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Effects of exogenous stress protein 70 on the functional properties of human promonocytes through binding to cell surface and internalization

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Abstract The presence of antibodies against the major stress protein, Hsp70, in patients with autoimmune diseases led us to hypothesize that Hsp70 may occur extracellularly, and could exert chaperoning and regulatory effects on various cells. We examined the action of pure Hsp/Hsc70 on the main physiological functions of human promonocytic U-937 cells. The protein was isolated from calf muscle and was shown to be a mixture of inducible Hsp70 (60%) and constitutive Hsc70 (40%) isoforms. It was observed that the addition of the protein up-regulated two major monocyte/macrophage differentiation markers, CD11c and CD23, by 20–35%, while it had no effect on CD14. The experiments performed to investigate the influence of Hsp/Hsc70 on the reaction of U-937 cells to differentiation stimuli demonstrated that the addition of the protein prior to PMA was able to inhibit binding of proper transcription factors to double-symmetry and cAMP-response elements of the *c-fos* early response gene promoter. Administration of exogenous Hsp/Hsc70 prior to treatment with the tumor necrosis factor-alpha significantly lowered the number of apoptotic and necrotic cells. In no case did the control protein, ovalbumin, taken in the same concentration give a comparable effect on U-937 cells. Since the Hsp/Hsc70 effects occurred within the first hour of co-incubation, and therefore they might be explained by its interaction with the cell surface, we assayed binding of the biotinylated protein to U-937 cells by immunoenzyme assay, flow cytometry and indirect immunofluorescence. Using these three techniques we were able to detect Hsp/Hsc70 bound to cells after a 20 min incubation. According to flow cytometry data, at this time 32% of cells were positively stained with streptavidin-FITC. Immunofluorescence studies demonstrated Hsp/Hsc70 bound to the cell surface after a 20 min incubation followed by induction of patch and cap-like structures. One hour later, the majority of the protein had been internalized by U-937 cells.

INTRODUCTION

The polypeptides belonging to the heat shock protein 70 (Hsp70) family are known to contribute to the main processes of cell physiology in normal conditions and under the influence of physico-chemical stressors or biogenic cytotoxic factors (Becker and Craig 1994; Morimoto et al 1994). In mammalian cells this family includes the Hsp70 inducible protein and constitutively expressed Hsc70 isoform both localized to the cytoplasm. There are several other polypeptides belonging to the Hsp70 family that are confined to mitochondria and endoplasmic reticulum (Feige and Polla 1994). All Hsp70 family members possess a so-called chaperoning activity

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that relates to their ability to bind misfolded, or newly synthesized, protein and to keep it in that state until the moment of transportation through intracellular membrane to the addressed compartments of the cell (Morimoto et al 1994; Rassow et al 1995). Release of a polypeptide from the chaperone requires ATP and other proteins, like Hdj1 and Hip (Freeman et al 1996).

During the last few years, the data appeared convincing that Hsps, thought to be intracellular polypeptides, might occur in the extracellular matrix. It was demonstrated that Hsp70 could be transferred from the surrounding glial sheath cells to the squid giant axon (Tytell et al 1986). Experiments with radioactively labeled proteins secreted by rat fibroblasts after heat shock showed that Hsp70 is among those polypeptides (Hightower and Guidon 1989). Numerous studies have demonstrated the occurrence of Hsp70 linked to tumor antigens on the surface of cancer cells and that the protein is capable of eliciting an antitumor specific immune response (reviewed in Multhoff and Hightower 1996).

The other results supporting the occurence of exogenous Hsp70 were obtained in immunological studies. Many autoimmune diseases cause high titers of antibodies against two stress proteins, Hsp60 and Hsp70 (Minota et al 1988; Engman et al 1990; Mustafa et al 1993). In order to induce the immune response, Hsp70 should be most probably found outside a cell. This could happen if the protein is released from living (as mentioned above) or from dead, lysed cells. Another possibility would be an infection by microorganisms and bacteria bearing their own Hsc/Hsp70 or its closely related analog, DnaK (reviewed in Morimoto et al 1994).

It is important that under certain conditions Hsp70 can occur in an extracellular matrix and, regardless of its origin, the protein may affect the cells present in blood or in another locale via its chaperoning activity.

Studies that were focused on the effects of an exogenous Hsp70 on various target cells were recently initiated by several groups. The data demonstrated that the major *Mycobacterium tuberculosis* antigen, Hsp70, introduced to human lymphocytes, exhibited a number of effects, including activation of proliferation and secretion of interleukins-2, -4 and -6 and γ-interferon (Beagly et al 1993; Launois et al 1993). When added to cultured macrophages, *Mycobacterium* Hsp70 stimulated synthesis of proinflammatory cytokines, interleukins-1 and -6 and tumor necrosis factor (TNF). The authors assumed that 'bacterial Hsps might modulate immunity by rapidly increasing cytokine synthesis in macrophages' (Retzlaff et al 1994). In another model, administration of exogenous bovine brain Hsc70 was found to prolong survival of neurons from dorsal root ganglion, after axotomy (Houenou et al 1996). In view of the above facts, it appears that the extracellular Hsp70 may modulate vital cellular functions and even protect cells against cytotoxic factors.

The aim of the present study was to investigate effects exhibited by exogenous non-bacterial Hsp70 on the process of differentiation and of programmed cell death in U-937 human premonocytes and to reveal possible ways by which the protein could induce those effects. The U-937 cell line was chosen because the pattern of its mature markers and the cell responses to inducers of maturation and apoptosis, phorbol ester and TNF-α, were well established during the last few years. Here we present the data showing that pure Hsp/Hsc70 preparation isolated from bovine red muscle was able to induce changes in the expression of mature surface markers and in the character of cellular response to TNF and to a known differentiation inducer, phorbol ester. The assays performed with the aid of biotinylated Hsp/Hsc70 demonstrated that the changes in U-937 cell functions may be explained by a transient association of the protein with the cellular membrane concomitantly with a possible stimulation of certain surface domains. Thereafter, labeled Hsp/Hsc70 was taken up by the cell in an endocytosis-like manner with a formation of typical patch and cap structures.

