Spontaneous apoptosis and expression of cell surface heat-shock proteins in cultured EL-4 lymphoma cells

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Abstract. The expression of heat-shock proteins (HSPs) is enhanced in stressed cells and can protect cells from stress-induced injury. However, existing data about the relationship between apoptosis and HSP expression is contradictory. In this paper, a mouse lymphoma cell death model system is used to detect simultaneously both the process of apoptosis and the level of HSP expression. The model was established after discovering that spontaneous apoptosis and spontaneous cell surface HSP expression occurs in EL-4 mouse lymphoma cells during normal optimal culture conditions. The data show that apoptotic EL-4 cells had higher levels of hsp25, hsp60, hsp70 and hsp90 exposed on the plasma membrane surface than viable cells. The level of surface HSPs was found to increase through several stages of early and late apoptotic death as measured by flow cytometry, with the highest levels observed during the loss of cell membrane phospholipid asymmetry. Heat shock and actinomycin D significantly increased the proportion of apoptotic cells in culture. However, hyperthermia only stimulated a weak and temporary increase in surface HSP expression, whereas actinomycin D strongly elevated the level of surface and intracellular HSPs, particularly in live cells. These results show an associative relationship between apoptosis and HSP expression. The relationship between the progression of cell death and HSP expression suggests a role for membrane HSP expression in programmed cell death.

INTRODUCTION

Stress-induced proteins (heat-shock proteins (HSPs)) perform an important protective role both in cells growing under optimal conditions and those exposed to less favourable environments. The known functions of HSPs include structural stabilization of newly synthesized polypeptides, prevention of protein aggregation, protection of proteins from denaturation by stress-inducing agents, and participation in the repair or degradation of polypeptides that have been denatured under stress (Schlesinger 1990, Craig, Weissman & Horwich 1994, Hartl 1996). HSPs are intracellular proteins and most of their known

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functions studied are connected with intracellular processes. Nevertheless, several workers have described the cell surface localization of HSPs. HSP association with the plasma membrane has been demonstrated for both normal and tumour cells (Poccia *et al.* 1992; Erkeller-Yeksel *et al.* 1992, Ferrarini *et al.* 1992, Camins *et al.* 1995, Multhoff *et al.* 1995, Altmeyer *et al.* 1996), virus-infected T lymphocytes (Di Cesare *et al.* 1992) and T cells undergoing apoptosis (Poccia *et al.* 1996). The functions of the plasma membrane associated HSPs are not clear, although there is evidence that they can stabilize lipid membrane and prevent denaturation of membrane-localized proteins during stress (Török *et al.* 1997). At the tissue level, HSPs might play a role as a marker of damaged cells in the immune system (Poccia *et al.* 1996).

Recently evidence has emerged concerning the role of HSPs in events leading to programmed cell death. Hsp70 antisense treatment has been shown to induce apoptosis of Molt-4 tumour cells (Wei *et al.* 1995). Bleomycin-induced apoptosis of human alveolar macrophages is preceded by a decrease in hsp72 expression (Hamilton *et al.* 1995). Accumulation of hsp70 in heat-treated Ehrlich carcinoma cells is believed to cause an increase in their resistance to apoptosis-inducing agents (Gabai *et al.* 1995). Constitutive expression of human hsp27 in murine L929 cells has been shown to block Fas/APO-1-mediated cell death (Mehlen, Schulze-Osthoff & Arrigo 1996). An increase in cellular levels of hsp27 or hsp70, induced either by mild heat shock or by stable transfection, also increases the resistance of U937 and Wehi-s cells to apoptotic cell death (Samali & Cotter 1996).

