Heat-shock protein expression on the membrane of T cells undergoing apoptosis

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SUMMARY

Heat-shock proteins (hsp) represent a highly conserved family of proteins, normally localized in the cytoplasm and nucleus, whose expression is induced in situations involving cell stress. This paper reports the unusual translocation of hsp to the cell membrane of T cells undergoing apoptosis. We observed that glucocorticosteroid-induced thymocyte death is associated to the surface expression of hsp 60 and hsp 70 in a discrete fraction of apoptotic cells. hsp surface expression is closely related to a thymic subset of immature $CD3^{low/-}$ T cells. The expression of surface hsp 60 appears early after treatment with dexamethasone (3 hr) whereas the membrane expression of hsp 70 follows different kinetics and peaks later. Morphological analysis of the hsp^{+} apoptotic cells suggest that this subset represents late-stage apoptotic cells at their minimal volume before fragmentation into apoptotic bodies. Membrane expression of hsp is also associated with apoptosis in peripheral blood mononuclear cells from AIDS patients cultured *in vitro*. Altogether, we show that a discrete fraction of cells undergoing apoptosis expresses membrane hsp 60 and hsp 70, supporting the hypothesis that apoptosis causes a radical alteration in the expression of cell surface molecules. Surface hsp expressed during apoptosis may constitute a novel immune-context able to generate packages of self- and exogenous antigens, originating from degradation of altered cells.

Heat-shock proteins (hsp) belong to a highly conserved family of proteins normally localized in the cytoplasm and nucleus.[1] On the basis of molecular weight and function, different types of hsp are recognized. The hsp of 60–70 000 MW (hsp 60 and hsp 70 respectively) are involved in polypeptide translocation and in the assembling/disassembling of molecular complexes.[1] hsp 70 synthesis is strictly related to the cell cycle,[2] and both hsp 60 and hsp 70 participate in oncogene activation.[3,4] hsp 70 is able to bind to a mutant of the protooncogene p53, a nuclear phosphoprotein, with characteriztics of a transcription factor that has been implicated both in cell transformation and apoptosis.[4] The transcriptional activation of the human hsp 70 promoter is regulated by p53,[5] and by another oncoprotein, the adenoviral product E1a.[6] Finally, the synthesis of hsp could be induced by many viruses, such as New Castle disease virus,[7] Sindbis virus,[8] vescicular stomatitis virus,[8] herpes simplex virus,[9] Epstein–Barr virus,[10] and human immunodeficiency virus.[11] However, the induction of hsp 70 genes is not a general consequence to the stress of viral infection but represents a highly specific response, both with regard to the inducing virus and with regard to the target gene.[12]

We previously noticed that intracytoplasmatic levels of hsp 60 and hsp 70 are increased after HIV-1 *in vitro* infection,[13] and T-cell lines chronically infected with HIV-1 express abnormal levels of membrane hsp 70.[14] More recently, Chouchane *et al.* confirmed that hsp expression on the surface of human T-cell leukaemia virus type I-infected cell lines.[15] Considering increased hsp synthesis an important part of the cell response to several infections, it is interesting to study the surface translocation of these molecules as a signal for altered/damaged cells at T-cell level.

This report aims to analyse the membrane expression of hsp 60 and hsp 70 on T cells undergoing apoptosis. Programmed cell death (PCD) is preceeded by chromatin cleavage of nucleosomes by specific endonucleases which lead to extensive DNA fragmentation into oligonucleosomal subunits and a decrease in DNA nuclei content.[16] Cell death by apoptosis is a general phenomenon occurring during embryogenesis, in the homeostatic control of organs in adults, and particularly in the elimination of self-reactive T lymphocytes in the thymus.[16] It has been recently described that apoptosis is

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Abbreviations: AO, acridine orange; DEX, dexamethasone; EB, ethidium bromide; HSP, heat-shock proteins; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PCD, programmed cell death; PI, propidium iodide.