MATERIALS AND METHODS

Reagents

Antibodies to monocyte/macrophage surface antigens, CD11c (LeuM5), CD14 (LeuM3), were purchased from Beckton and Dickinson (Mountain View, CA, USA), to CD23 – from DAKO (Denmark). Phorbol myristate acetate (PMA), NHS-biotin derivatives, streptavidin-peroxidase conjugate, extravidin-FITC conjugate, secondary antibodies (antimouse) labeled with FITC or TRITC, protease inhibitors and reagents for electrophoresis were purchased from Sigma (St Louis, MO, USA), DAKO and Pharmacia (Uppsala, Sweden). Recombinant human TNF-α was obtained from 'Fermentas' Biotechnology Company (Vilnius, Lithuania).

Cells

Human promonocytic U-937 cells were cultured at the density 2–4 × 10^5 cells per ml in RPMI 1640 medium supplemented with glutamine, antibiotics and 10% fetal calf serum (Gibco BRL, USA). Cell viability after the administration of a certain inducer was monitored with the aid of Trypan blue staining; cell growth rate was measured by counting cells with the aid of a Burkit chamber.

Proteins

Hsp/Hsc70 was isolated from bovine red muscle using chromatography on Q-Sepharose FF (Pharmacia) and on

ATP-agarose (Sigma) affinity gel (Welch and Ferramisco 1985; Guerriero et al 1989). Residual ATP and ADP were removed by ammonium sulfate precipitation (60%) in the presence of 5 mM EDTA and, following resuspension, the protein was dialysed against Tris-buffered saline. To remove possible contamination of lipopolysaccharide the preparation was passed through the polymixin-coupled Detoxi Gel column (Pierce, Rockford, NY, USA) and was finally filtered through 0.22-um pore size cellulose membrane. Ovalbumin (Sigma) was employed as a control protein. Protein concentration was determined by the dye-binding method (Bradford 1976). Monoclonal antibody 2H9 recognizing only the inducible Hsp70 form (Lasunskaia et al 1997) was employed for immunoblotting.

Effects of exogenous Hsp/Hsc70 on U-937 cells

The U-937 cells were incubated with the pure Hsp/Hsc70 for time periods as indicated in the Results section, washed with ice-cold phosphate-buffered saline (PBS) and processed as stated below. Changes in surface antigen pattern after incubation with Hsp/Hsc70 and/or PMA were detected with a help of FACScan Flow Cytometer (Beckton and Dickinson). After washing, the cells were allowed to react with the antibodies to CD11c, CD14 and CD23 markers followed by a FITC- or TRITC-labeled secondary antibody.

In experiments concerning the possible influence of Hsp/Hsc70 on activation of c-fos early response gene, the cells were treated with PMA and Hsp/Hsc70 for 20 min. Nuclear extracts from stimulated and control cells were obtained according to the protocol of Schreiber et al (1989) and stored at −70° C. Protein concentration was made equal in each sample. Two [^{32}P]-labeled oligonucleotides resembling the double-symmetry element (DSE, -317; -298: GATGTCCATATTAGGACATC), and cAMP-responsible element (CRE, -62; -54: TGACGTTTA) of the c-fos promoter (Treisman 1986; Sassone-Corsi et al 1989) were employed for the gel-shift assay. The DNA-binding reaction was performed at room temperature; polyA-polyU-copolymer was used as a non-specific competitor. DNA-protein complexes were separated by electrophoresis in 4% polyacrylamide gel (Schreiber et al 1989).

To assess the potential protective effect of Hsp/Hsc70, the cells were incubated with 200 IU/ml of TNF together with 100 ug/ml Hsp/Hsc70 or ovalbumin. The percentage of apoptotic cells was estimated by reference to typical pattern of nuclei staining and presence of apoptotic bodies as revealed by Acridine Orange staining (Gregory et al 1991).

Interaction of biotinylated Hsp70 with U-937 cells

Labeling of proteins was performed with the aid of NHS-biotinamidocaproate and NHS-iminobiotin (Sigma) according to protocol supplied by the company. Usually 200 ug of a biotin derivative was employed to label 1 mg of protein; labeling was performed at room temperature for 2 h and was terminated by the addition of 3M tris. The conjugates were dialysed against PBS to remove free biotin.

Enzyme immunoassay

U-937 cells were incubated with 20 ug/ml of biotinylated Hsp/Hsc70 or control protein, ovalbumin, for various time intervals. After incubation, the cells were washed with cold PBS and 1% serum albumin solution in PBS, and samples were taken for immunoenzyme assay performed as follows. Cells were placed onto the surface of 96-well microtitration plate previously coated with poly-l-lysine and fixed in cold 96% ethanol for 15 min. Non-specific interactions were blocked by incubation in 0.1% Tween-20, PBS, followed by development for 60 min using streptavidin-peroxidase complex. The results were determined using a Titertek Multiscan microplate reader (Labsystems Oy, Turku, Finland).

Flow cytometry analysis

Cells were exposed to 20 ug/ml biotinylated Hsp/Hsc70 or ovalbumin for various time intervals and thoroughly washed with ice-cold PBS and 1% albumin, followed by fixation with 4% paraformaldehyde. Fixed cells were permeabilized with 0.1% Triton X-100, incubated with extravidin-FITC for 30 min and washed 3 times with PBS containing 3% fetal calf serum. Analysis was performed with the aid of ELITE/ESP flow cytometer (Coulter Electronics, Miami, FL, USA). Combination of forward light scatter and 90° scatter were analyzed to eliminate debris. Clumped cells were mostly excluded by gating. For each point from 5×10^3 cells were monitored.

