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Expression of heat shock protein 70 blocks thymic differentiation of T cells in transgenic mice

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SUMMARY

Heat shock protein 70 (HSP70) is involved not only in protein folding, but also in processes of differentiation and cell-cycle progression. Recently, HSP70 has been implicated in mediation of functions of some immunosuppressive agents. To study the role of HSP70 in differentiation of haematopoietic cells, we generated transgenic mice using the human inducible *hsp70* gene fused to the mouse H-2K promoter. These mice develop a T-cell deficiency that is characterized by thymic hypoplasia and a significant reduction in peripheral T cells. The total number of thymocytes is about 100-fold less than that in normal mice. The majority of the thymocytes are immature T cells that express neither CD4 nor CD8 molecules, indicating that T cells are affected at an early stage of thymic differentiation. Expression of the transgenic HSP70 was detected both in bone marrow cells and in thymocytes. Furthermore, injection of normal bone marrow cells into the T-cell deficient mice led to the generation of mature T cells indicating that the T-cell deficiency was caused by the action of HSP70 in T cells. The blockage of differentiation occurred only in T cells, both αβ- and γδ-T-cell receptor (TCR)-bearing cells, but not in B cells, granulocytes, and monocytes. The observations suggest that HSP70 may inhibit a cellular process that is essential for the differentiation of early stage T cells. Further experiments using this model system will widen our understanding of HSP70 and its function on a molecular level.

INTRODUCTION

It has been observed that the expression of heat shock proteins (HSPs) is regulated during various differentiation processes including embryogenesis, organogenesis, and haematopoiesis.[1,2] Members of the inducible heat shock protein 70 (HSP70) family, a major family of HSPs, have been examined for their role in these processes. HSP70 is expressed during differentiation processes including embryogenesis, spermatogenesis, ocular differentiation and haematopoiesis.[3–6] Although the role of HSP70 in these processes is not clear yet, some of the changes appear to be associated with alterations in the proliferative status of the cells and with cell-cycle regulation.[7,8]

Previous analysis of the role of some immunosuppressive agents suggested that members of the HSP70 family may work as immunophilins, or immunosuppressant binding proteins. Like cyclophilins and FK506 binding proteins, HSPs are ubiquitous, are involved in protein folding and trafficking, and bind exogenous drugs. Deoxyspergualin (DSG), a synthetic analogue of spergualin, exerts potent immunosuppressive effects in many models of T-cell dependent immune responses, such as antibody production after challenge with T-cell dependent antigen, delayed-type hypersensitivity, and allograft rejection.[9,10] A cellular component which bind to DSG was found to be constitutive heat shock protein 70 (HSC70).[11] It has been well documented that soluble human leucocyte antigen (HLA) class I molecules induce unresponsiveness to some allografts.[12] This observation led to the identification of HLA-derived peptides which inhibit differentiation of precursor T cells into mature cytotoxic T lymphocytes (CTLs) or lysis by CTL.[13,14] These immunosuppressive peptides were shown to bind to HSC70 and HSP70[15] It is interesting that only inhibitory peptides bind to HSC70 and HSP70 whereas non-inhibitory peptides with similar sequences do not bind. Based on these observations, it has been suggested that HSP70 may represent a new class of immunophilin.

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Abbreviations: HSP, heat shock protein; HSP70, inducible heat shock protein 70; HSC70, constitutive heat shock protein 70; LPS, lipopolysaccharide; IEL, intraepithelial lymphocytes; DN, double negative; DP, double positive; SP, single positive.

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The expression of HSPs in T cells is differentially regulated and depends upon the developmental state of the cell. When HSP synthesis after heat shock was examined in mouse thymocytes at three developmental stages, adult thymocytes, which are primarily $CD4^+$ $CD8^+$ (double positive; DP), terminated the induction of the HSP70 faster than early embryonic thymocytes, which are $CD4^-$ $CD8^-$ (double negative; DN), or mature spleen T cells, which are $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ (single positive; SP). Furthermore, DP thymocytes were more sensitive to hyperthermia than either the DN or SP thymocytes with respect to apoptotic cell death.[16] The data suggest that either the restriction or the promotion of HSP70 synthesis at a specific stage of T-cell differentiation is necessary for the proper development of functional T cells.