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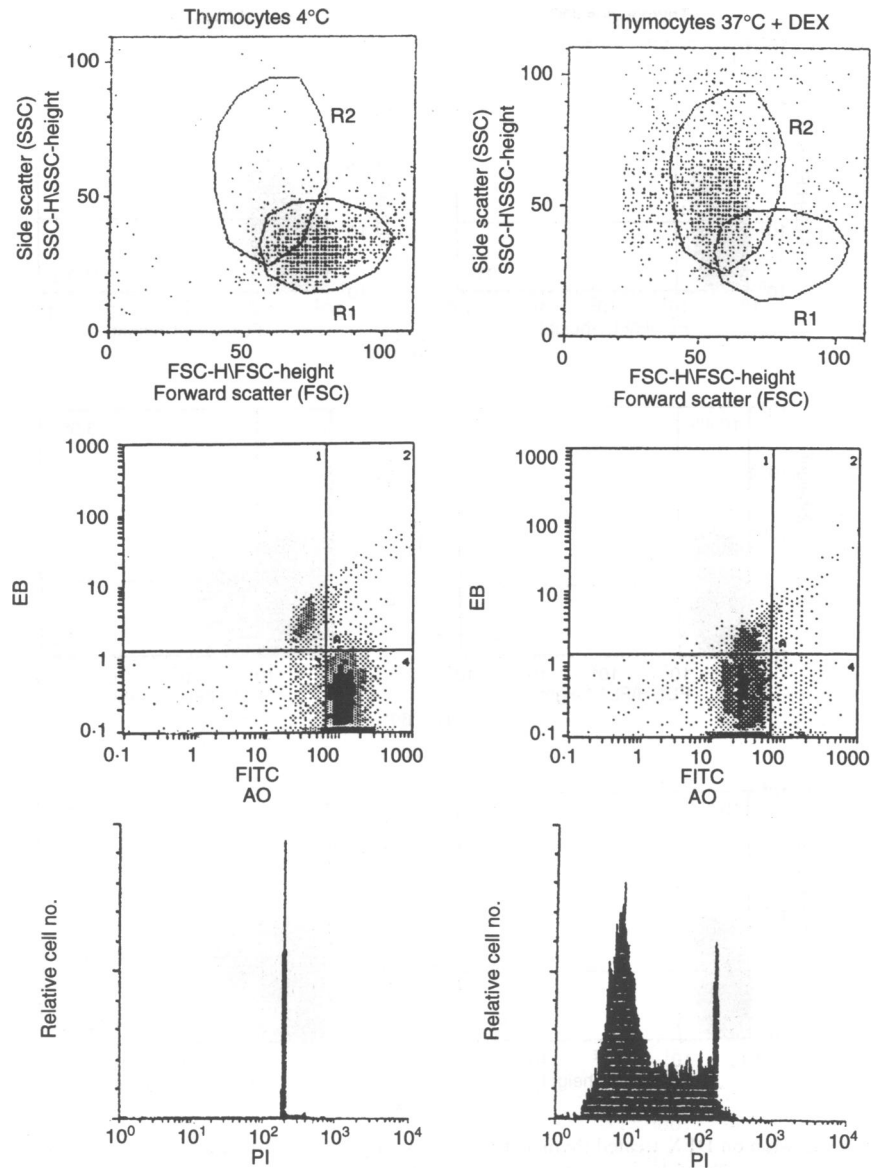