Immunofluorescence

After incubation with biotinylated Hsp/Hsc70 or ovalbumin, cells were fixed and permeabilized as described for flow cytometry. U-937 cells were plated onto poly-l-lysine-coated cover slips and the latter were incubated with extravidin-FITC. Immunofluorescence was detected in a Zeiss Axioscop fluorescence microscope (Karl Zeiss, Germany). Incubation of untreated cells with streptavidin-FITC alone revealed no visible staining.

RESULTS

The first part of this study was performed to analyze possible changes caused by the extracellular Hsp/Hsc70 on various activities of human premonocytic U-937 cells.

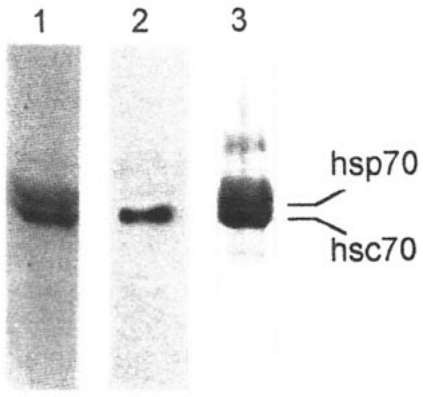

Fig. 1 Analysis of Hsp/Hsc70 preparation from bovine red muscle. Lane 1 – protein isolated from bovine thigh muscle, gel stained with Coomassie, 10 ug/lane; lane 2 – sample from lane 1 stained after blotting with monoclonal antibody 2H9 specific for Hsp70 inducible form, 20 ng/lane, lane 3 – biotinylated Hsp/Hsc70 stained after blotting with Streptavidin-peroxidase, 30 ng/lane.

The electrophoretic analysis showed that the protein preparation isolated from bovine thigh muscle (slow red type, Guerriero et al 1989) contained Hsp70 (inducible), and Hsc70 (constitutive) isoforms (Fig. 1). The purity of the preparation was no less than 97%. The results of Western blotting using the 2H9 monoclonal antibodies recognizing only the inducible member of Hsp70 family (Lasunskaia et al 1997) confirmed the identity of the lower band. The ratio of Hsp70 to Hsc70 was 60:40. After biotinylation two major bands were always observed, reflecting the modified protein isoforms (Fig. 1).

Effects of exogenous Hsp/Hsc70 on U-937 cell differentiation

From the analysis of cell growth dynamics after treatment with 100 ug/ml Hsp/Hsc70 we found that protein elicited a weak cytotoxic effect. Approximately 15% of the cell population died after a 72 h incubation with exogenous Hsp/Hsc70. In control cells treated with ovalbumin, the level of cell death was 3–5% (Fig. 2).

One of the significant indicators of U-937 cell differentiation to monocyte/macrophage phenotype is the pattern of surface markers. It was found that the addition of Hsp/Hsc70 in a concentration of 50 or 100 ug/ml to U-937 cell culture resulted in elevated expression of two markers; the induction was observed within the first hour of co-incubation. Twenty four hours after application of the protein, the percentage of CD11c-positive cells increased from 12% in untreated cells to 34%, and the percentage of CD23-positive cells increased from 32% to 69% (Fig. 3). The plateau value for CD11c antigen resulting from the action of well-established macrophage phenotype inducer, PMA (Öberg et al 1993) was substantially

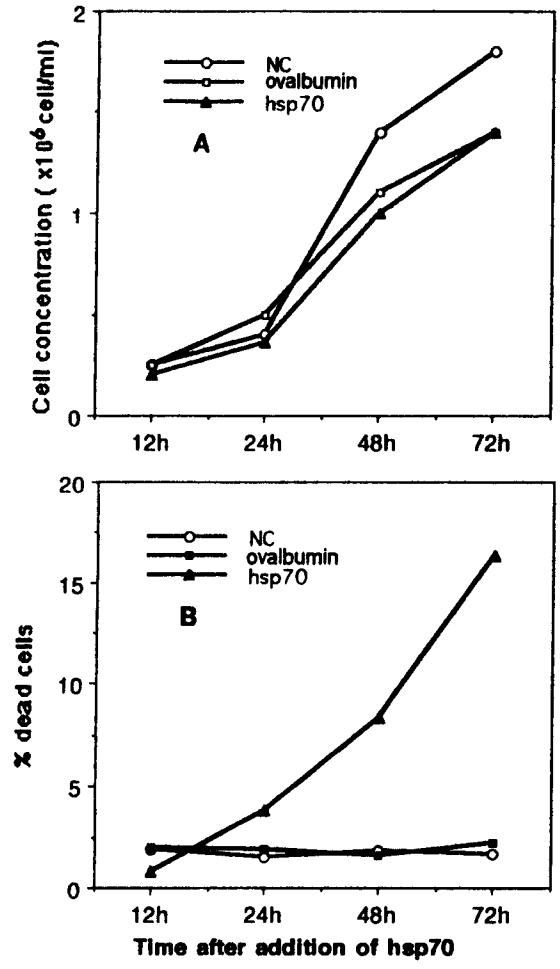

Fig. 2 Effect of exogenous Hsp/Hsc70 on cell growth and viability of U-937 cells. The U-937 cells incubated for time ranges as indicated with purified Hsp/Hsc70, 100 ug/ml, ovalbumin, 100 ug/ml, or no added protein (NC) were stained with Trypan blue. (A) cell concentration, (B) percentage of dead cells.

higher – 62% of positive cells (Fig. 4). Neither Hsp/Hsc70 nor ovalbumin had any effect on CD14 marker expression (data not shown). In order to prove that the Hsp/Hsc70 actually interferes with the pattern of mature antigens, we treated the cells first with the protein, and then with PMA. It was found that the expression of the antigens was markedly reduced to 41% for CD11c and 44% for CD23, when compared with cells stimulated with PMA alone (Fig. 4). Finally, Hsp/Hsc70 gave no effect on cells pre-induced with phorbol ester (data not shown) proving that extracellular Hsp70's action was restricted to immature cells with a changing pattern of surface domains, and not to those with established domains. Control treatment with ovalbumin at the same concentration as Hsp/Hsc70, did not influence the U-937 cell surface antigen pattern.

