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Heat shock-enhanced T cell apoptosis with heat shock protein 70 on T cell surface in multicentric Castleman's disease

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SUMMARY

We report here that T cells from patients with multicentric Castleman's disease (MCD) are sensitive to hyperthermia. T cells from two of three patients with MCD revealed DNA ladder formation and chromatin condensation following heat shock (30 min at 41·5°C). Peripheral blood mononuclear cells (PBMC) from the same MCD patients exhibited high levels of spontaneous apoptosis after 72 h in culture and elevated apoptosis after heat shock, as evaluated by a quantitative flow cytometric assay. Heat shock protein 70 (hsp70) was detected on the cell surface of T cells in all three patients after heat shock. Furthermore, hsp70 was detected on T cells in the two MCD patients with apoptosis even in the absence of heat shock. T cells from normal samples did not show either heat-shock-induced expression of cell-surface hsp70 or apoptosis. Thus, heat shock treatment augmented hsp70 expression on the cell surface of T cells and enhanced apoptosis. Our studies suggest that hyperthermia may influence the clinical course of MCD.

Keywords heat shock hsp70 T cell apoptosis multicentric Castleman's disease

INTRODUCTION

Castleman's disease (CD), also referred to as angiofollicular lymph node hyperplasia (AFH), was first described in 1956 [1]. The disease is thought to be benign and appears most frequently as a solitary mass in the mediastinum.

Multicentric Castleman's disease (MCD), which has systemic manifestations and resembles AFH histologically, has been reported [2,3]. However, CD and MCD have recognizable clinical differences [4]. MCD may resemble acquired immunodeficiency syndrome (AIDS) in several ways [5]: first, patients with MCD face a 20–30% risk of developing malignant disease, most frequently Kaposi's sarcoma or lymphoma [3,6]; second, lymph node changes similar to that seen in MCD were described recently in a small number of AIDS patients [7,8]; third, dysimmunity similar to that seen in AIDS was observed in MCD [5]; fourth, apoptosis of T cells seen in AIDS was found in MCD [9]. Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism [10]. Oxidative stress may be a mediator of the apoptosis associated with HIV infection and this is correlated with the broad-based loss of antioxidant defences and excess production of reactive oxygen species (ROS) during disease [11]. The low levels of antioxidant render T cells in HIV infection susceptible to death by hyperthermia [12], a prevalent oxidative stress in clinical medicine [11]. We hypothesized that hyperthermia-induced T cell apoptosis may be an important feature in MCD.

Increased expression of hsp70 is believed to exert a protective effect against apoptosis [13], and cellular damage is associated with increased expression of this protein [14,15]. hsp70 expression serves as a marker of damaged cells [14], and evidence suggests that cell-surface hsp70 is upregulated by heat shock [16]. Many observations also suggest that hsp70 may have a role in antigen presentation [16–22], and thus hsp70 on the surface of T cells may be an important signal for phagocytic recognition of cells undergoing apoptosis [16,23].

We report here that heat-shock-enhanced apoptosis of T cells occurs in patients with MCD, and is associated with an increased expression of cell-surface hsp70.

PATIENTS AND METHODS

Patients

Patients with MCD were diagnosed according to lymph node pathology and clinical criteria [4]. Their main clinical and biological characteristics are summarized in Table 1. All patients were seronegative for HIV-1 and anti-human T cell lymphotrophic virus type-1 (HTLV-1) antibodies as tested by Western blot analysis. Only patient 1 had been treated and the regimen was 10 mg prednisolone per day. Controls were 20 normal healthy donors.

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Table 1. Clinical characteristics of MCD patients

Patient no.	Age/sex	Systemic manifestation	WBC ($\times 10^9/l$)	Lymph (%)	$CD4^+$ (%)	$CD8^+$ (%)	Hb (g/dl)	CRP (mg/dl)	IgG (mg/dl)
1	50/F	+	4·4	12	30	42	11·9	3·2	3400
2	55/M	+	4·3	19	52	20	6·0	6·4	6800
3	52/M	+	6·3	38	30	32	12·1	1·2	3900

Systemic manifestations included generalized lymphadenopathy and plasma cell proliferation in organs other than the lymph nodes. Lymph, Lymphocytes; Hb, haemoglobin; CRP, C-reactive protein.

Mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized peripheral blood by centrifugation through Ficoll–Hypaque. The collected cells were washed with PBS.