In contrast, several authors have produced evidence suggesting a positive correlation between the level of HSP expression and the susceptibility of cells to apoptosis (Beere *et al.* 1993; Arata, Hamaguchi & Nose 1995; Chant, Rose & Morris 1996; Galea-Lauri *et al.* 1996). Such positive correlation between apoptosis and the expression of HSP is consistent with the observation that cell stress can frequently induce apoptosis. The ability of oxidative stress to induce cell death in a variety of cell types has been examined in detail (Buttke & Sandstrom 1994, Nosseri, Coppola & Ghibelli 1994, De Laurenzi *et al.* 1995, Lotem *et al.* 1996, Xu *et al.* 1997). The effects of other stresses have also been shown to be similar (Cosulich & Clarke 1996, Borthers & Cidlowski 1996, Verheij *et al.* 1996, Der *et al.* 1997, Pourzand *et al.* 1997). It therefore seems clear that HSPs induced by stress are unable or insufficient to prevent concomitantly induced programmed cell death. The reason for the absence of a protective effect of HSPs in such situations is unknown. It has been proposed that the increased expression of HSPs and apoptosis are, respectively, the first and second stages of cell reaction to an elevated level of stress. HSPs may therefore afford some protection at early stages of stress induced damage but are unable to provide complete protection once an apoptotic cascade is triggered (Samali & Cotter 1996). However, as mentioned above, the sensitivity of cells to apoptosis can increase for cells with elevated HSP levels (Beere *et al.* 1993, Arata *et al.* 1995, Chant *et al.* 1996, Galea-Lauri *et al.* 1996).

The nature of the relationship between apoptosis and HSP expression is therefore unclear. Existing data suggest that HSPs may play a role both in prevention and in the development of apoptosis. In this paper, a simple apoptotic model is used to demonstrate strong co-ordination between the onset of apoptosis and HSP expression. This model is based on the spontaneous apoptosis of the mouse lymphoma cell line EL-4 *in vitro* and the spontaneous expression of HSPs on the surface of these cells. The results show a positive correlation between apoptosis and expression of HSPs. This strong associative relationship between HSP expression and cell death support a role for HSP upregulation in the modulation of apoptosis.

MATERIALS AND METHODS

Cell culture and separation of adherent and non-adherent populations of EL-4 cells

Mouse EL-4 lymphoma cells were maintained in a CO₂ incubator by *in vitro* passage in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM l-glutamine, 20 mM HEPES and 50 µg/ml gentamicin (complete medium; all reagents from Sigma, St Louis, MO, USA). Cells were passaged every 3 days. Cell number was determined using a haemocytometer and viability was assessed by their ability to exclude trypan blue.

In vitro cultures of EL-4 lymphoma usually contain both adherent and non-adherent cells. Non-adherent cells were obtained by gently swirling the flask and pouring off the cells. The remaining adherent fraction was incubated in phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ for 30 min at 4°C to facilitate their removal from the walls of the flask.

Flow cytometric analysis of HSP expression and cell death

Analysis of cell size by forward scatter (FS), granularity by side scatter (SS), indirect surface and intracellular immunofluorescence and analysis of apoptosis in cell stained with propidium iodide (PI) and annexin V-fluorescein isothiocyanate (FITC) were all performed using an EPICS ELITE fluorescence-activated cell sorter (Coulter Corporation, Hialeah, FL, USA). For HSP immunolabelling, cells were transferred from culture medium into PBS supplemented with 1% FCS and 0.05% NaN₃ and were incubated with an antibody specific for a cell surface marker for 45 min at 4°C. The cells were then washed and incubated under the same conditions with a secondary anti-immunoglobulin antibody, coupled with a fluorescein (FITC) or phycoerythrin (PE) fluorophore. Labelled cells were washed twice and measured immediately or kept in 1% paraformaldehyde (in PBS) until analysis. The HSP antibodies used were mouse monoclonal anti-HSP25 (clone IAP-9, IAP-28), anti-HSP60 (clone LK1, LK2), anti-HSP70 (clone BRM-22) and anti-HSP90 (clone AC-16) (all obtained from Sigma). An isotype-matched control antibody (IgG1, Dakopatts, Denmark) was used in each experiment. The secondary antibodies used were either FITC or PE-conjugated sheep-anti-mouse IgG F(ab)₂ (Sigma). For each analysis more than 3–10 × 10³ cells were analysed. For evaluation of the level of expression of HSPs, the arithmetic mean level of cell fluorescence was used. The relative level of expression of HSP expression is presented as: [mean fluorescence intensity (HSP) – mean fluorescence intensity (control)].