In order to study the role of the inducible HSP70 in thymopoiesis, separate from other members of the HSP family, we generated transgenic mice that expressed the inducible human HSP70 under the control of the mouse H-2K promoter. We used the H-2K promoter because previous transgenic studies showed that the H-2K promoter directs high level expression of transgenes in lymphoid organs.[17,18] Transgenic mice developed thymic hypoplasia and immunodeficiency due to the lack of mature T cells in the thymus and the periphery. Our transgenic mice provide a novel model system in which to study the role played by the inducible HSP70 during cellular differentiation of haematopoietic cells.

MATERIALS AND METHODS

Monoclonal antibodies

The fluoroscein isothiocyanate (FITC)- or phycoerythrin (PE)-labelled rat anti-CD4 monoclonal antibody (RM4–5), FITC- or PE-labelled rat anti-CD8 (53–6·7), PE-labelled anti-TCR-αβ (H57–597), FITC-labelled anti-TCR-γδ (GL3), anti-Thy-1 (G7), anti-H-$2K^q$ (KH114), anti-H-$2D^q$ (KH117), and anti-H-$2K^b$ (AF6–88·5) were purchased from Pharmingen (San Diego, CA). Anti-HSP70 antibody (HSP72/73) was purchased from Santa Cruz Biotechnology, Inc. FITC-labelled mouse antirat antibody and FITC-labelled rat antimouse antibody were purchased from Jackson Immunoresearch Lab. (West Grove, PA).

Construction of transgenic vector

The pH 2·3 plasmid containing human *hsp70* gene (provided by Dr R. I. Morimoto, North-western University) was digested with *Bam*HI and *Eco*RI. This 2·3 kb gene fragment with its own polyadenylation signal was cloned downstream of the mouse H-2K promoter (provided by Dr C. O. Jacob, University of Southern California).

Western blot analysis

Preparation of cell lysates and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) were done as described.[19] After SDS–PAGE, the proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) as described by the manufacturer. The membranes were then incubated in a blocking solution [5% nonfat dried milk and 0·1% Tween-20 in Tris-buffered saline (TBS)] for 1 hr, incubated for 1 hr with anti-HSP70 antibody, washed three times with 0·1% Tween-20 in TBS in 5 min interval, incubated with 0·1 μg/ml horseradish peroxidase-labelled goat antimouse immunoglobulin (Pierce, Rockford, IL), and washed again three times with 0·1% Tween-20 in TBS. Bands were visualized using ECL detection reagents (Amersham, Arlington Heights, IL) and by exposure to X-ray films.

Flow cytometric analysis

Flow cytometric analysis of lymphoid cells was performed on a FACStar^{plus} (Becton-Dickinson, Mountain View, CA). Cell staining for flow cytometric analysis was performed as described previously.[20] Briefly, cells (5×10^5) were first stained with a FITC- or PE-labelled monoclonal antibody (0·5 mg in 50 ml) against cell surface antigen, washed twice with phosphate-buffered saline (PBS) with 0·5% fetal bovine serum (FBS) and 0·1% NaN_3, and analysed with flow cytometry. Data were analysed with Lysis II program.

Generation of bone marrow chimeras

Bone marrow cells were expelled from the femurs of a 2-month-old donor mouse by injecting culture medium with a 1-ml syringe with a 26-gauge needle. Approximately 5×10^5 cells were intravenously injected into recipient mice. Transgenic mice with T-cell deficiency were injected without irradiation, while C57BL/6 mice were irradiated with a single-dose (900–1100 rads using Cs^{137}) 2 hr ahead of the injection.

RESULTS

The linearized transgenic construct (H-2K/hsp70) (Fig. 1a) was microinjected into the male pronucleus of fertilized mouse eggs derived from FVB females mated to syngeneic males. We generated five transgenic lines of mice and confirmed that they carried the transgene by Southern hybridization and polymerase chain reaction (PCR) analysis of genomic DNA taken from the tails. Expression of the transgene was detected in the brain, thymus, spleen, liver, and lung by Western analysis (Fig. 1b). Reverse transcription (RT)–PCR analysis also confirmed the expression of the transgene in the transgenic mice (data not shown). In normal mice, low-level expression of HSC70 and HSP70 was detected in almost all tissues except heart, in which no expression was detectable of either HSC70 or HSP70. Expression of the transgene was highest in the spleen. Less amount of expression of the transgene was