Isolation of T cell-rich fraction

PBMC were transferred into a small tube and mixed with a 100-fold excess of sheep erythrocytes (Japan Biological Materials Center, Tsukuba, Japan) in 1·0 ml of PBS with 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, Santamore, NSW, Australia). The suspension was incubated at 37°C for 20 min with continuous shaking; centrifuged at 180 ***g*** for 5 min, and then incubated for 1 h at 4°C without shaking. Erythrocyte rosette-forming cells were isolated by Ficoll–Hypaque gradient centrifugation. Hypotonic Tris-buffered solution (1 ml) was added to the rosette-forming cells to remove residual sheep erythrocytes. The rosette-forming cells were designated as the T cell-rich fraction and contained 85–90% $CD3^+$ cells as determined by FACS analysis.

Monoclonal antibody

CD3 (Leu-4 FITC), control antibody (IgG2a FITC; IgG1 PE) (1/10 dilution; all antibodies from Becton Dickinson, Mountain View, CA), hsp70 (1/20 dilution; monoclonal anti-heat shock protein 70 antibody (IgG1) from Affinity BioReagents, USA) and PE-conjugated rabbit anti-mouse immunoglobulin (1/50 dilution; from MBL, Japan) were used.

Cell viability

Cell viability was assessed by trypan blue dye exclusion staining, and 300 cells per sample were examined by light microscopy.

Flow cytometry

The lymphocyte phenotype was determined by two-colour analysis, using a FACScan (Becton Dickinson) [5]. PBMC were incubated for 30 min on ice with MoAb. After washing twice, the stained cells were incubated with PE-conjugated rabbit anti-mouse immunoglobulin for a further 30 min. After two additional washes, the stained cells were incubated with FITC-conjugated MoAb for 30 min. Finally washed cells ($0·5–1.0 \times 10^4$) were analysed for each MoAb and all subsequent analyses were performed on samples of the cell population within the lymphocyte window. In the control experiments, cells were incubated with isotype-matched antibodies or PE-conjugated rabbit anti-mouse immunoglobulin.

DNA fragmentation assay

DNA fragmentation was determined according to the method of Groux *et al*. [24]. PBMC (2×10^6) from MCD patients and normal controls (n=10) were cultured in 1 ml RPMI with 10% FCS for the 24 h period after heat shock (41·5°C for 30 min), and lysed in 1 ml hypotonic lysing buffer (5 mM Tris, pH 7·4, 5 mM ethylene diamine tetra-acetic acid (EDTA), 0·5% Triton X-100). The lysates were centrifuged at 12 000 ***g*** for 15 min. Supernatants were deproteinized by sequential extractions in phenol/chloroform, chloroform/iso-amyl alcohol (24:1) and finally precipitated at −20°C in 50% isopropanol/130 mM NaCl. DNA was electrophoresed in 2% agarose gels and was stained by ethidium bromide.

Electron microscopy

PBMC and the T cell-rich fraction were incubated for 24 h after heat shock. Cells were fixed in 2% glutaraldehyde in 0·1 M sodium phosphate buffer, pH 7·4, for 24 h at 4°C. After centrifugation, pellets were postfixed in 1% aqueous osmium tetroxide for 2 h, dehydrated, and embedded in Epoxy resin. Sections were stained with uranyl acetate lead citrate before examination with a Hitachi H-7100 electron microscope (Hitachi, Hitachi, Japan).

Flow cytometric method to evaluate apoptosis

A flow cytometric assay was used to quantify apoptosis according

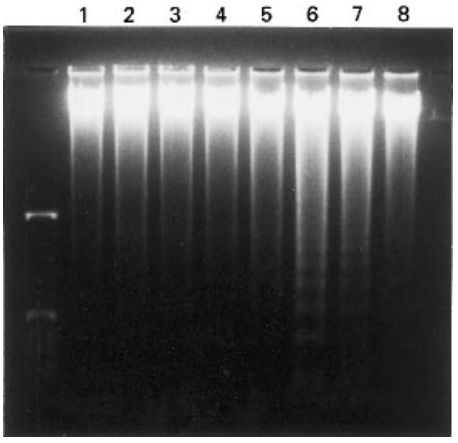


Fig. 1. Apoptosis of T cells from multicentric Castleman's disease (MCD) patients. DNA fragmentation in peripheral blood mononuclear cells (PBMC) from MCD patients and controls after 24 h incubation in the absence or presence of heat shock (30 min at 41·5°C). Control PBMC without heat shock (lane 1); patients 1, 2, and 3 without heat-shock (lanes 2–4); control PBMC with heat-shock (lane 5); patients 1, 2, and 3 with heat-shock (lanes 6–8). Lane 6 and lane 7 show a characteristic DNA fragmentation pattern in multiples of a 200 base pair unit by agarose gel electrophoresis.