Figure 1. Cytofluorimetric analysis of DEX-treated thymocytes. This figure shows one representative experiment out of seven. Thymocytes were obtained from 8-weeks-old C57BL/6 mice. The cells were kept in complete medium (RPMI-1640 enriched with 10% heat inactivated fetal bovine serum, 1% glutamine and antibiotics) at a concentration of 10^7 cells/ml for 18 hr at 4° (left panels). To induce apoptosis, the thymocytes were cultured in the presence of 0·5 μM DEX (Sigma Chemical Company, St Louis, MI), for 18 hr at 37° (right panels). PCD (apoptosis) was quantified by double staining with ethidium bromide (EB) and acridine orange (AO). Briefly, cells were stained with EB (10 μg/ml) which enters dead cells, and AO (3 μg/ml) which requires an active transport through the cell membrane and thus stains nucleic acids of living cells (both EB and AO were purchased from Sigma Chemical Company). The fluorescence was immediately acquired on a FACScan flow cytometer and analysed with the Lysis II software (Becton-Dickinson). Apoptosis was also quantified by DNA labelling with propidium iodide (PI). This fluorescent compound is an intercalant dye that allows DNA quantification. Cells were resuspended in 1 ml of hypotonic fluorocrome solution (PI 50 μg/ml in 0·1% sodium citrate plus 0·1% Triton X-100, Sigma Chemical Company), kept at 4° in the dark overnight, and the fluorescence of nuclei was analysed by flow cytometry. The forward scatter (FSC) and side scatter (SSC) of particles were simultaneously measured and the small percentage of low fluorescent detritus was eliminated by gating. Cell viability was always controlled by trypan-blue dye exclusion.
Top panels: dot plots representing FSC versus SSC, which indicate the cell size and granulosity. Apoptotic cells are located in region 2 (R2 cells). Middle panels: dot plots showing the staining with AO, a molecule that requires an active transport to pass trough the cell membrane. Only dead cells are stained with EB. Bottom panels: analysis of DNA content by PI staining. The hypodiploid DNA content of DEX-treated thymocytes is typical for DNA fragmentation that normally occurs during apoptosis.

also induced by viral and bacterial infection.[17,18] Interestingly, heat treatment produces DNA fragmentation and cell death directly in a proportion of mouse thymocytes, while surviving cells become resistant to apoptosis.[19] Moreover, other agents able to induce the expression of hsp genes produce a temporary resistance to a subsequent challenge by other forms of stress, suggesting a role for hsp in preventing apoptosis.[19] In the present work we studied hsp expression on apoptotic cells using

