum diluted in PBS. Next, 100 µl NHS was added and the cells were further incubated for 1 h at 37°C. HI-NHS served as control. The cells were then washed with PBS containing 10% FCS and were mixed in 0.2% Trypan blue (Fluka, Buchs, Switzerland) in PBS. Percent cell lysis was determined microscopically based on Trypan blue inclusion or exclusion. Percentage of mortality in the presence of HI-NHS or without antibodies was usually 1–3%.

Northern blotting

Analysis was essentially performed as described in Sambrook et al. (30). Briefly, RNA was extracted from 10×10⁶ K562 cells after cell disruption in 4 M guanidinium isothiocyanate/12% β-mercaptoethanol and ultracentrifugation over cesium chloride. RNA concentration was determined by absorbance at A₂₆₀, and quality controlled by agarose gel electrophoresis and examination of ribosomal RNA. RNA (15 µg) was fractionated in agarose gel and transferred to Hybond C-extra nitrocellulose membrane (Amersham, Piscataway, NJ). The membrane was heated for 2 h at 80°C and hybridized with [α-³²P]CTP-labeled DNA probes for 24 h at 42°C in roller bottles (Hybaid, Middlesex, UK). Following washes, the membrane was exposed to X-ray film (Kodak) and the autoradiogram was scanned in a densitometer (Dinko and Renium, Jerusalem, Israel). A 2.4 Kb hsp70 DNA probe was prepared by restriction enzyme digest (BanHI + HindIII) of the HSP70-1 plasmid (31). The 1 kb hsc70 DNA probe was prepared by PCR of genomic DNA (32) of B35 B lymphoblastoid cells using synthetic oligonucleotide primers 1 and 2 (primer 1: 5′-CCGCTGTGTTTCCACCAGC-3′; primer 2: 5′-TCATTAGCC-AAGCTTTGGT-3′). The two DNA probes were excised from low gelling temperature Sea Plaque agarose (FMC BioProducts, Rockland, ME) gel and melted at 65°C, just before labeling. Probe labeling with [α-³²P]CTP was performed by using the Multiprime DNA labeling system (Amersham).
Analysis of cellular hsp70 and hsc70 by Western blotting

K562 cells (1 × 10⁶) were mixed in 50 μl sample buffer composed of Tris buffer, pH 6.8, containing 1 mM dithiotreitol, 3% SDS, 10% glycerol and 0.04% bromophenol blue, and heated for 3 min at 95°C. Protein concentration was determined using the methods of Bradford (33). SDS-PAGE was performed according to Laemmli (34) on 4–20% acrylamide gradient gels. Proteins were transferred to nitrocellulose membrane (Amersham) in a Tris–glycine buffer, pH 8.3 (35). Electro-transfer was performed for 2 h at 200 mA. In order to block non-specific binding, the paper was incubated with 5% Blocking reagent (Amersham’s ECL kit) and 5% BSA in PBS for 1 h at room temperature. The hsp70 and hsc70 bands were detected with the MA3-009 mAb and the rabbit anti-hsc70(623–643) antibodies respectively. The second antibodies used were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham’s ECL kit). Following extensive washing, the peroxidase was detected using an ECL kit and film (Amersham).

Flow cytometry

K562 cells (1 × 10⁶) were first treated with or without antibody and NHS or HI-NHS and washed. For immunofluorescence with mouse mAb, the cells were stressed with rabbit anti-K562 cells antiserum. In immunofluorescence assays utilizing rabbit anti-hsp70/hsc70, the cells were stressed with mouse anti-K562 antiserum. This prevented binding of the FITC-conjugated antibodies used to activate complement. The cells were reacted with an anti-hsp70 or –hsc70 antibody diluted (1:100–1:200) in PBS/FBS for 1 h on ice. After washes, the cells were reacted with FITC-conjugated antibodies diluted 1:100 in PBS/FBS for 1 h on ice. FITC-conjugated AffiniPure F(ab’)2 fragment donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was used to detect the rabbit first antibody, whereas FITC-conjugated AffiniPure goat anti-mouse IgG (Jackson) was used to detect the mouse mAb. The cells were washed, resuspended in 0.6 ml PBS containing 2% PBS and analyzed in a Coulter FACS (Beckman Coulter, Fullerton, CA).

Statistical analysis

Statistical significance was analyzed by using the two-sided unpaired Student’s t-test.