Analysis of intracellular HSPs was carried out by the method described by Clevenger, Bauer & Epstein (1985). Briefly, cells were sequentially treated by 0.5% paraformaldehyde for 30 min and then with 0.1% Triton X-100 in phosphate-buffered saline at 4°C for 3 min followed by standard staining with indirect immunofluorescence. The value of level of intracellular HSPs was calculated as described above for surface HSPs.

For flow cytometric analysis of apoptosis, cells were fixed in 70% ethanol for 30 min, then washed twice in PBS and resuspended in a solution containing 20 µg/ml propidium iodide and 1 mg/ml RNase A (Sigma). Samples of 3–10 × 10⁵ cells were kept for up to 2 days at 4°C before the analysis of DNA content. Apoptotic cells differed from live cells by having lower PI fluorescence intensity (Telford, King & Fraker 1991, Darzynkiewicz *et al.* 1992). In a number of experiments, identification of HSP expression on the surface of live, apoptotic and dead cells was measured using FITC-conjugated annexin V (PharMingen, San Diego, CA, USA) simultaneously with PI exclusion (Vermes *et al.* 1995). Briefly, cells stained as described above by anti-HSP antibodies and secondary PE-conjugated antibodies were washed twice with cold PBS and then resuspended in annexin V-FITC in a Ca²⁺-containing

binding buffer at a concentration of 1×10^6 cells/ml. After incubation with annexin V-FITC according to the manufacturer's instructions, PI was added at 2 $\mu\text{g}/\text{ml}$ and the samples analysed by flow cytometry for FITC, PE and PI expression. Live cells were double-negative for annexin V-FITC and PI, apoptotic cells were annexin V-FITC-positive and PI-negative and advanced apoptotic and necrotic cells were double positive.

DNA isolation and electrophoresis

The detection of DNA fragmentation was performed as previously described (Collins *et al.* 1992). Briefly, cells were washed in PBS and resuspended at approximately $5 \times 10^6/\text{ml}$ in 200 μl buffer containing 10 mM Tris (pH 8.0), 0.15 M NaCl, 10 mM EDTA, 0.1% SDS. Cell lysates were incubated with 250 $\mu\text{g}/\text{ml}$ proteinase K for 3 h at 37°C, followed by RNase A (DNase-free) treatment (0.25 mg/ml) for 1 h at 50°C. DNA was then harvested by standard phenol/chloroform extraction followed by ethanol precipitation (Sambrook, Fritsch & Maniatis 1989). DNA samples in 20 μl of TE (10 mM Tris pH 8.0, 0.1 M EDTA) were electrophoresed at 5 V/cm in a 1.5% agarose gel, in 0.5 $\times$ TBE (45.5 mM, Tris; 45.5 mM, borate and 1 mM, EDTA) containing 1 $\mu\text{g}/\text{ml}$ ethidium bromide.

Exogenous inducers of apoptosis

Heat shock and actinomycin D (Sigma) were used as inducers of apoptosis in EL-4 cells. Heat shock was induced by incubating 1×10^6 cells/ml at 43°C for 40 min. Control cells were incubated in for the same time period at 37°C. Actinomycin D treatment (5 $\mu\text{g}/\text{ml}$) was performed in complete medium in a 24-well tissue culture plate at a cell density of $1 \times 10^6/\text{ml}$, for the indicated period of incubation at 37°C after which the cells were washed and prepared for analysis.