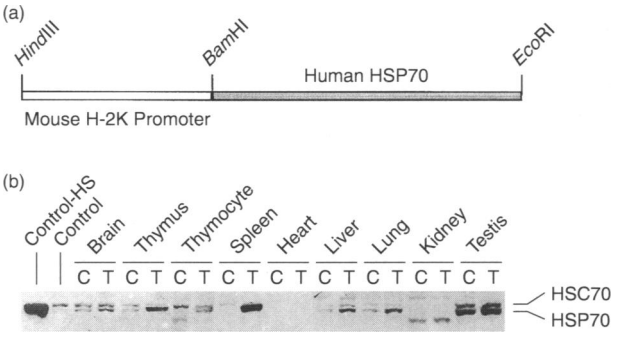

Figure 1. Partial restriction map of the transgenic vector (a) and Western analysis of the transgene expression pattern (b). Rabbit anti-HSP70 polyclonal antibody which can recognize both HSC70 and HSP70 was used. Proteins isolated from normal and heat shocked human cells were used as controls. C, control mouse; T, transgenic mouse.

detected in the thymic organ and in isolated thymocytes (Fig. 1b).

Thymic hypoplasia in association with a lack of peripheral T cells was observed in three transgenic lines (line 3, 11, and 35). These mice had only a rudimentary thymic organ (Fig. 2a,b) with 100-fold reduction in the number of thymocytes (Table 1). Histological analysis of the thymic rudiments of these mice revealed the lack of a well-developed medulla and a corticomedullary boundary and also a marked decrease in cellularity (Fig. 2c,d). In most of the transgenic mice with thymic hypoplasia, we found inflated spleens accompanied by an increase in spleen cell numbers as compared to non-transgenic mice (Table 1). Other lymphoid organs, such as Peyer's patches and mesenteric lymph nodes (LNs), were missing in these mice.

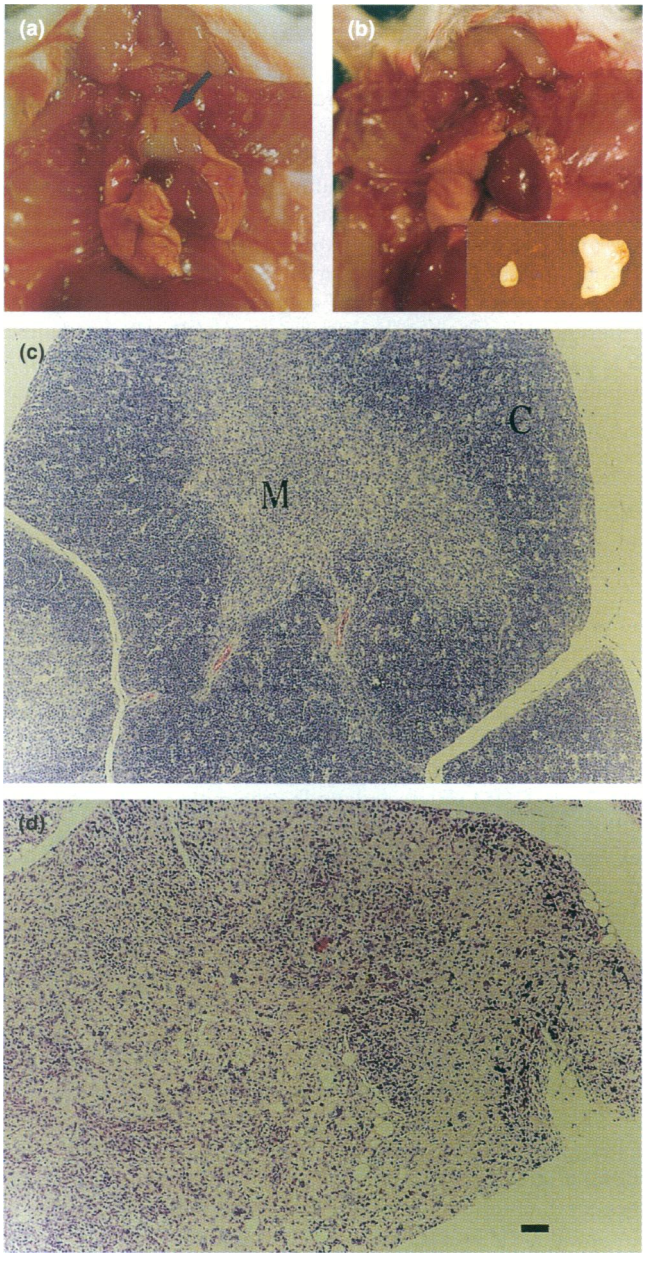

Figure 2. Gross microscopic findings in the transgenic mice with thymic hypoplasia. Photograph of intrathoracic cavity of an age- and sex-matched normal mouse (a) and a transgenic mouse with thymic hypoplasia (b). Normal thymus is indicated by an arrow in (a). Isolated thymic tissues were compared in the lower right panel in (b). Haematoxylin-eosin staining of thymus from a normal mouse (c) and a transgenic mouse with thymic hypoplasia (d). M, medulla; C, cortex. Scale bar, 125 μm.