Results

Effects of DSG, alcohols and hemin on complement-mediated lysis

DSG inhibits the activity of hsc70 by binding to its C-terminal EEVD regulatory domain (36,37). K562 cells were pretreated for 1 h at 37°C with DSG, 1 or 5 μM in PBS or just with PBS. Then, the cells were treated first with rabbit anti-K562 antibodies for 30 min on ice followed by NHS, as a source for complement for 1 h at 37°C. To reach about 20% cell death in the control, PBS pretreated cells, the rabbit antiserum was diluted 1:16 and NHS was diluted 1:2 with PBS. After a wash with PBS, the percent lysis was determined by Trypan blue exclusion. As shown in Fig. 1, pretreatment with DSG sensitized the K562 cells to lysis by complement, which increased from 20 up to 80%. Even a shorter pretreatment with DSG for 10 min was sufficient to produce a significant (P < 0.005) increase in cell lysis (not shown). In contrast to the effect of DSG, cell treatment with alcohols was shown to increase expression and activity of heat shock proteins, including that of hsc70 (38). The effect of two alcohols, ethanol and butanol, on cell sensitivity to complement-mediated lysis was tested. K562 cells were grown for 24 hr in culture medium containing 20 mM HEPES, pH 7.4, by itself or supplemented with 25 mM ethanol or butanol. The cells were then washed and challenged with antibodies (various dilutions) and NHS (1:2). Cells treated with ethanol (Fig. 2A) or butanol (Fig. 2B) were significantly more resistant to complement-mediated lysis relative to control cells. Hemin can induce erythroid differentiation of K562 cells, activation of the heat shock factors and increased synthesis of hsp70 (39,40). Pretreatment of K562 cells with 30 μM Hemin for 3 h at 37°C made them more resistant to killing by antibody and complement (P < 0.001) (Fig. 2C). Thus, inhibition of the cognate hsc70 is associated with enhanced sensitivity to complement while activation of hsc70 or hsp70 is associated with reduced sensitivity to complement. This finding was studied further.

Effect of heat shock and Azetidine on complement-mediated lysis

The effect of heat (42°C) on the amount of hsp70 and hsc70 mRNA and protein in K562 cells was examined. Cells cultured at 42°C for 30–60 min were washed and extracted. mRNA was isolated and analyzed by Northern Blotting with 32p-labeled hsp70 and hsc70 probes. As shown in Fig. 3(A), the levels of both hsp70 mRNA and hsc70 mRNA were increased in heated K562 cells. Based on densitometry analysis, the increase in hsp70 mRNA was 6- to 10-fold, whereas that of hsc70 mRNA was only 2- to 2.5-fold. This is in good agreement with previous analyses of the effect of heat on hsp70/hsc70 mRNA levels in other cell types (41). Analysis of the hsp70/hsc70 protein amount in heated K562 cells, 30–90 min
Heat shock and azetidine induce hsp70 synthesis and cell resistance to complement. (A) K562 cells were kept for 1 h at 42°C (42) or 37°C (C). Also, K562 cells were treated with rabbit anti-K562 antibodies and HI-NHS (HI) or NHS for 60 min at 37°C under sublytic conditions. Total RNA extracted from the cells (same amount of 18S and 28S) was analyzed by Northern blotting with hsp70- or hsc70-specific probes. Autoradiograms of the hsp70 and hsc70 mRNA bands are shown. (B) K562 cells were kept for 2 h at 37°C (C) or for 30 min at 42°C followed by 90 min at 37°C [42(37)] or complement. K562 cells were treated: (A) with 25 mM ethanol in culture medium for 24 h at 37°C; (B) with 25 mM butanol in culture medium for 24 h at 37°C or (C) with 30 µM hemin in culture medium for 3 h at 37°C. The cells were then challenged with antibody (at the various dilutions indicated) and NHS (1:2), and percent lysis was determined by Trypan blue exclusion. Controls included cells cultured without alcohol or hemin. Results are means ± SD of four replications and experiments are representative of three independent experiments. P values shown are for antibody dilutions 1:10 (A), 1:5 (B) and 1:20 (C).