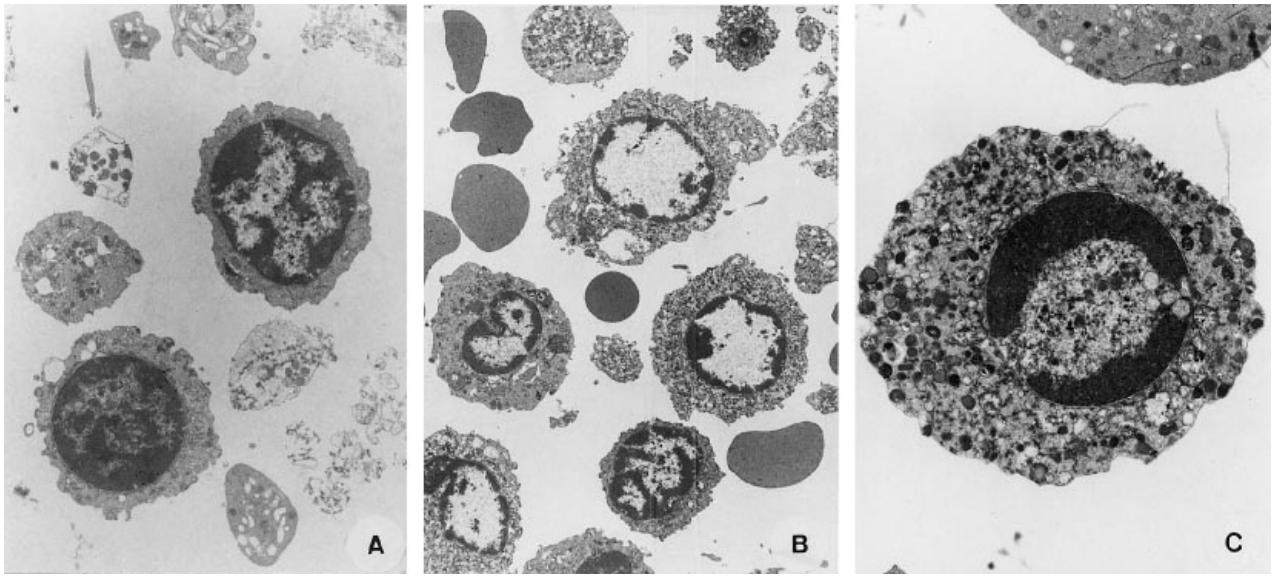


Fig. 2. Electron micrographs of peripheral blood mononuclear cells (PBMC) from (A) normal control and (B) a multicentric Castleman's disease (MCD) patient (patient 2) at 24 h incubation after heat stress (30 min at 41·5°C). In (B), the chromatin has collapsed down into cresents along the nuclear envelope, resulting in nuclear chromatin condensation. (C) An electron micrograph of a T cell-rich fraction from an MCD patient (patient 2) also showed the same findings.

to the method of McCloskey [25]. Aliquots of PBMC were exposed to heat shock (41·5°C) or control temperature (37·5°C) for 30 min. Following 72 h incubation in RPMI with 10% FCS, 1×10^6 cells were harvested and fixed in 70% EtOH for 1 h at 4°C. The cells were washed and resuspended in 0·5 ml RPMI to which 0·5 ml RNase solution (1 mg/ml) was added, followed by 1 ml propidium iodide solution (PI, 100 μg/ml). The samples were mixed gently and maintained at 4°C in the dark overnight. PI fluorescence of each sample was assayed using a FACScan machine (Becton Dickinson).