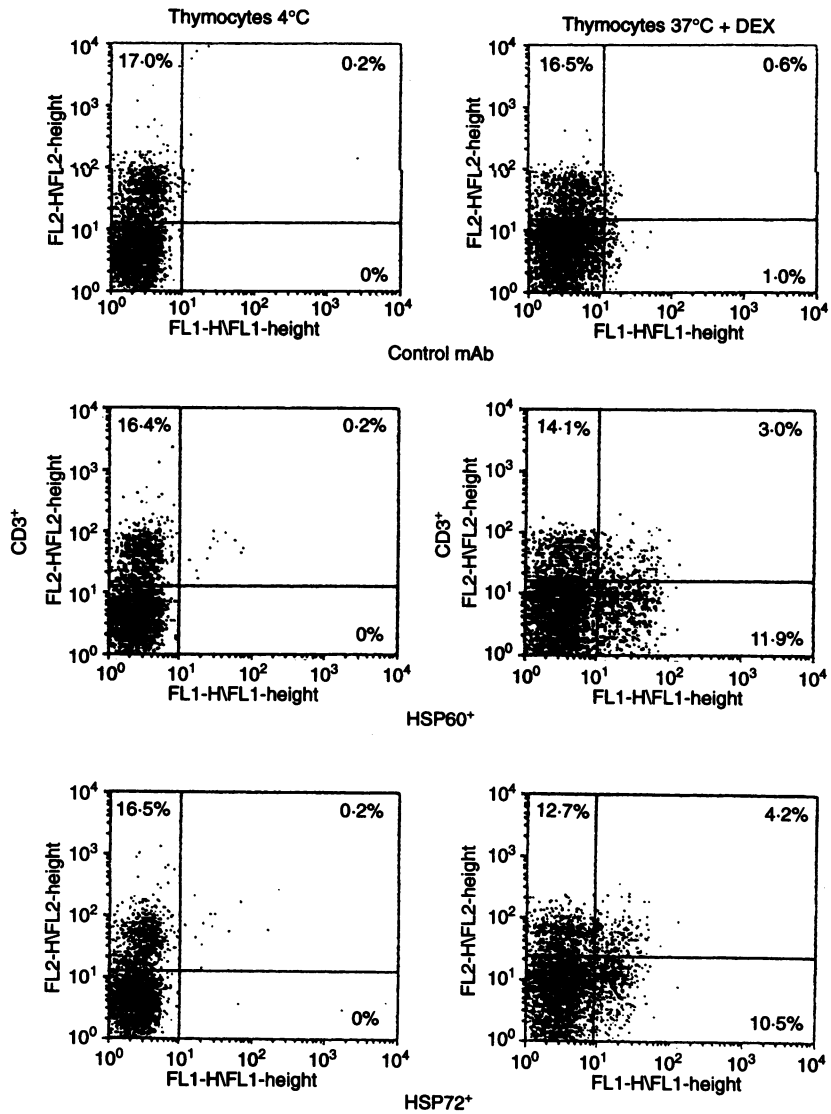


Figure 2. hsp surface expression on DEX-treated thymocytes. Control thymocytes (left panels) and DEX-treated cells (right panels) were analysed by flow cytometry. The following mAbs were used: ML30 (IgG_1) recognizes the 275–295 aa of 65 000 MW hsp from several mycobacteria species and cross-reacts with human hsp 60 (this mAb was kindly obtained from Professor J. Ivanyi). hsp 70 has been detected by a mAb specific for eucaryotic hsp 70 (IgG_1 RPN1197, Amersham, UK). An anti-measles mAb (IgG_1, Biosoft, Paris, France) was used as isotype control. As second antibody a fluoresceinated (FITC) goat anti-mouse mAb (GAM-FITC, Coulter, Miami, FL) was used. Mouse thymocytes were double stained for the expression of surface hsp and CD3 molecules. CD3 expression was detected using a mAb directly coupled to phycoerythrine (PE) (clone 145–2C11, Pharmigen, San Diego, CA). All samples were finally washed twice, fixed in 1% paraformaldehyde and immediately acquired using a flow cytometer (FACScan, Becton-Dickinson). For each sample, 10^4 viable cells were gated following size (FSC) and granularity (SSC) criteria and analysed with the Lysis II software (Becton-Dickinson).

as models dexamethasone (DEX)-treated thymocytes and *in vitro* cultured peripheral blood mononuclear cells (PBMC) from acquired immune deficiency syndrome (AIDS) patients.

DEX as well as other glucocorticosteroids can strongly induce apoptosis on thymocytes.[16,19] We characterized apoptosis on thymocytes in more detail using a combination of cytofluorimetric techniques in order to study cell morphology, membrane functionality and DNA content simultaneously. Figure 1 shows that in comparison to untreated thymocytes DEX-treated thymocytes have a reduced forward scatter (FSC) and a higher side scatter (SSC) which indicate a lower size and an increased granulosity (top panels). This observation is consistent with membrane blebbing that occurs during apoptosis. These cells share a reduced size and an increased granulosity typical for apoptosis (R2 cells, Fig. 1, top-right panel). Untreated thymocytes are able to incorporate acridine orange (AO), showing that membrane functionality is preserved, since AO represents a molecule that requires an active transport for passing through the cell membrane (Fig. 1, middle panels).[20] On the contrary, DEX-treated thymocytes failed to incorporate AO showing that the membrane functionality was significantly compromised. Moreover, the main proportion of