Flow-cytometric analysis of the transgenic mice with severe thymic hypoplasia indicated that these mice had suffered a substantial reduction in phenotypically mature T cells in the thymus, spleen, and the peripheral blood. In the thymus (Fig. 3a), a great reduction in cells expressing either CD4 or CD8 was observed. Cells expressing high levels of TCR-αβ or TCR-γδ, were not detected. These data suggest that the differentiation of a majority of thymocytes was blocked at an early stage.

We then investigated the expression of other early T-cell differentiation markers including heat-stable antigen (HSA), CD44, and CD25. It has been shown that the pre-T cells, which seed the thymus at embryonic day 13, express CD44 and HSA but not CD25.[21–23] The level of expression of HSA and CD44 is high in pre-T cells and decreases as the T cells mature. The level of expression of CD25, which is not expressed in pre-T cells, briefly increases during the early stage as the pre-T cells start the thymic differentiation. Flow-cytometric analysis of thymocytes from our transgenic mice with T-cell deficiency indicated that a majority of these cells expressed high level of CD25 and HSA (Fig. 3b). This indicates that the blockage of differentiation was occurred at an early stage: after up-regulation of CD25 and before the down-modulation of CD25. The high level expression of CD44 in some of the thymocytes also indicates the early stage block in differentiation. Previous analysis of thymocytes obtained at embryonic day 14/15 had been negative for the expression of CD3, CD4 and CD8 but positive for the expression of CD25, CD44 and HSA.[24] These data suggest that the expression of HSP70 resulted in the accumulation of cells that retained the phenotypic characteristics of early stage T cells in the thymus.

We analysed the splenic B-cell population in the transgenic mice with T-cell deficiency to find out whether expression of HSP70 affected differentiation of B cells. The level of expression of CD45R (B220) and surface immunoglobulin M (IgM) was normal, suggesting that differentiation of B cells is not affected in these mice (Fig. 4). This suggests that the expression of HSP70 does not have a significant effect on the differentiation of B cells. In the peripheral blood, the concentration of white blood cells (WBCs) was reduced to 40% of that of normal blood and the ratio of lymphocytes to granulocytes was significantly decreased in the transgenic mice with T-cell deficiency (1·2 : 1) as compared to normal mice (2·5 : 1).

We generated bone marrow chimeras in an effort to find out whether the defect that caused the blockage of T-cell differentiation lay within the T cell or the non-T-cell compartment. We tested the transgenic mice with T-cell deficiency 2 months after injection of bone marrow cells derived from C57BL/6 mice (H-2^b haplotype) along with non-transgenic litter mates which were irradiated and then injected with bone marrow cells derived from C57BL/6 mice. Both the transgenic mice with T-cell deficiency and the non-transgenic litter mate supported the differentiation of thymocytes (Fig. 5). The presence of H-$2K^{b+}$ cells in $CD4^+$ and/or $CD8^+$ population indicates that the donor bone marrow cells had developed

Table 1. Cell count and pathological descriptions of the transgenic mice with T-cell deficiency. Cell numbers were counted from 6 mice each. Age-and sex-matched normal mice were used

Mice	Age*	Cell number		Pathology
		Thymocytes	Splenocytes	
Normal	4–6	184·7 (12·9)†	107·7 (33·1)	–
Transgenic	4–6	1·5 (0·8)	165·3 (75·3)	Thymic hypoplasia, T-cell deficiency

*Age in weeks after birth, †cell numbers ($\times 10^6$). Numbers in parenthesis are standard deviations.

successfully into SP thymocytes. Most of the DN cells did not express H-2K^b molecule suggesting that they were residual DN cells of the recipient mouse. Injection of bone marrow cells isolated from the transgenic mice with T-cell deficiency into a sublethally irradiated C57BL/6 mouse resulted in no mature T cells in the thymus as expected (data not shown). The analyses of the bone marrow chimeras therefore indicate that the cause of the blockage of T-cell differentiation in the transgenic mice lay in a defect in the T cells.