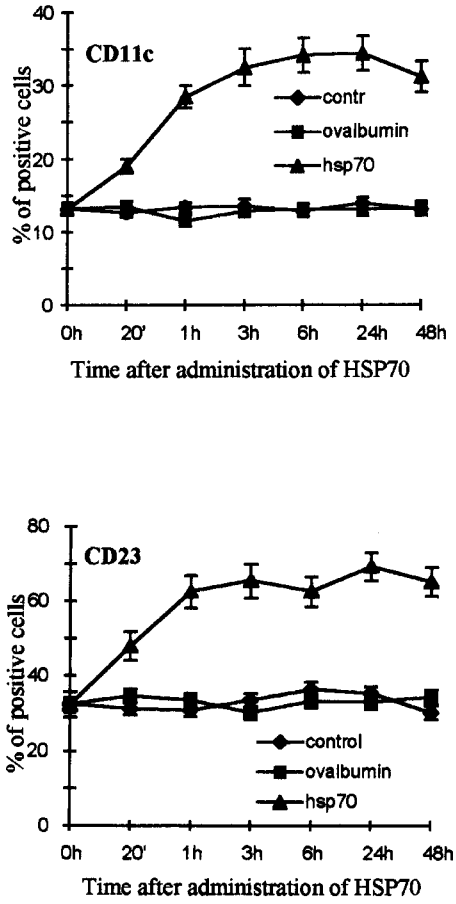

Fig. 3 Time-course of CD11c and CD23 appearance in U-937 cells incubated with Hsp/Hsc70. The cells were incubated in the presence of 100 ug/ml Hsp/Hsc70 or ovalbumin and then processed with FACScan fluorimeter in order to detect the pattern of CD11c and CD23 mature markers. The data are presented as percentage of positive cells and the results of three independent experiments are shown.

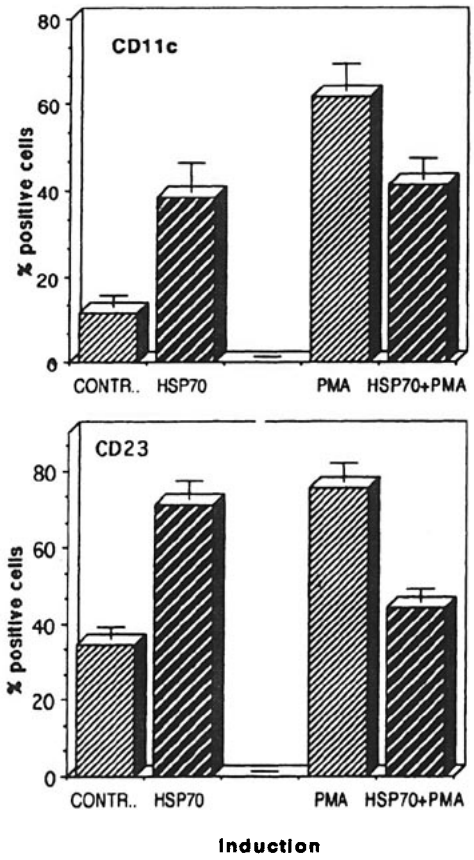

Fig. 4 Effect of exogenous Hsp/Hsc70 and phorbol ester on differentiation markers of U-937 premonocytes. The cells were incubated alternatively with Hsp/Hsc70 100 ug/ml, PMA 10 ng/ml or with the Hsp/Hsc70 first and 3 h later with PMA. The data are given as percentage of positive cells.

Exogenous Hsp/Hsc70 inhibits DNA-binding activity of cAMP-responsive and double-symmetry factors

One of the first events in monocyte/macrophage differentiation appears to be an activation of c-fos proto-oncogene by transcription factors that transiently bind specific DNA sequences, particularly cAMP-responsive (CRE) and double-symmetry (DSE) elements (Treisman 1986; Sassone-Corsi et al 1989). The analysis of DNA-binding activity of the appropriate transcription factors in nuclear extracts isolated from differently stimulated cells was carried out with the aid of the gel mobility shift method. Two concentrations of Hsp/Hsc70 were tested, 25 and 100 ug/ml, and they were found to give similar effects. As expected, induction of U-937 cells with PMA strongly affected CRE and DSE oligonucleotide mobility, while Hsp70 alone had no influence on the former (Fig. 5). The addition of Hsp/Hsc70 to the medium containing PMA for 10 or 20 min was found to abolish DNA-binding activity of both factors (Fig. 5). Control protein, ovalbumin, had no effect on PMA-induced shift of CRE and DSE oligonucleotide mobility (data not shown).