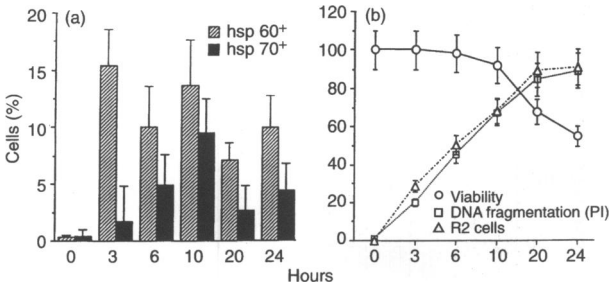


Figure 3. Kinetic of hsp surface expression on DEX-treated thymocytes. DEX-treated thymocytes were analysed by flow cytometry at different time points. Error bars represent standard deviations of three experiments. Panel A: kinetic of hsp 60 and hsp 70 surface expression on DEX-treated thymocytes. Panel B: kinetic of DNA fragmentation (PI staining), membrane blebbing (R2 cells) and cell viability (trypan-blue exclusion). All parameters were analysed as described in Figs 1 and 2.

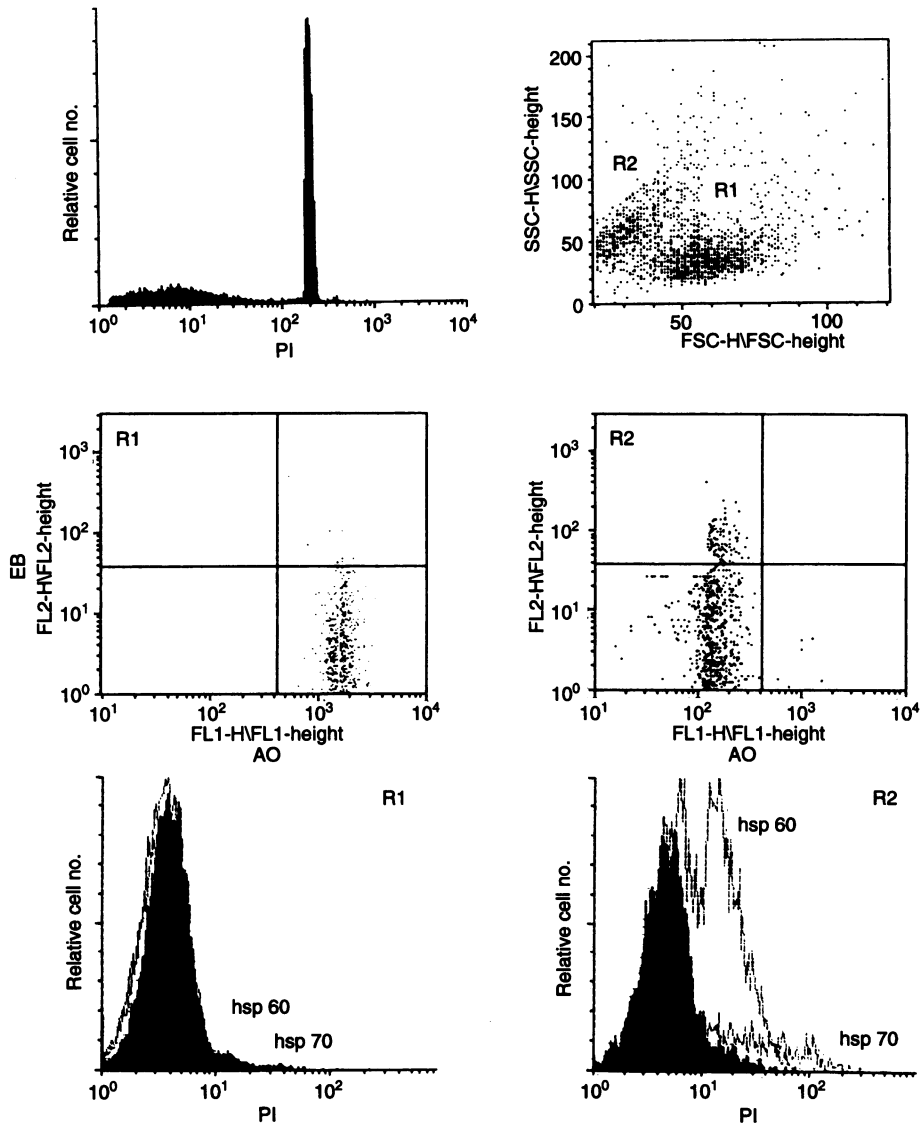
both untreated and DEX-treated thymocytes is still alive, since they are negative to the staining with EB, an intercalant dye that stains exclusively nucleic acids of dead cells (Fig. 1, middle panels).[20] The apoptotic phenotype of DEX-treated thymocytes was finally confirmed by propidium iodide (PI) staining. Fig. 1 (bottom panels) shows a large fraction of DEX-treated thymocytes (80%) with hypodiploid DNA content typical for DNA fragmentation which normally occurs during apoptosis.