RESULTS

Apoptosis of T cells

T cells in two MCD patients (patients 1 and 2) had two features characteristic of apoptosis at 24 h after heat shock. First, gel electrophoresis of the isolated DNA showed a fragmentation pattern in multiples of a 200 base pair oligonucleosome length unit (Fig. 1). We found no DNA fragmentation in normal control PBMC (n=10). Without heat shock, DNA fragmentation was not observed in both MCD patients and normal controls. Second, electron microscopy of PBMC and the T cell-rich fraction

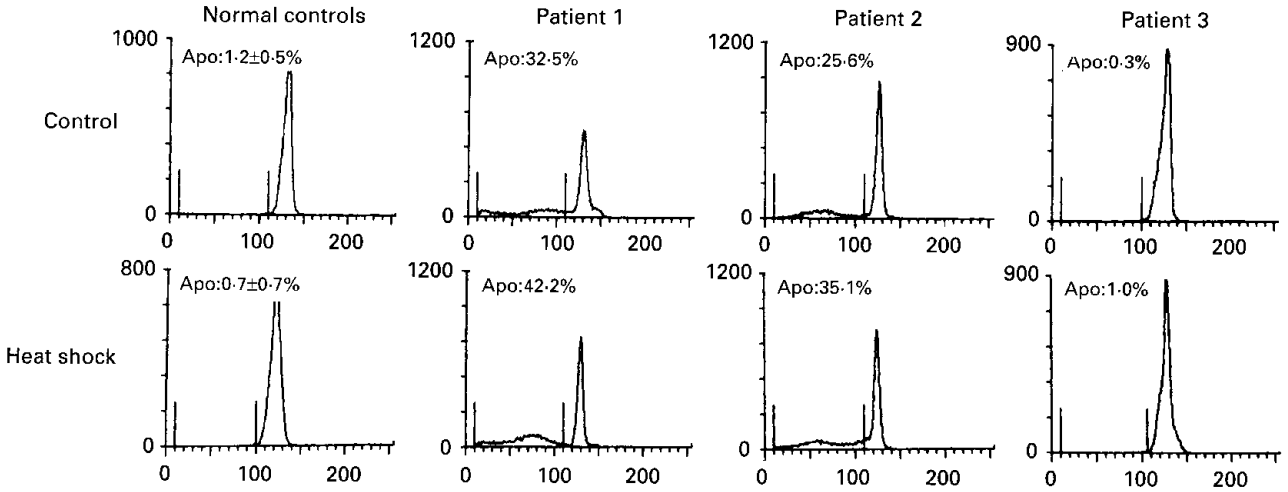
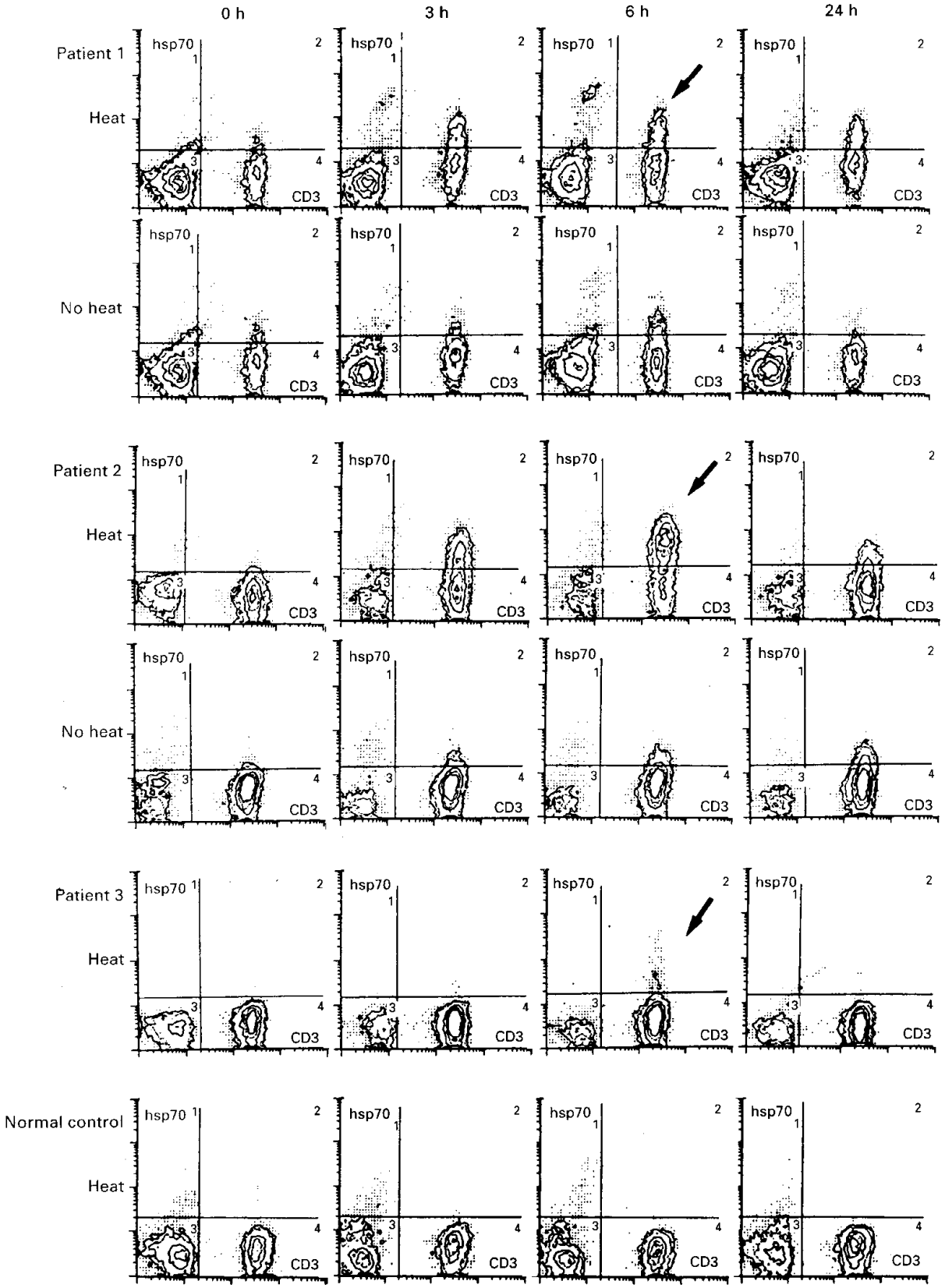