After heating, clearly indicated that hsp70 synthesis was up-regulated, while hsc70 synthesis was unaffected (Fig. 3B). After 30 min treatment at 42°C, the amount of hsp70 increased only slightly (1.6-fold) [Fig. 3B, 37(42)]. However, 90 min later, the amount of hsp70 protein was 2.7-fold higher in heated cells than in control cells [Fig. 3B, 42(37)]. To determine the effect of heating on cell sensitivity to lysis, K562 cells were cultured for 30 min at 42°C, further cultured for 90 min at 37°C and subjected to complement-mediated lysis. Cells were first treated with mouse anti-K562 antibodies (diluted 1:100) and then with NHS diluted 1:8 or 1:16 for 1 h at 37°C. As shown in Fig. 3(C), heated cells showed increased resistance to complement-mediated lysis both with NHS 1:8 (Fig. 3C, 1 and 2) and NHS 1:16 (Fig. 3C, 3 and 4).

Azetidine, an amino acid analog, was shown to activate synthesis of hsp70 (42) and hsc70 (43) mRNA in human and mouse cells. K562 cells were cultured for 14 h at 37°C in the presence of 5 mM azetidine. This treatment was not toxic to the cells, as determined by Trypan blue exclusion, but led to a marked growth arrest of the cells (70–90% inhibition). Western blotting analysis of hsp70 and hsc70, clearly demonstrated that azetidine-treated cells contain much more hsp70 than control cells (10- to 40-fold increase), but the same level of hsc70 protein (Fig. 3B). Azetidine-treated cells were also found to be more resistant to complement-mediated lysis than control cells (Fig. 3C).
K562 cells treated with sublytic doses of antibody and complement become, within 20–60 min, resistant to lytic complement doses (4). This is not associated with induction of hsp70 or hsc70 synthesis, as suggested by mRNA analyses (Fig. 3A; NHS versus HI). Hence, we focused on the behavior of constitutive hsc70. Initial experiments (not shown) suggested that the stress response produced by low doses of the complement membrane attack complex caused a reduction in cytoplasmic free hsc70 and an accumulation of hsc70 at the plasma membrane, where the complement-mediated damage occurs. Rabbit antibodies specific to the C-terminus of hsc70 (amino acids 623–643) were used in flow cytometry analyses to demonstrate the appearance of hsc70 on the surface of K562 cells treated with sublytic doses of complement (Fig. 4). These antibodies bound more strongly to cells treated with antibody and NHS than to cells treated with antibody and HI-NHS. Normal rabbit antibodies bound similarly to NHS- and HI-NHS-treated cells (Fig. 4, insert). Another polyclonal rabbit antiserum directed against the C-terminal portion of hsc70 (amino acids 556–568), SPA-816, reacted more strongly with complement-activated cells than did normal rabbit serum or SPA-811 antiserum that is selective for hsp70 amino acids 556–568 (Fig. 5A).

We next investigated whether antibodies directed against the N-terminal portion of hsc70 selectively bound to the complement-stressed cells. MA3-007 is a mouse mAb directed against the N-terminal portion (amino acids 122–264) of the hsp70 family members, including hsc70. As shown in Fig. 5B, MA3-007 antibodies bound to complement-stressed K562 cells more strongly than either an isotype-matched MOPC-31C mAb or the MA3-006 mAb directed to the C-terminal portion (amino acids 504–617) of hsp70 family members, including hsc70. As both MA3-007 and MA3-006 bound to complement stressed cells, it appears that both the N- and C-terminal portions of hsc70 on the plasma membrane are accessible to antibodies.

To rule out the possibility that the shift in peak position results from non-specific antibody binding to dead cells, the antibody-labeled cells were double labeled with ViaProbe (7-aminoactinomycine D). 7-Aminoactinomycin D penetrates into dead cells with a damaged plasma membrane and binds to nuclear DNA. This permits identification of the dead cells in a second channel of the cell sorter and removal of the signal received from dead cells. Results shown in Fig. 5(A) were not corrected, but those shown in Fig. 5(B) are of viable cells only. Data presented in Fig. 5(C and D) show that the dead cells contribute to the appearance of a small shoulder or a second peak of more intense fluorescence, but not to peak shift. This is mainly due to the fact that the experiments were performed under sublytic conditions with only 10–20% cell death. For some unclear reason, MA3-006 antibodies bound to dead cells stronger than MA3-007 antibodies. Similar to SPA-811 (Fig. 5A), two other antibodies selective to hsp70 (MA3-009 and RPN1197) did not bind to complement-stressed cells (data not shown). This is probably due to the fact that the cells contain very little hsp70 relative to hsc70.