Control thymocytes and DEX-treated cells were double stained with anti-mouse CD3 monoclonal antibodies (mAbs) coupled to phycoerithrine (PE), and with control or anti-hsp mAbs. The latter expression was detected by a two-step labelling with fluoresceinated (FITC) goat anti-mouse mAb. Thymocytes kept at 4° do not express surface hsp (Fig. 2, left panels). On the contrary, following 18 hr of treatment with DEX (Fig. 2, right panels), we found a discrete subset of thymocytes expressing surface hsp 60 (13% ± 2) and hsp 70 (12% ± 2). The percentage of hsp^- surface-expressing cells was always lower than the percentage of cells undergoing apoptosis (65% ± 11), suggesting that hsp expression is a transitory phase that occurs during cell degradation. Interestingly, surface hsp expression is closely related to $CD3^{low/-}$ T cells (Fig. 2, right panels), probably because this thymic subset is more prone to undergo apoptosis than mature $CD3^{high}$ T lymphocytes.[21] We analysed by flow cytometry the size/granulosity distribution of hsp^+ cells by gating on this cell population. hsp^+ DEX-treated thymocytes have a lower size (FSC) and an increased granulosity (SSC) if compared with untreated thymocytes (median FSC: 54·6 ± 2·7 versus median FSC: 75·6 ± 2·9; median SSC: 55·8 ± 1·4 versus median SSC: 29·0 ± 1·5). Interestingly, hsp^+ DEX-treated thymocytes have a smaller size and a reduced granulosity than apoptotic hsp^- DEX-treated thymocytes (median FSC: 60·6 ± 1·4 versus FSC: 54·6 ± 2·7; median SSC: 55·8 ± 1·4 versus median SSC: 83·4 ± 3·9). Both hsp 60^+ and hsp 70^+ cells show a reduced size compared with the remaining hsp^- apoptotic cells, suggesting that hsp expression is linked to membrane blebbing and cell zeiosis before fragmenting into apoptotic bodies.

We compared surface hsp expression on DEX-treated thymocytes with other parameters related to apoptosis, such as DNA fragmentation, membrane blebbing and cell viability. As shown in Fig. 3 (panel A), hsp expression is an early phenomenon that becomes evident already after 3 hr of treatment with DEX. At this time point, the percentage of hsp 60^+ cells correlates with the percentage of hypodiploid cells present in culture and with the percentage of cells displaying the apoptotic morphology of reduced size and increased granulosity (Fig. 3, panel B). This fraction of hsp 60^+ cells has proven also to be viable by trypan-blue exclusion (Fig. 3, panel B) and remained constant during all the apoptotic process, slowly decreasing only after 10 hr (Fig. 3, panel A). Thus, this cell subset may represent a step of cell degradation that normally occurs during apoptosis. Interestingly, the expression of surface hsp 70 displayed a clearly different kinetic characterized by a peak of expression after 10 hr of DEX treatment (Fig. 3, panel A), suggesting that expression of surface hsp 60 and hsp 70 occurs in consequence to distinct processes in apoptotic cells.