Fig. 3. Influence of heat shock on *in vitro* apoptosis of peripheral blood mononuclear cells (PBMC) from normal controls and multicentric Castleman's disease (MCD) patients. The percentage of apoptotic cells was determined after 72 h incubation after heat shock by flow cytometry using propidium iodide. PBMC from patients 1 and 2 revealed high levels of spontaneous apoptosis which were elevated by heat shock. Normal control data are expressed as the mean ± s.d. for 10 individuals. Patient 1, patient 2, and patient 3 correspond to the patient numbers in Table 1.


0 h
3 h
6 h
24 h
Patient 1
Heat
No heat
Patient 2
Heat
No heat
Patient 3
Heat
Normal control
Heat
hsp70
CD3
1
2
3
4

(Fig. 2) from the two patients revealed heat shock-induced nuclear chromatin condensation after heat shock.

Trypan blue dye exclusion demonstrated that there were >97% viable cells at the time of assay and that viability was independent of heat-shock treatment.

Quantification of apoptotic cells

Quantification of apoptosis was performed by flow cytometric analysis of PBMC from three MCD patients and controls at 72 h after heat shock (Fig. 3). Heat shock enhanced the apoptosis of PBMC from patients 1 and 2. Spontaneous apoptosis in culture without heat shock was observed for PBMC from patients 1 and 2. Spontaneous apoptosis was not observed in normal controls, and heat shock did not induce apoptosis in normal controls.

hsp70 expression on the cell surface of T cell after heat stress

To analyse the effect of heat stress on T cells, the expression of hsp70 on the cell surface of $CD3^+$ T cells from MCD patients and controls (n=20) was investigated using samples isolated at 0 h, 3 h, 6 h, and 24 h after exposure to 41·5°C for 30 min (Fig. 4). Heat-shock treatment of normal T cells could not induce the expression of surface hsp70 ($0{\cdot}3 \pm 0{\cdot}2\%$) at 6 h incubation after heat shock. These data are consistent with the lack of expression of hsp70 ($0 \pm 0\%$) on the cell surface of normal PBMC (n=14) at 4 h incubation following heat shock (41·8°C, 200 min) [16]. In the absence of heat treatment, hsp70 antigen appeared spontaneously on T cells in two MCD patients with apoptosis of T cells. We detected increased levels of hsp70 antigen on the cell surface of T cells after heat treatment in all three MCD patients. hsp70 antigen appeared on both CD4 and CD8 single positive T cells (data not shown). PE-conjugated rabbit anti-mouse immunoglobulin (secondary antibody) alone without hsp70 antibody was not detected after heat treatment on T cells in all samples.