Exogenous Hsp70 impedes cytotoxic effects of TNF-α

It was recently established that U-937 premonocytes, as well as many other cells, respond to TNF, which is known to elicit strong cytotoxic effect and causes cell death mainly by apoptosis (Wong and Goeddel 1994; Wright et al 1992). In our experiments, the cells were exposed to TNF alone or in combination with Hsp/Hsc70 100 ug/ml, or ovalbumin 100 ug/ml. To the control probes, the latter two were added alone. Hsp/Hsc70 in contrast to ovalbumin was found to reduce the number of cells stimulated to undergo apoptosis (Fig. 6). As revealed by Trypan blue staining, the presence of Hsp/Hsc70 in U-937 cell culture inhibited TNF-mediated cytotoxic effect via both necrosis and apoptosis. Ovalbumin was shown not to influence TNF-induced cytotoxicity (Fig. 6).

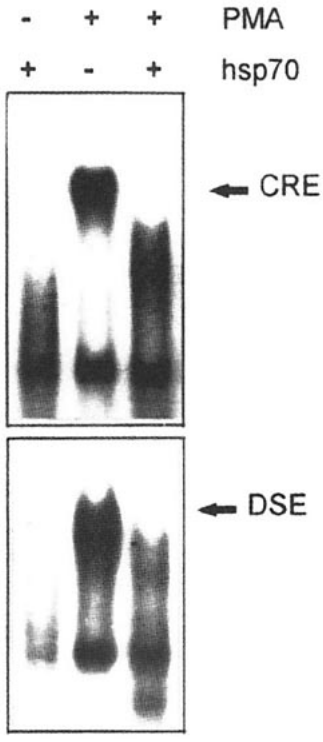

Fig. 5 Inhibitory effect of extracellular Hsp/Hsc70 on PMA-induced activation of DNA-binding of appropriate transcription factors to cAMP-responsive and double-symmetry elements of *c-fos* early response gene. The U-937 cells were incubated 20 min with Hsp/Hsc70 25 ug/ml, with PMA 10 ng/ml, or with a mixture of both. After incubation, cell nuclear fraction was isolated and employed in the gel-shift assay in combination with [^{32}P]-labeled oligonucleotide probes of cAMP-responsive and double-symmetry elements of *c-fos* gene promoter. After the binding reaction, DNA-protein complexes were separated from free labeled probe by electrophoresis in 4% acrylamide gel.

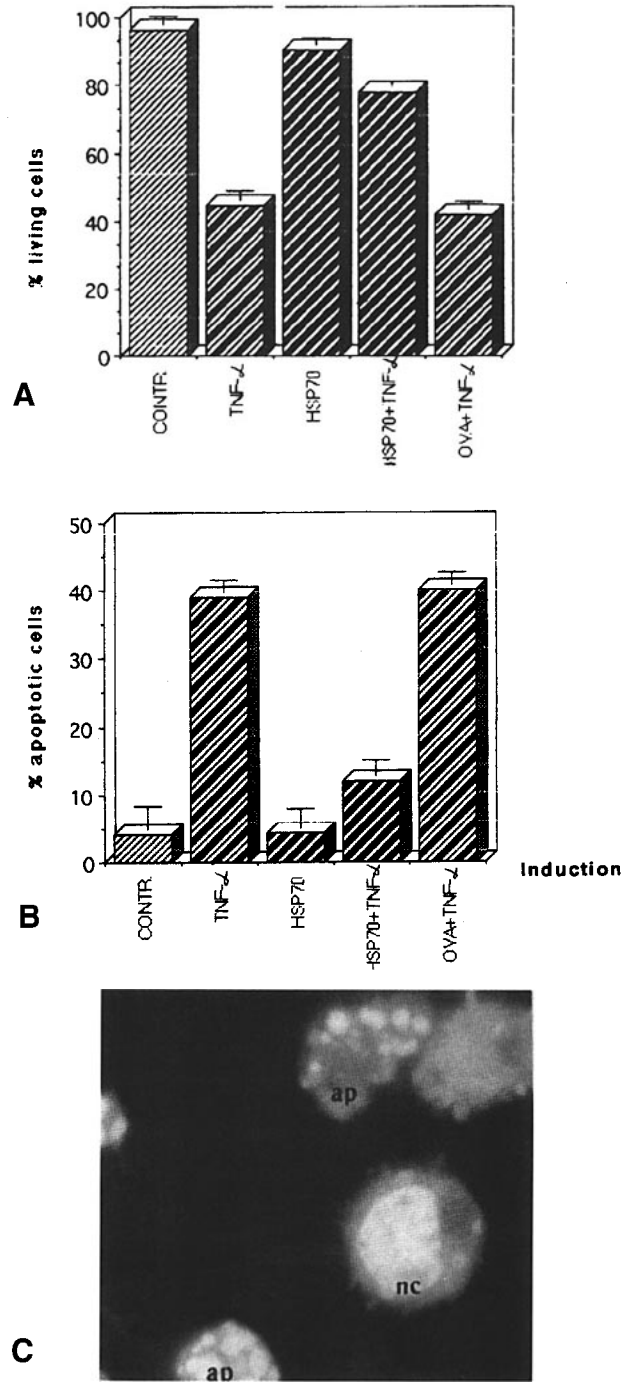

Fig. 6 Protection of U-937 cells from TNF-mediated cytotoxicity by the exogenous Hsp/Hsc70. The cells were treated either with TNF 200 un/ml or Hsp/Hsc70 100 ug/ml alone, or both (see text for details); after 48-h treatment the percentage of dead cells was measured (A), as well as of apoptotic cells (B). An example of apoptotic cells with fragmented nuclei is shown on micrograph (C).

Binding and internalization of exogenous Hsp70

Processes such as differentiation or apoptosis are triggered by certain signaling systems; the latter activity is initiated from the interaction of a signal with an appropriate surface domain. The results of above studies led us to investigate whether Hsp/Hsc70 could temporarily interact with the cell surface. The following quantitative experiments were designed to detect the protein endocytosed by the cell and/or associated with the cell surface. Biotinylated Hsp/Hsc70 or ovalbumin was incubated with U-937 cells for different time intervals and the cells were processed using three techniques. The results of the enzyme immunoassay indicated that labeled Hsp70 became associated with the cell within 40 min of co-incubation (Fig. 7). The amount of cell-bound label peaked at 1 h, reached a plateau lasting 6 h, and then progressively declined.