PCD has been widely described *ex vivo* in the course of HIV infection,[17,20] and the present analyses confirm that PBMC from AIDS patients, cultured for 24 hr in complete medium, spontaneously undergo an apoptotic process characterized by reduction of the DNA content (36% ± 15 of hypodiploid nuclei). Figure 4 shows the typical hypodiploid DNA pattern obtained by PI staining of PBMC from AIDS patients after 24 hr of culture *in vitro* (top-left panel). Moreover, apoptotic cells are always located in region 2 (R2 cells, Fig. 4, top-right panel). The staining with EB and AO (middle panels), previously described, show that R1 cells correspond to normal living cells (which incorporate AO but not EB) whereas R2 cells mainly appear as apoptotic cells (which are not stained by both AO and EB). In parallel, we observed the unusual localization of hsp on the cell surface (hsp 60: 46% ± 12 of cells; hsp 70: 25% ± 10 of cells) that is linked to PCD of PBMC from AIDS patients (R2 cells) as shown in Fig. 4, bottom panels. hsp^+ cells were mainly CD3 T cells since more than 90% of R2 cells were CD3 positive (data not shown).

This paper shows that thymocyte death chemically induced by DEX, and apoptosis occuring in HIV-1 infection, are correlated with the expression of surface hsp 60 and hsp 70. The expression of hsp on the cell membrane appears early in the course of apoptosis, with a different kinetic of expression for hsp 60 and hsp 70 respectively. Thus, the membrane translocation of hsp is not only a consequence of hsp neosynthesis and membrane alteration, but follows two distinct pathway specific for hsp 60 and hsp 70. Interestingly, it has been recently observed that some surface markers, e.g. CD4, CD8, and heat-stable antigen, are down-regulated in apoptotic thymocytes.[22] On the contrary, other markers such as T-cell receptor (TCR)-β/CD3, CD69 and CD25 are upregulated on the membrane of apoptotic thymocytes,[22] suggesting that the expression of particular molecules on the cell surface may reflect a breakdown in the intracellular trafficking leading to the accumulation of markers on the cell membrane. Our results confirm that apoptosis causes a radical alteration in the expression of molecules on the cell surface. Since it is known that hsp are normally induced in situations involving cell stress,[1] it should be considered that surface expression of these molecules may be due to the altered intracellular pathway that occurs during apoptosis. The observation that hsp membrane-expressing cells represent only a discrete subset of apoptotic cells with a smaller volume suggests that hsp^+ cells are in late stages of the



	% of cells hsp 60+	% of cells hsp 72+	% of apoptotic nuclei
Cultured PBMC from healthy donors	0·4 ± 0·2	1·0 ± 0·1	4·5 ± 2·1
Cultured PBMC from HIV patients	**46 ± 12**	**25 ± 10**	**36 ± 15**
Thymocytes at 4°C	0·2 ± 0·1	0·1 ± 0·1	3·2 ± 0·3
Thymocytes at 37°C + Dex	**13 ± 2·5**	**12 ± 1·7**	**65 ± 11**

Figure 4. hsp surface expression on human peripheral blood mononuclear cells from AIDS patients cultured *in vitro*. All panels concern AIDS patients, since no apoptosis is detectable in cells taken from healthy donors and cultured under identical conditions (as shown in the bottom table). Human peripheral blood mononuclear cells (PBMC) from healthy donors or AIDS patients were isolated by standard Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) gradient and cultured for 24 hr at a concentration of 10^6 cells/ml in complete medium before analysis. Top-left panel: apoptosis characterized by the typical hypodiploid DNA pattern obtained by PI staining (36% ± 15 of hypodiploid nuclei). Top-right panel: reduced size and an increased granulosity typical for apoptosis (R2 cells). Middle panels: the EB and AO staining show that R1 cells correspond to normal living cells (which incorporate AO but not EB) whereas R2 cells mainly appear as apoptotic cells (which are not stained by both AO and EB). Bottom panels: these histograms describe the unusual localization of hsp on the cell surface of apoptotic R2 cells. In the bottom part of the figure the mean percentage of apoptosis and hsp-expressing cells are reported. All parameters were analysed as described in Figs 1 and 2.