RESULTS

Spontaneous apoptosis in cultures of EL-4 cells

Flow cytometric analysis of EL-4 cells cultivated under conventional conditions for various lengths of time revealed the presence of two morphologically distinct cells populations. After 20 h of incubation the majority of cells (55–65%) had the typical morphological features (size and granularity) of activated T lymphocytes (Figure 1a). The second population showed decreased cell size and increased granularity (Figure 1b), a recognized characteristic of apoptotic cells (Swat, Ignatowicz & Kisielow 1991, Darzynkiewicz *et al.* 1992). Cytometric detection of apoptosis in cells of the same culture stained with propidium iodide showed an apoptotic population equivalent in percentage to the cell population with reduced size (Figure 1b). Viable and apoptotic EL4 cells could therefore be easily distinguished by flow cytometry using both scatter and DNA dye binding characteristics. This apoptotic subpopulation did not contain necrotic cells, as verified by preparing necrotic EL4 cells with lethal heat treatment (50°C for 20 min). The resulting light scatter characteristics of necrotic EL-4 cells (Figure 1d) differed from those for viable and apoptotic cells (Figure 1c). Necrotic cells were approximately the same size as live cells but showed increased granularity, similar in level to apoptotic cells. The morphological characteristics of apoptotic and necrotic cells in this model were therefore sufficient to confirm the absence of necrosis in the apoptotic subpopulation. Viable, apoptotic and necrotic subpopulations can therefore be quantified using this technique.

It is known that adherent cells become detached during apoptosis (Wyllie, Kerr & Currie 1980). This characteristic was exploited to physically separate viable from apoptotic EL-4

Figure 1. Flow cytometric analysis of E-4 cell apoptosis based on scatter characteristics (a) and PI fluorescence (b) of mouse lymphoma cells EL-4 cultivated *in vitro*, and comparison of scatter characteristics of viable, apoptotic and necrotic cells (c and d). Cells (2×10^6) in 1 ml complete medium were plated into a 24-well tissue plate and cultivated for 20 h before analysis. The cultivation of EL-4 cells leads to appearance of two morphologically distinguished cell pools. One of them containing the majority of cells has the typical morphological features (size, forward scatter and granularity, side scatter) of activated T lymphocytes (a, viable). Cells of another pool were smaller and had increased granularity (a, apoptotic). The percentage of cells in these subpopulations was similar to the percentage of live and apoptotic cells among cells of the same culture detected by flow cytometry of PI stained cells (b). To induce cell necrosis, EL-4 cells were incubated at 50°C for 20 min before measuring forward and side scatter. Control cells were incubated in the same conditions at 37°C. In heated sample the majority of cells (necrotic cells) were in a region (d, necrotic) distinct from the viable and apoptotic subsets (d, viable and apoptotic). The percentage cells in the apoptotic region following heat treatment (d, apoptotic, 21%) was approximately the same to that for control (c, apoptotic, 18.5%) indicating that necrosis-inducing condition did not result in cells with apoptotic morphology.

cells by harvesting adherent and non-adherent fractions of cells from cultures in order to obtain cell suspensions enriched with live or apoptotic cells. Flow cytometric analysis confirmed that the majority of adherent cells had the morphological characteristics of live cells (Figure 2a) and that the non-adherent fraction showed enrichment of apoptotic cells based on decreased size and increased granularity (Figure 2b). Flow cytometric analysis of PI-stained cells showed a small percentage of apoptotic cells in the adherent fraction (Figure 2c) and a much greater percentage of such cells in the non-adherent fraction (Figure 2d). The presence of apoptotic cells in the non-adherent fraction was further confirmed by electrophoretic analysis of DNA isolated from the cell fractions (Figure 2e,f). DNA fragmentation typical of apoptotic cells was found in the non-adherent fraction; in contrast, the adherent fraction showed little fragmented DNA (Figure 2f).