To obtain a quantitative measure of hsc70 expression on stressed cells, Dako’s QIFI calibration beads bearing known amounts of mouse mAb were labeled with the same dilution of FITC-conjugated donkey anti-mouse IgG as used with the K562 cells and analyzed by flow cytometry. The resulting histogram representing five bead populations bearing: 3800, 17,000, 60,000 200,000, and 550,000 mAb/bead respectively is shown in Fig. 6(A). The linear calibration curve obtained from plotting the number of mAb per bead versus the peak

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**Fig. 4.** Expression of hsc70 on the surface of complement-stressed K562 cells. K562 cells were treated with mouse anti-K562 antibodies (30 min on ice) followed by HI-NHS or NHS (1 h, 37°C). After washes, the cells were reacted on ice with rabbit anti-hsc70(623–643) (1:500) followed by FITC-labeled goat anti-rabbit IgG and analyzed by flow cytometry. Insert shows reactivity of these cells with normal rabbit serum and FITC-goat anti-rabbit IgG.

**Fig. 5.** Surface hsc70 labeling and removal of dead cells signal. K562 cells were activated by a sublytic dose of mouse anti-K562 antibodies (A) or rabbit anti-K562 antibodies (B–D) and NHS. Percent cell death, as determined by Trypan blue exclusion, was ≤20%. Then, the cells were washed and reacted with ViaProbe which labels dead cells (B–D). The cells were analyzed by flow cytometry in two channels, one adjusted for FITC and one for ViaProbe. The cell population labeled by ViaProbe was removed from the histogram plotting cell number versus relative fluorescence intensity, thus showing only viable cells. Histograms in (A) and (B) show specific labeling of hsc70 on complement-stressed cells with rabbit polyclonal SPA-816 antibodies (A), and with monoclonal MA3-007 and MA3-006 antibodies (B). Controls included normal rabbit serum (A) and MOPC-31C (B) and hsp70-directed SPA-811 polyclonal antibodies (A). (C and D) Histograms of cell labeling with MA3-007 and MA3-006 before and after removal of dead cells (labeled by arrows).
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viable cells expressed more hsc70 on their surface. The quantity of cell bound mAb is shown in the table in Fig. 7(C). When looking at the mean peak position, the number of bound MA3-007 increased from 3200 to 13,700 mAb/cell, whereas MA3-006 increased from 3000 to 5500 mAb/cell. Also shown in the table are the numbers of mAb bound per cell at the maximal range of the histograms. This demonstrates that some of the complement-stressed cells bound up to about 50,000 MA3-007 mAb or 18,000 MA3-006 mAb/cell.

Discussion

The results presented here demonstrate, for the first time, that heat shock proteins are involved in cell protection from complement-mediated damage. This is supported by the following findings. (i) Pretreatment with DSG sensitizes K562 cells to lysis by antibody and complement (Fig. 1). DSG is an immunosuppressant, known to bind to hsc70 at its regulatory C-terminal end and to block its activity (36,37,44). (ii) Up-regulation of hsc70 synthesis by treatment with ethanol, butanol and hemin increases K562 cell resistance to complement-mediated lysis (Fig. 2). (iii) Up-regulation of hsp70 synthesis by heat treatment or with Azetidine (an amino acid analogue) also increases K562 cell resistance to complement-mediated lysis (Fig. 3). Heat and Azetidine treatments increase the hsp70 protein content in K562 cells by 2.7- and 40-fold respectively, but have no significant effect on the level of hsc70 protein content (Fig. 3B). It is, therefore, conceivable that both hsp70 and hsc70 have the capacity to confer on K562 cells resistance to the damaging effect of complement. Yet, the concentration of hsp70 in unstressed cells is much lower than that of hsc70, suggesting that basal cell resistance to complement will depend more on hsc70 than on hsp70 activity. It is reasonable to assume that any condition that induces a heat shock response may have a supportive effect on the capacity of cells and tissues to withstand a complement injury. This includes environmental stress (heat shock, amino acid analogues, oxygen free radicals, etc.), pathophysiological states (inflammation, ischemia, tissue injury, etc.), and normal development and differentiation (13).