The results of immunofluorescence revealed a complex pattern of biotinylated Hsp/Hsc70 interaction with U-937 cells. Within the first 5 min of co-incubation the whole cell surface was stained. Thereafter, bound Hsp/Hsc70 aggregated into patch-like structures that in turn gave rise to larger complexes resembling caps

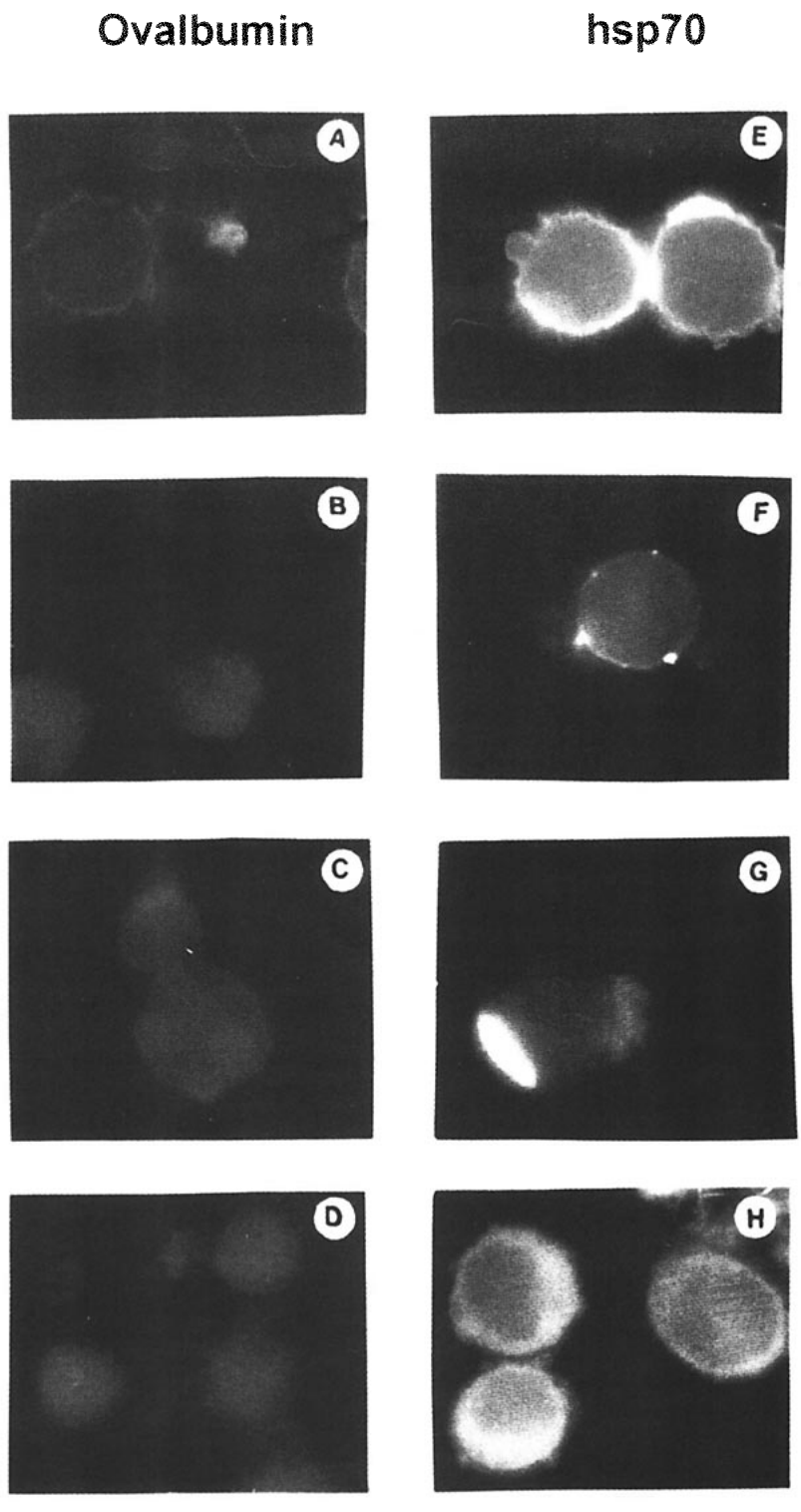

Fig. 8 Pattern of exogenous Hsp/Hsc70 interaction with U-937. The cells were incubated with ovalbumin 20 ug/ml (A–D) or Hsp/Hsc70 20 ug/ml (E–H) for the following time intervals: 5 min (A, E), 20 min (B, F), 40 min (C, G) and 3 h (D, H). After fixation the cells were plated onto poly-l-lysine coated cover slips, permeabilized and stained with extravidin-FITC. The fluorescence was visualized with the aid of Zeiss Axioplan microscope.

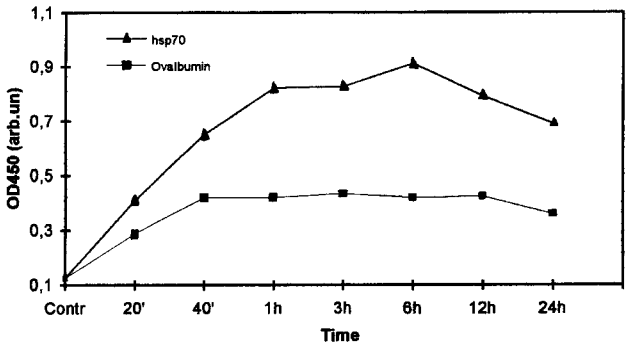

Fig. 7 Time-course of biotinylated Hsp/Hsc70 binding to U-937. The cells were incubated for times indicated with biotinylated Hsp/Hsc70 20 ug/ml or ovalbumin 20 ug/ml. After washing, the cells were applied to a 96-well microtitration plate, fixed, permeabilized and stained with the aid of streptavidin-peroxidase. The absorbance at 450 nm was measured, and one representative experiment (total four) is shown.