In addition to the viable, apoptotic and necrotic subpopulations described above, a subpopulation was also observed occupying an intermediate position in scatter characteristics between the viable and apoptotic fractions. (Figure 1; Figure 3a, region 2). This pool of cells forms a connection or 'neck' between regions of live and apoptotic cells and most constitutes an early stage of apoptosis. Indeed, 'neck' cells are negative for annexin V-FITC labelling and PI uptake (Figure 3c), suggesting that they are at a stage of cell death upstream of plasma membrane asymmetry loss. As expected, cells identified as apoptotic by scatter characteristics were annexin V-FITC positive and PI-negative (Figure 3d). These results show that in the frame of this model at least three successive stages of apoptosis can be easily distinguished and analysed simultaneously in a sample by multiparametric flow cytometry.

The phenomenon of spontaneous apoptosis in EL-4 cell culture is not a result of a lack of serum factors, depletion of culture medium or change in pH. Spontaneous apoptosis occurred even in the presence of large quantities of FCS (up to 20%, data not shown). The largest fraction of apoptotic death occurred early in the culture period, prior to significant cell expansion and exhaustion of medium (Figure 4a). Moreover, the percentage of apoptotic cells actually decreased late in the culture period, despite a significant increase in total cell number (Figure 4a) and depletion of growth medium. Furthermore, plating EL-4 cells at increased densities resulted in decreased spontaneous cell death, again suggesting that nutrient depletion was not the cause of the observed apoptosis (Figure 4b). The greatest levels of apoptotic death were observed at the point in cell culture where cell density was the lowest. This suggests that spontaneous apoptosis in low density cultures of EL-4 cells may be caused by a lack of autocrine/paracrine cell factors.

Expression of HSPs on the surface of live and apoptotic EL-4 cells

Cultured viable EL4 cells were found to express low constitutive levels of surface hsp25, hsp60, hsp70 and hsp90 as measured by flow cytometry. In contrast, the apoptotic EL-4 fraction demonstrated elevated levels of the above HSPs. Expression involved all cells and not just a proportion of the population, since indirect immunolabelling of HSPs resulted in the displacement of the resulting histogram to a higher level of fluorescence, without changing its shape (Figure 5). This allowed an estimation of the level of surface HSP expression in a gated cell population using the difference between the mean fluorescence intensities (MFIs) of samples labelled with HSP antibodies and corresponding samples labelled with isotype-matched controls. This approach showed that the level of HSP expression changed for four successive stages of cell status: viable cells, an earliest stage of apoptosis detected by scatter ('neck' cells), the stage of plasma membrane asymmetry loss corresponding with early apoptosis (annexin V-FITC positive, PI-negative cells, these being a portion of the low size/high granularity scatter population) and more advanced apoptotic cells (annexin V-FITC positive, PI-positive cells,

Figure 2. (a–d) Forward vs. side scatter and PI DNA content analysis of adherent (a, c) and non-adherent (b, d) subsets of cultured EL-4 cells. Cells (1×10^6) in 5 ml complete medium were plated into 25 cm² tissue culture flask and cultivated for 48 h prior to separation as described in *Materials and methods*. The majority of cells in the adherent fraction have the morphological features of viable cells (a). The majority of non-adherent cells have decreased size and increased granularity characteristic of apoptosis (b). Analysis of PI fluorescence showed that the adherent and non-adherent subpopulations consisted primarily of PI-high (viable) and PI-low (apoptotic) cells, respectively (c and d). (e,f) Gel electrophoresis of whole genomic DNA extracted from adherent (e) and non-adherent (f) fractions, showing the presence of internucleosomal DNA fragmentation in the non-adherent (presumably apoptotic) cell fraction.