apoptotic process, before cell fragmentation into apoptotic bodies. However, the factors controlling the redistribution of surface hsp on apoptotic cells remain unclear. We previously described the cell surface localization of hsp on the cell membrane of transformed or infected cells[13,14,23,24] and this observation was confirmed by several groups,[25–27] but how these proteins are transported to the cell membrane is still an open question. Immunoprecipitation experiments showed that the whole molecules are expressed on the cell surface.[25,26] Since hsp are known to have strong protein-binding capacities,[1] it is reasonable to suggest that some hsp are transported to the cell surface after binding to other proteins.[28] However, lipid anchorage could not be excluded for hsp surface localization.

We observed the appearance of hsp membrane-expressing cells among apoptotic cells from PBMC of AIDS patients cultured for 24 hr in complete medium. Thus, hsp surface expression induced by HIV-1 infection[13,14] can be seen as a consequence of apoptosis. High levels of apoptosis were recently described *in vivo* in lymphoid organs from HIV-1-infected individuals.[29] Apoptosis occurring during infections may reflect the increased turnover of activated lymphoid cells and represent a natural system to restore homeostasis. Nevertheless, evidence is emerging that apoptosis of viral or bacterial infected cells may constitute a protective mechanism aiming to destroy the infected cells and to block the spread of infectious agents.[17,18] Moreover, it should be considered that hsp may modulate the antigen presentation of viral and tumour antigens, and hsp 70 directly participates in antigen processing by interacting with major histocompatibility complex (MHC) molecules.[28] It can be then hypothesized that hsp bind to cellular antigens originating from cell alteration, and surface-expressed hsp on apoptotic cells may be then involved in the processing and presentation of cell-degradation products. The expression of surface hsp 70 was recently described on peripheral monocytes, suggesting that this surface-expressed molecule could play a role in antigen presentation since anti-hsp antibodies block the proliferative response of T cells to monocytes pulsed with tetanus toxoid.[30] However, we observed that freshly elutriated monocytes lack the expression of hsp 70, whereas this expression could be detected on *in vitro* cultured monocytes from AIDS or healthy donors, and correlates with spontaneous apoptosis (data not shown). The physiological role of monocytes undergoing apoptosis in antigen presentation remains to be elucidated.

Another important consequence of the surface translocation of hsp 60 and hsp 70 on apoptotic cells could be the formation of immunocomplexes of hsp/antibodies that may be detected by both T cells and monocytes/macrophages. The immune response to microbial hsp has been postulated to represent a possible link with autoimmunity,[31] and immunization of animals with the mycobacterial hsp 65 can prevent autoimmune disorders.[31] Considering that apoptosis may generate novel packages of exogenous and self-antigens,[32] our findings may have implications on the possible role of hsp in the induction of autoimmune phenomena. Moreover, non-MHC-restricted $\gamma\delta$ T lymphocytes have been shown to recognize cell-surface-expressed hsp 60 on human lymphoma cells,[26] and hsp 70 act as an immunogenic determinant on heat-treated sarcoma cells recognized by non-MHC-restricted natural killer (NK) cells.[33] Since $\gamma\delta$ T lymphocytes strongly react to non-peptidic cell metabolites[34] as well as to hsp 60 expressed on the surface of Daudi lymphoma cells,[26] we suggest that hsp present on the surface of altered cells may bind non-peptidic cell metabolites able to stimulate the immune response of MHC-unrestricted lymphocytes. Thus, membrane hsp expression on altered cells could provide new evidence for a role of these surface molecules in both protective immune response and autoimmunity.

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