Treatment of K562 cells with sublytic complement confers on the cells resistance to lytic MAC doses (4). In this respect, it was disappointing to find that sublytic MAC, within the time frame of the experiment (1 h), has no significant effect on hsp70 and hsc70 mRNA synthesis (Fig. 3A). Thus, hsc70 is supporting basal cell resistance to complement, but is not involved in the elevated resistance induced by sublytic MAC. This also suggests that complement stress does not induce the immediate heat shock transcription factor response required for up-regulation of hsc70 and hsp70 synthesis. It is still possible that sublytic MAC is inducing de novo synthesis of other heat shock proteins. The identity of the mechanisms involved in the complement-induced protection is still not resolved. The possibility that sublytic MAC is inducing a rapid expression of the complement regulatory membrane proteins was also ruled out (4,45). Some insight into the signaling ensuing after MAC insertion has been achieved. Calcium ion influx, protein kinase C activation and ERK activation are essential stages in the complement-induced protection phenomenon (4,9,10). However, how they lead to enhanced

Fig. 6. Quantitation of bound mAb. K562 cells were treated with rabbit anti-K562 antibodies followed by HI-NHS (negative control) or NHS. The cells were washed and reacted with MA3-007 (A), MA3-006 (C) or MOPC-31C (D). Then, the cells and mAb-containing QIFi beads were coated with FITC-labeled donkey anti-mouse IgG. The cells and the beads were analyzed by flow cytometry using the same window setting. The peak fluorescence position of each of the five bead populations (shown in A) was plotted versus the number of mAb they bear per bead (determined by Dako). This linear titration curve (B) was used to calculate the number of mAb bound per cell at the peak or at the maximal fluorescence position.
Data to be described elsewhere demonstrates that the surface hsc70 found in complement-stressed cells is contained within large molecular complexes. Extracellular surface hsp70 has been described before in unstimulated human tumor cells (41,47). This has recently been verified by an electron microscopy study of unstimulated and heated mouse NIH 3T3 fibroblasts and rat H9c2 myocytes (48). No increase in hsp70 surface expression upon heat shock was seen in the latter study. A direct effect of stress on cell surface expression of hsp70 was proposed in analyses of dog neutrophils (49) and human melanoma cells (50). We could not detect hsp70 or hsc70 on unstimulated K562 cells. To rule out the possibility that the surface hsc70 is the result of dead cell staining, dead cells were labeled with 7-aminoactinomycin D and their fluorescent signal was separated from that of viable cells (Fig. 5). Anti-hsc70 antibodies bound tightly to dead cells and observations under a fluorescent microscope showed that the labeling was primarily cytoplasmic. In contrast, viable intact cells had only surface labeling.

Four different antibodies identified hsc70 on the surface of viable complement-stressed cells: two mouse mAb (MA3-007 and MA3-006) and two polyclonal rabbit antibodies [SPA-816 and the home-made antibodies directed to hsc70(623–643)]. MA3-007 (clone 5A5) recognizes an epitope in the ATPase N-terminal domain of hsc70 (and hsp70) (amino acids 122–264) (51) and MA3-006 (clone 3A3) binds to the C-terminal peptide binding domain of hsc70 (and hsp70) (amino acids 504–617) (Affinity Bioreagents). SPA-816 is specific to the C-terminal peptide binding domain of hsc70 (amino acids 556–568) (17) and according to StressGen’s specifications does not bind to hsp70. The home-made rabbit anti-hsc70(623–643) antibodies are selective to the C-terminal end of hsc70. Apparently, a large portion of the hsc70 molecule is exposed on the surface of complement-stressed cells. This is slightly different from the findings of Multhof and colleagues (52) who analyzed the extracellularly localized epitope of hsp70 on human colon carcinoma cells CX+.

Unstressed CX+ cells bound the anti-hsp70 antibodies directed to the C-terminal epitopes (MA3-006, MA3-009 and RPM1197) but not MA3-007 (52). They concluded that at least the C-terminal region 504–617 has to be localized extracellularly. Unlike CX+ cells, complement-stressed K562 cells did not bind the hsp70-selective mAb MA3-009. Taken together with the finding that SPA-811, hsp70-selective rabbit antibodies, do not bind to complement-stressed cells (Fig. 5A), our results indicate that hsc70, but not hsp70, is translocated upon complement stress, to the K562 cell surface. This may merely reflect the higher concentration of hsc70 in K562 cells or could imply distinct functions for hsc70 and hsp70 in complement stress. Further experimentation is required to resolve this question.