Figure 3. Flow cytometric analysis of apoptosis in EL-4 cells using simultaneous annexin V-FITC and PI viability staining. Cultured cells were labelled with annexin V-FITC and PI as described in *Materials and methods*. Flow cytometric analysis for scatter showed three populations (corresponding to viable (region 1), early apoptotic or 'neck' cells (region 2), and apoptotic cells (region 3)). Annexin exposure and PI permeability were low for viable and 'neck' cells (cytograms (b) and (c), respectively). Cells characterized as apoptotic by scatter formed populations of annexin V-positive, PI negative cells (early apoptotic) and double-positive cells (advanced apoptotic)(cytogram (d)).

corresponding with the rest of the small size/high granularity scatter population). Live cells (based on scatter) had lower surface HSP than 'neck' cells. The highest levels of HSPs were observed in the low size/high granularity apoptotic subpopulation (Figure 6a). When cells were labelled with annexin V-FITC and PI, the double-negative population showed the lowest HSP expression (as shown for hsp70 in Figure 6b). Annexin V-FITC-positive PI-negative cells showed higher hsp70 expression, while the double-positive advanced apoptotic cells showed lower hsp70 expression than the single positive population but still higher than viable cells. This data demonstrates tight co-ordination between the onset of EL4 cell apoptosis and HSP expression, prior to the most terminal stages of cell death. This apparent increase in HSP expression was not the result of increased antibody non-specific binding to apoptotic cells; the level of MHC class I expression as measured by immunolabelling declined throughout the EL-4 cell apoptotic process (data not shown).

Effect of stress on apoptosis and HSP expression in cultured EL-4 cells

Heat shock and actinomycin D treatment of EL-4 cells resulted in an increase in the proportion of apoptotic cells and in a change in surface HSP expression. Heat-shock (43°C for 40 min) induced the increase in the percentage of apoptotic cells ($12.4 \pm 2.3\%$) and weakly increased the density of hsp60 and hsp70 on the surface of apoptotic cells. Hsp25 and hsp90 expression did not change significantly (Figure 7a). After a 2-h recovery period at 37°C the number of apoptotic cells increased to $24.7 \pm 5.1\%$ but the level of HSP expression became approximately the same as or lower than the control sample (Figure 7a).

Actinomycin D had a more pronounced effect than hyperthermia and resulted in both a considerable increase in the proportion of apoptotic cells (Figure 7b) and expression of surface hsp25, hsp60 and hsp70 in both viable (Figure 7c) and apoptotic (Figure 7d) cells during the first 4 h of incubation. This was followed by a decrease in this expression below the control level (cells incubated without actinomycin D) after 24 h and 30 h of incubation. Changes in hsp90 expression were less pronounced (Figure 7c,d) with actinomycin D treatment.

Elevated HSP surface expression following treatment with the transcription inhibitor actinomycin D could potentially be explained either by an increase of HSP translation and/or by translocation of existing proteins from the cytoplasm to the cell membrane. To assess

Figure 4. (a) Kinetics of percentage apoptotic cells vs. total number of cells in EL4 cultures. Cells (2×10^6) in 1 ml complete medium were plated onto 24-well tissue plates and harvested at the indicated timepoints. Percentages of apoptotic cells for indicated times were determined by flow cytometry as described in the *Materials and methods*. Total cell number was estimated by using a haemocytometer. Results are the means of three representative experiments (SEM). (b) Relationship between EL-4 culture density and percentage of apoptotic cells. Cells were plated at the indicated cell densities in 1 ml complete medium. Percentage of apoptotic cells within cultures was measured by flow cytometry after 24 h of incubation. Results are the means of four representative experiments (SEM).

Figure 5. Flow cytometric analysis of surface expression of hsp25, hsp60, hsp70 and hsp90 on viable EL-4 cells. Cells were incubated for 24 h before analysis. HSP expression was measured on viable cells only by gating on scatter morphology. Cell labelling was carried out using unconjugated primary anti-HSP antibodies (or an isotype-matched control) followed by FITC-conjugated anti-mouse IgG secondary antibody.