DISCUSSION

The present study showed that hyperthermia enhanced the apoptosis of T cells in two patients with MCD and that T cells in three MCD patients expressed hsp70 through heat shock. We propose that expression of hsp70 on the T cell surface may be correlated with apoptosis of T cells.

Patients with MCD face 20–30% risk of developing malignant disease, most frequently Kaposi's sarcoma or lymphoma [3,6], and the development of malignancy may be due to profound immunological defects [4]. Atrophy of the T cell-dependent areas in the lymph nodes of MCD has already been reported [26], and data from our group show that the immunological disturbances in MCD were similar to those seen in HIV infection in Table 1 and [5]. Thus T cell dysfunction, NK cell dysfunction, polyclonal B cell activation, and dysregulation of IL-6 secretion by monocytes are features of both MCD and HIV infection [27–30]. In MCD patients, cell dysfunction also results in apoptosis [9], a shared feature of HIV infection [24,31–33].

Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism [10]. The hypothesis that oxidative stress may be a mediator of apoptosis during HIV infection is supported by the broad-based loss of antioxidant defences and excess production of reactive oxygen species (ROS) during HIV disease [11]. Low levels of antioxidant lead to rapid generation of free radicals which in turn render T cells susceptible to apoptotic death [12]. Hyperthermia may also induce cell damage [12], and concomitant increases in ROS levels. When this occurs in MCD, apoptosis with a similar increase in cell-surface hsp70 may occur, so explaining an apparent reduction of T cell numbers.

Increased expression of hsp70 is believed to exert a protective effect against apoptosis [13,34]. Cell damage is associated with increased expression of hsp70 [14,15]. We observed that heat treatment augmented hsp70 expression on the T cell surface independently of CD4 or CD8, which may be correlated with apoptosis of T cells.

Furthermore, several observations suggest that hsp70 could function in antigen presentation. First, hsps are molecular chaperones which can pass through the cell membrane and therefore can interact with the immune system [17]. Second, the hsp70 gene maps within the major histocompatibility complexes (MHC), which suggests a role in immune surveillance [18]. Third, the presence of autoantibodies against hsp70 in various autoimmune conditions has been reported, implicating an antigenic role in these disorders [19–21]. Fourth, it is reported that antibody to p53 was dependent on the complex between p53 and hsp70 [22], thus hsp70 may play an important role in the antigenic presentation of p53 [22].

Phagocyte recognition of damaged or apoptotic cells is important to protect tissues from the potentially harmful consequences of exposure to intracellular toxins. Surface-expressed hsp on stressed or human HIV-infected lymphoid cells represent the target for antibody-dependent cellular cytotoxicity [23], and therefore hsp70 may have a role in the elimination of stressed cells [23,34]. A similar mechnanism may occur in MCD, and expression of hsp70 could result in the elimination of T cells. It is clear from our findings that apoptosis can be easily induced in MCD patients by heat shock. The clinical correlate of heat shock is fever and this may affect the clinical course of MCD. Indeed, MCD patients are susceptible to fatal infections [35].

MCD is a rare disease and it is difficult to study large groups of patients. For the three patients examined here, T cells from patients 1 and 2 showed identical characteristics of spontaneous and heat shock-induced apoptosis accompanied by expression of surface hsp70, whereas cells from patient 3 closely resembled normal controls. It is interesting to note that the clinical courses for patients 1 and 2 were poor and therapy was required (patient 2 received prednisolone after the study), but patient 3 remained asymptomatic.

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Fig. 4. Effect of heat-shock on surface hsp70 of T cells in multicentric Castleman's disease (MCD) patients. hsp70 was detected in the absence of heat-shock in two of three MCD patients. hsp70 was detected in all three patients after heat-shock (see arrows). Heat-shock treatment of normal T cells (n = 20) could not induce the expression of hsp70 ($0{\cdot}3 \pm 0{\cdot}2\%$) on the cell surface at the time of 6 h incubation after heat shock. The quadrants were set to contain >98% of the isotype matched FITC and PE control antibodies. (Heat = 30 min at 41·5°C, No heat = non-heated, Pt.1, Pt.2, and Pt.3 correspond to the patient numbers of Table 1; Normal control = healthy control).

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