(Fig. 8). Within 3 h of incubation, cell surface was cleared almost completely, and the staining was observed inside the cell (Fig. 8). Cells treated with biotinylated ovalbumin had a much weaker staining pattern, which was only observed within the first hour of incubation, suggesting a non-specific association (Fig. 8).

To quantitate immunofluorescence results, we performed flow cytometric analyses of U-937 incubated with biotinylated Hsp/Hsc70 or ovalbumin. The kinetics of Hsp/Hsc70 binding determined by this technique agreed with that obtained by immunoenzyme assay and immunofluorescence. Cells binding biotinylated Hsp/Hsc70 were detected during first 5 min of incubation; the label reached a plateau approximately 20 min later. It was found also that only a part of the cell population was able to internalize biotinylated Hsp/Hsc70, i.e. at least 33% of the cells were positive for Hsp/Hsc70 after 3 h of incubation (Fig. 9). For ovalbumin, this percentage was never more than 10%.

DISCUSSION

The first part of our study was performed in order to elucidate effects of pure Hsp70 introduced into the U-937 cell culture system. The rationale for the study follows from the observations that the extracellular chaperone can influence cellular physiological functions (Johnson and Tytell 1993, B. A. Margulis and I. V. Guzhova, unpublished observations) and, among other effects, may protect sensory neurons injured by axotomy (Houenou et al 1996). It was sufficient that this activity of exogenous Hsp70 arises from its ability to bind to misfolded polypeptides. To confirm that the Hsp/Hsc70 preparation used in the present study was active, it was tested in several ways. Electrophoresis and immunoblotting showed that the preparation was no less than 97% pure and consisted of a mixture of the two isoforms, where the major component was identified as an inducible Hsp70 form. Functional tests, one measuring the shift in fluorescence output after the addition of ATP to Hsp/Hsc70 in solution (Ha and McKay 1995), and the other carried out to estimate the binding to denatured substrate, carboxymethylated lactalbumin (Wawrzynow and Zylicz 1995), demonstrated that the protein preparation possessed chaperonic activity (B. A. Margulis and V. M. Bozhkov, unpublished observations). However, by the current methods it is still not possible to define exactly the activity of Hsp/Hsc70. The high concentration of the protein employed in some of our experiments, 100 ug/ml, reflected the fact that probably only a part of the protein was in active form with respect to its property to bind to other polypeptides.

In studying the effects of exogenous Hsp/Hsc70, we found that the protein can change the patterns of differentiation and apoptosis in U-937 promonocytic cells. Firstly, Hsp/Hsc70 was shown to up-regulate expression of two surface antigens, CD11c and CD23, although the change in the former was not so significant, as in the case of phorbol ester, a traditional inducer of differentiation to macrophage phenotype. It seems that the fast recruitment of the two antigens induced by both factors, Hsp/Hsc70 and PMA, occurred by similar or at least interfering pathways. The data supporting this were obtained in experiments where incubation with Hsp/Hsc70 preceded induction with PMA; in this case, Hsp/Hsc70 considerably reduced the level of CD11c and CD23 expression, compared to that achieved with PMA alone. The fact that expression of CD14 antigen was not affected by Hsp/Hsc70 could show that a certain selectivity exists in the protein targets. In spite of the changes in mature markers, no morphological pattern of differentiation induced by Hsp/Hsc70 was observed.

Secondly, Hsp/Hsc70 was found to inhibit the DNA-binding activity of two transcription factors recognizing DSE and CRE elements in promoter region of c-fos gene, known to be induced at the early phase of differentiation (Pelech and Sanghera 1992). As in the first case, during short-term exposure to Hsp/Hsc70, the cells could not incorporate the protein, and we relate its effect mainly to the interaction with the specific or non-specific sites on the outer cellular membrane. The most probable regulatory pathways for the transduction of phorbol ester-induced signal is one mediated by protein kinase C. We may only speculate that the Hsp/Hsc70, by masking surface structures, made phorbol-stimulated domains, whose endpoints are nuclear regulatory proteins, inaccessible with a simultaneous inhibition of the whole protein kinase C-dependent mechanism (Pelech and Sanghera 1992).