whether the elevation of surface HSP expression was connected with increased synthesis of these proteins or with a depletion of their cytoplasmic pool, we measured the level of intracellular hsp70 in the total population of EL-4 cells incubated with actinomycin D for 4 h. Actinomycin D induced a strong elevation in cytoplasmic hsp70 in EL-4 cells, approximately 6–7 times higher than the cell surface density of this protein. This is in contrast with untreated cells, where cytoplasmic and surface hsp70 concentrations were approximately equal (Figure 8a). These results suggest that enhanced levels of surface HSPs in EL-4 cells after actinomycin D treatment were not connected with depletion of the intracellular pool of these proteins. However, the increased level of surface HSP expression in apoptotic cells (Figure 6) might have been connected with a depletion of cytoplasmic HSPs during apoptosis. Indeed, comparison of the level of intracellular hsp25, hsp60, hsp70 and hsp90 in adherent (viable) and non-adherent (apoptotic) fractions of cultured EL-4 cells showed that in the former the content of cytoplasmic HSPs is higher than in the latter by

Figure 6. Level of surface hsp90, hsp70, hsp60 and hsp25 expression in morphologically gated live, 'neck' and apoptotic EL-4 cells (a) and surface hsp70 expression of different populations of annexin V-FITC/PI stained cells (b). Cells were incubated in standard conditions for 24 h before analysis. Expression of surface HSPs was measured by flow cytometry as illustrated in Figure 5. Relative level of HSP expression was determined as described in *Materials and methods*. Data are representative of three experiments.

approximately 7, 4, 5, 4 times, respectively (Figure 8b). This suggests that the process of apoptosis in EL-4 cells was accompanied by translocation of intracellular HSPs to the plasma membrane.

DISCUSSION

Spontaneous apoptosis of different types of cells in culture has been described by a number of authors (Moss *et al.* 1985, Lynch, Nawaz & Gerschenson 1986, Soloff *et al.* 1987, Servomaa & Rytomaa 1988, Van der Bosch *et al.* 1990, Kluck *et al.* 1993). The phenomenon can be related to depletion of nutrients and growth factors in culture media, and changes in physico-chemical parameters associated with high cell density. In contrast, the present data indicates that spontaneous programmed cell death (apoptosis) is observed in EL-4 mouse lymphoma cells cultivated under optimum conditions in complete medium, over a wide range of cell density. Moreover, in this cell culture model, apoptosis increased with a reduction of

Figure 7. The influence of heat-shock (a) and actinomycin D (Act D) (b–d) on expression of surface hsp90, hsp70, hsp60, hsp25 in viable and apoptotic EL-4 cells. Cells were heat-shocked by incubation for 40 min at 43°C followed by 2 h at 37°C. Actinomycin-D (5 µg/ml) treatment was performed in complete medium at a cell density of 1×10^6 /ml for 4 h at 37°C. After treatment cells were prepared for flow cytometry analysis as described in *Materials and methods*. Expression of surface HSPs was measured for viable (a as indicated, c) and apoptotic (a as indicated, d) cells gated by scatter morphology. Level of HSP expression was determined as described in *Materials and methods*. Percentage of apoptotic cells (b) was determined as the proportion of cells in the apoptotic gate. Data are representative of two comparable experiments.

Figure 8. (a) The effect of actinomycin D (Act D) on surface and intracellular hsp70 expression in EL-4 cells. Cells were incubated with 5 $\mu\text{g}/\text{ml}$ actinomycin-D for 4 h followed by immunofluorescence labelling and flow cytometric analysis as described in *Materials and methods*. For all samples with cytoplasmic and surface hsp70 labelling MIF was calculated for total cell population including both viable and apoptotic cells. The data are representative of three comparable experiments. (b) Intracellular expression of hsp25, hsp60, hsp70 and hsp90 in adherent and non-adherent fractions of cultured EL-4 cells. Samples of adherent and non-adherent cells were prepared as described in *Material and methods*. Intracellular concentration of the HSPs was measured using flow cytometric analysis as described in *Materials and methods*. The data are representative of three comparable experiments.

the density of cultivated cells (Figure 4b), despite increasing the culture nutrients. The effect of low cell density on apoptosis may be connected with a deficit of autocrine/paracrine factors within the cell culture system, further supporting the conception of social control of cell survival (Raff 1992). The present hypothesis that the process of apoptosis in this system is regulated by autocrine/paracrine factors is supported by the data of Fratelli *et al.* (1995) which demonstrated that apoptosis in EL4-6.1 cells is regulated by the autocrine factor interleukin (IL)-1. These results led us to consider spontaneous apoptosis of EL-4 cells as a convenient basis for the study of mechanisms of induction of programmed cell death involving cell-cell interactions.