Assuming an antibody/hsc70 binding ratio of 1:1, the number of bound mAb (Fig. 7C) may be converted to number of hsc70 molecules/cell. The problem is that MA3-007 mAb gave higher fluorescence intensity profiles than MA3-006 mAb. One possible explanation is that the epitope recognized by the MA3-006 mAb (the peptide binding domain) is adjacent to the plasma membrane or in contact with another protein and partly hidden from the mAb. Alternatively, MA3-007 mAb may have a higher binding affinity to hsc70 than MA3-006.

Fig. 7. Correlation between hsc70 surface expression and degree of complement stress. K562 cells were treated with rabbit anti-K562 diluted 1:60, 1:40, 1:30, 1:24, 1:20 or 1:16 with PBS (30 min on ice) and then with NHS diluted 1:2 (1 h at 37°C). As determined by Trypan blue exclusion, this produced cell death ranging from 6 up to 59.7% (C). The cells were washed and reacted with MA3-007 (A) or MA3-006 (B) antibodies, followed by FITC-labeled donkey anti-mouse IgG and ViaProbe. The seven histograms shown in (A) and (B) represent, from left to right, the seven samples shown in the table in (C). Sample 1 is of control cells treated with NHS but not with anti-K562 antibodies. Values of mean (MnX) and maximal (MaxX) peak positions were used to calculate (with plot shown in Fig. 6B) the number of mAb bound per cell of each sample (C).
The fact that immediate cell fixation with paraformaldehyde after labeling with antibodies gave the same difference between MA3-007 and MA3-006 staining supports the first possibility. The mean net number of hsc70 molecules per viable cell, as estimated from MA3-007 mAb labeled cells in group 6 (Fig. 7C, 25% lysis) is 11,600 with many viable cells bearing up to 54,800 hsc70 molecules/cell.

How heat shock proteins protect K562 cells from complement-mediated lysis is still an open question. They may limit, directly or indirectly, the number of MAC deposited on the K562 cells. However, it is more likely that they act to reduce the lytic effects of the MAC. Heat shock proteins are known to perform many house-keeping functions, each of which can support damage repair during MAC attack. Two of the early morphological changes that occur in cells bearing lytic doses of MAC are cell swelling due to osmotic shock, and mitochondrial swelling and damage (53). This is counteracted by the shedding of plasma membrane vesicles containing the MAC (8), the pumping out of potassium ions to balance the influx of sodium ions (54), and membrane and organelle repair. The machinery executing these processes has not yet been identified and it is suggested that heat shock proteins play a major role. hsc70 could be part of the machinery transporting replacing units to and across the damaged plasma membrane. This may resemble hsc70’s activity in axonal transport (18,55) and in nuclear targeting (56). Alternatively, hsc70 could perhaps form the ion channels restoring the ionic balance to the damaged cell. In this regards, Arispe and de Maio (57) have just demonstrated that recombinant hsc70 can incorporate into artificial lipid bilayers and form a transmembrane ATP-dependent cationic channel. Furthermore, it has been shown that hsp70 can activate potassium channels in U937 cells (58). Finally, it is believed that MAC removal by plasma membrane vesiculation or internalization is one of the major mechanisms of cell protection from complement-mediated lysis. The possibility that hsc70 is accelerating these processes is supported by the electron microscopic data of Kurucz et al. (48) suggesting that hsp70 is extracellularly associated with the clathrin-coated pit and by the observation that hsp70 is associated with the exosomes of maturing reticulocytes (59).

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>DSG</td>
<td>deoxyspergualin</td>
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<tr>
<td>HI</td>
<td>heat inactivated</td>
</tr>
<tr>
<td>hsc70</td>
<td>cognate form of hsp70</td>
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<tr>
<td>hsp70</td>
<td>70 kDa heat shock protein</td>
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</tbody>
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References

Antibodies against 70-kD heat shock cognate protein inhibit heat shock factor channel formed by Hsc70 in acidic phospholipid membranes.


Adams, P. M. and Stein, S. A. 1993. Basic and inducible hsp70s are associated with the transferrin receptor in exosomes from maturing reticulocytes. J. Cell Biol. 120:35.