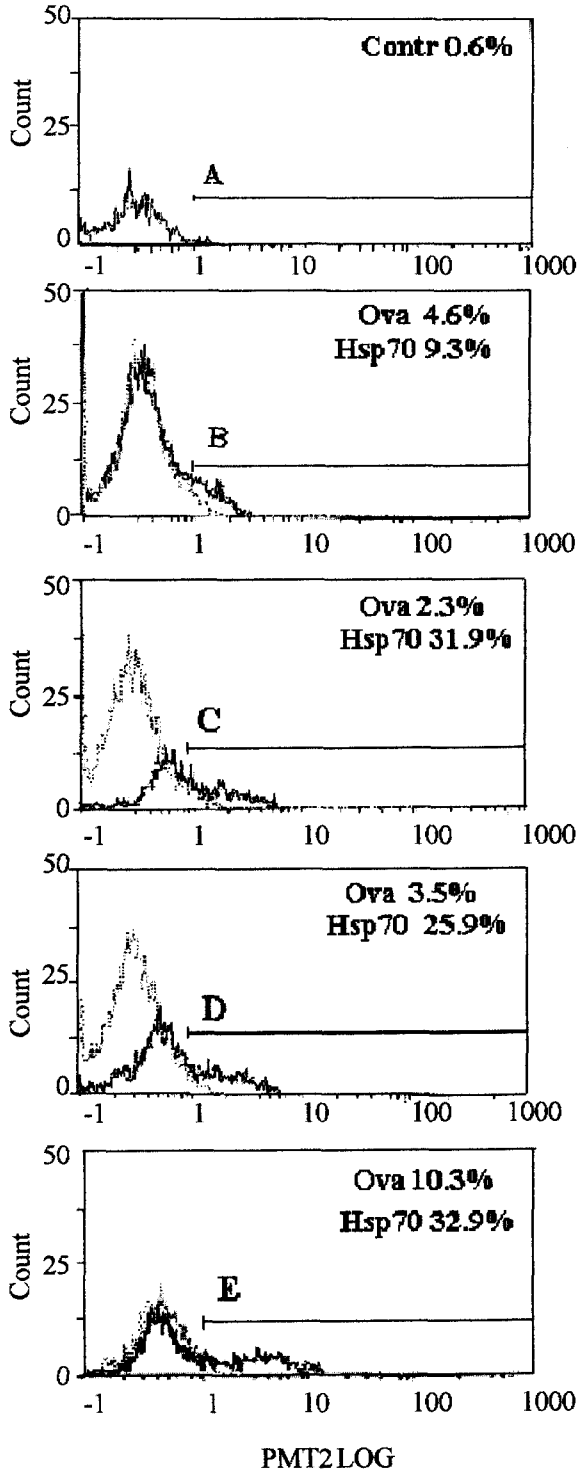

Fig. 9 Flow cytometry analysis of Hsp70-binding to U-937 cells. After the incubation with biotinylated Hsp/Hsc70 25 ug/ml or ovalbumin 25 ug/ml, for following time intervals: 5 min (B), 20 min (C), 40 min (D) and 3 h (E) the cells were fixed, permeabilized, stained with extravidin-FITC and processed with the aid of Coulter fluorimeter; (A) control, cells treated with extravidin-FITC. The profiles for Hsp/Hsc70 (solid line) and for ovalbumin (dotted line) were overlaid. Percentage of positive cells is presented in upper right corner. One representative experiment is shown.

We also demonstrated a protective effect of exogenous Hsp/Hsc70 on U-937 cells induced to apoptosis with TNF-α. Although the data support the theory of Hsp70-mediated cell protection against factors inducing apoptosis and/or necrosis, the majority of results are related to the protein that has been induced or transferred to a cell by gene transfection or by liposomes (Jäättelä 1993; Jacquier-Sarlin et al 1994; Margulis et al 1991), i.e. the protein became intracellular. For the case when the Hsp70 was applied exogenously, we have two possible explanations of the protein effect. First, Hsp/Hsc70 might interact with TNF-α molecule itself or type B TNF receptor, the latter activity results in apoptosis in HL-60 and U-937 cells (Greenblatt and Elias 1992). The interaction could lead to the suppression of one or both of those. Secondly, the protein, being internalized by a cell, might block or interfere with the TNF-induced signal transfer to the nucleus. Since the protective effect of exogenous Hsp70 may be of potential interest for the therapies of traumas and other injuries, more careful studies are needed to understand this effect.

Hypothetically, all three effects of Hsc70/Hsp70 introduced into U-937 cell culture exogenously can be explained by an interaction of the protein with certain domains on the cell surface. Interestingly, heat shock proteins have been shown to make pores in artificial biomembranes (Alder et al 1990) and, recently, a similar preparation of Hsp/Hsc70 was found to induce specific K^+-channels on the U-937 cell surface (Negulyaev et al 1996).

To analyze the pattern of the chaperone–cell interaction, we biotinylated Hsp/Hsc70, as well as a control protein, ovalbumin, and added them to U-937 cells. The results of immunoenzyme assay showed that the process of binding to a cell surface took less than an hour, and that the amount of label reached a plateau that lasted several hours. According to flow cytometry data, only 33% of the whole cell population was able to bind and internalize Hsp/Hsc70. This probably means that only the cells existing at a certain phase of cell cycle efficiently bind the protein and internalize it. The results of immunofluorescence gave more details concerning the interaction between the chaperone and U-937 cells. Within the first few minutes the whole membrane was stained indicating rapid and uniform association of the protein with the cell surface. After that, separate domains were formed followed by overall clustering of patch-like structures and their conversion into cap-like formations. This process took 40 min, and its details and time-course agree well with the schedule expected of endocytosis. Random binding of exogenously introduced Hsc70 to a cell surface has been reported by Johnson and Tytell (1993). However, their procedure did not allow the detection of patch- and cap-like structures. We suggest that the distinction in cell-binding pattern of Hsp70 lies in

differences on cell types used as models. The study cited above was carried out employing differentiated smooth muscle cells, while U-937 promonocytic cells used in our experiments are known to possess rich and alternating patterns of surface domains. To our knowledge, the fact that Hsp70 chaperone can be endocytosed is presented here for the first time. The endocytosis-mediated pathway of Hsp/Hsc70 internalization may be explained by the fact that the protein can bind several lipid components of plasma membranes (Guidon and Hightower 1986; Boulanger et al 1995). This property of Hsp-like proteins has been suggested to help in the formation of ion-conducting pores in biomembranes as mentioned previously.

We suggest that the Hsp/Hsc70 introduced to U-937 cell culture can affect surface structures and by this stimulate various signaling systems within the first 10–20 min after its addition. Thereafter, the protein is transported inside the cell and probably digested by lysosomal enzymes, as observed by the protein level reduction in enzyme immunoassay.

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