Spontaneous and induced expression of surface HSPs has been demonstrated for tumour and normal cells (Erkeller-Yeksel *et al.* 1992, Ferrarini *et al.* 1992, Poccia *et al.* 1992, Camins *et al.* 1995, Multhoff *et al.* 1995, Altmeyer *et al.* 1996), including infected T lymphocytes (Di Cesare *et al.* 1992) and T cells undergoing apoptosis (Poccia *et al.* 1996). Such a phenomenon was discovered to be inherent to mouse EL-4 thymoma cells cultured *in vitro* under optimal tissue culture conditions (Figure 5). The expression of surface HSPs was not detected in mouse thymocytes or in mouse CTLL-2 T cells by the methods described in this paper (data not shown). Live and apoptotic EL-4 cells differed in the level of expression of HSPs (Figure 6). Using the model, a relationship between the development of apoptosis and surface HSPs expression was analysed. The process of programmed death in EL-4 cells was accompanied by an elevation of density of surface hsp25, hsp60, hsp70 and hsp90. The expression reached a maximum during the loss of plasma membrane asymmetry, followed by some decrease in the terminal phase of apoptosis. The data indicated that the intracellular pool of these proteins is depleted during apoptosis (Figure 8b), suggesting HSP translocation from the cytoplasm to plasma membrane. Such a phenomenon has been described for other surface markers of thymocytes (Kishimoto, Surh & Sprent 1995). The authors considered the upregulation of surface markers on apoptotic thymocytes as a reflection of a breakdown in intracellular trafficking leading to accumulation of the proteins on the cell surface. In contrast with this, it is unlikely that the accumulation of protective proteins (HSPs) on the cell surface is just the result of a breakdown in intracellular trafficking. HSPs can stabilize lipid membranes (Török *et al.* 1997), suggesting that they might protect the plasma membranes of dying cells from destruction connected with proteolysis of cytoskeleton components. In addition, the elevated density of surface HSPs might serve as identifying markers of apoptotic cells for phagocytic cells.

Expression of HSPs therefore accompanies apoptosis without providing protection of the cells from programmed death. There is considerable evidence that enhanced synthesis of HSPs can protect cells from apoptosis (for example Gabai *et al.* 1995, Mehlen *et al.* 1996, Samali & Cotter 1996). Therefore an increase of HSPs expression could be considered a logical cellular reaction to prevent apoptosis. This is nevertheless unlikely in the present model, since actinomycin D-induced elevation of HSP synthesis did not protect EL-4 cells from apoptosis (Figure 7b,c,d). On the contrary, such a treatment induced strong expression of HSPs in cells resulting in their enhanced apoptosis. Similar positive correlation between expression of HSPs and apoptosis is described in a number of works (Beere *et al.* 1993, Arata *et al.* 1995, Chant *et al.* 1996, Galea-Lauri *et al.* 1996, Poccia *et al.* 1996). Although the mechanism of this phenomenon is unknown, one explanation of these results is that elevated HSP expression may play a role in the progression of cell death. Indeed, the results show that after the triggering of apoptosis a redistribution of cell HSPs is observed. These proteins are translocated from cytoplasm to cell plasma membrane. As mentioned above, such translocation might be aimed at the prevention of plasma membrane destruction that could result from degrading the cytoskeleton. At the same time, a role of HSPs as effectors of apoptosis might be connected with their participation in the

process of intracellular proteolysis. Thus, it can be supposed that in addition to the known ability of HSPs to prevent programmed cell death they may play a role in apoptotic death after its triggering. The data presented here demonstrate a new approach to the study of relationships between apoptosis and HSP expression.

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