Because the differentiation block in the transgenic mice with T-cell deficiency appears to have occurred at an early stage in T-cell differentiation, it seemed possible that the expression of the transgene began at a prethymic stage. To test this hypothesis, we analysed the expression of HSP70 in the bone marrow where pre-T cells originate. Bone marrow cells isolated from normal mice did not express any detectable level of HSP70. Bone marrow cells isolated from the transgenic mice expressed high level of HSP70 (Fig. 6). The data suggest that the T-cell deficiency and high level expression of HSP70 in the bone marrow are related.

DISCUSSION

The physiological relevance of the expression of HSP70 in the thymus may be found in Selye's theory on stress response. Selye found that in response to different stress inducers (e.g. cold, heat, restraining immobilization, toxic chemicals, severe infections) the body reacts with a characteristic stress syndrome encompassing adrenal enlargement, gastrointestinal ulcer, and thymic involution.[28] Incubation of thymocytes at 43° for 20 min induced DNA fragmentation and cell death with a concomitant increase in the level of HSPs.[29] Furthermore, non-lethal heat treatment (42° for 20 min with a 1-hr recovery) of mice results in reduction of the number of thymocytes by half with marked reduction in CD4/CD8 double positive thymocytes (our unpublished observation). As HSP70 is one of the major molecule induced under stress conditions, it is likely that HSP70 is involved in mediating the responses to heat shock or stress.

Expression of HSP70 was detected in the bone marrow, thymus, spleen, liver and lung (Figs 1 and 6). We did not detect any apparent abnormality in other organs except lymphoid organs. Detailed analysis detected the expression of HSP70 in various haematopoietic cells, but differentiation of the cells other than T cells seemed not to be blocked in the transgenic mice with T-cell deficiency. The occurrence of T-cell deficiency in three independent transgenic mice lines was strong evidence that the pathology was a consequence of the presence and expression of the transgene rather than the result of same insertional event.

The HSP70 transgenic mice that have T-cell deficiency died within 4 months after birth. Autopsies revealed that these mice had suffered severe hypervascularization and inflammation in the abdominal cavity. These abnormalities may be related to chronic intestinal inflammation as found in interleukin (IL)-2 or IL-10 knockout mice and T-cell receptor mutant mice.[30–32] It has also been found that the presence of B cells is required for the development of the pathology. Perturbation of the immune system in our transgenic mice, which are deficient in T cells but do have B cells, can thus be expected to cause similar pathology in the intestinal track. The increase in the number of B cells in the spleen could be the result of inflammatory changes in B cells in the intestinal track.

The development of T-cell deficiency appear to occur during the embryonic development of the mice since histological analysis of the thymic organ and flow cytometric analysis of the thymocytes isolated from newborn mice were essentially same as the adult mice. During the mouse embryogenesis, the about one-hundred pre-T cells that seed the thymus each day undergo a 10^5-fold expansion over a 2-week period before they go through the thymic selection process.[33] In day 14/15 embryos, pre-T cells in the fetal thymus express IL-2, CD25 (IL-2Rα), CD54 (intercellular adhesion molecule-1; ICAM-1), CD59 (Ly-6A/E), HSA, and CD44. These molecules are also expressed when peripheral T cells are activated.[24] Expression of these molecules in the fetal thymocytes indicate that the mechanism of activation of immature thymocytes and mature thymocytes may similar in spite of the lack of TCR complex in immature thymocytes. Thymocytes from our transgenic mice with T-cell deficiency appear to be stopped at a point before the clonal expansion, since the total number of thymocytes is 100-fold less than normal thymocytes and phenotypic characteristic of these cells remained similar to those of day 14/15 thymocytes (our unpublished observation). The expression of HSP70 in the bone marrow cells of the transgenic mice suggest that the effect of HSP70 expression started in pre-T-cell stage of T-cell differentiation in the bone marrow.

What is the mechanism responsible for the HSP70 mediated block of T-cell differentiation? HSP70 may interact with and interfere with the signalling event which is responsible for the clonal expansion of early stage thymocytes. Block of the clonal expansion of early stage thymocytes will lead to a block of the further differentiation process. As stated before, HSP70 may function as an immunophollin.[9–14] Grp-78, a member of HSP70 family, was shown to be associated with PP1γ2, a testis-specific protein serine/threonine-phosphatase type 1

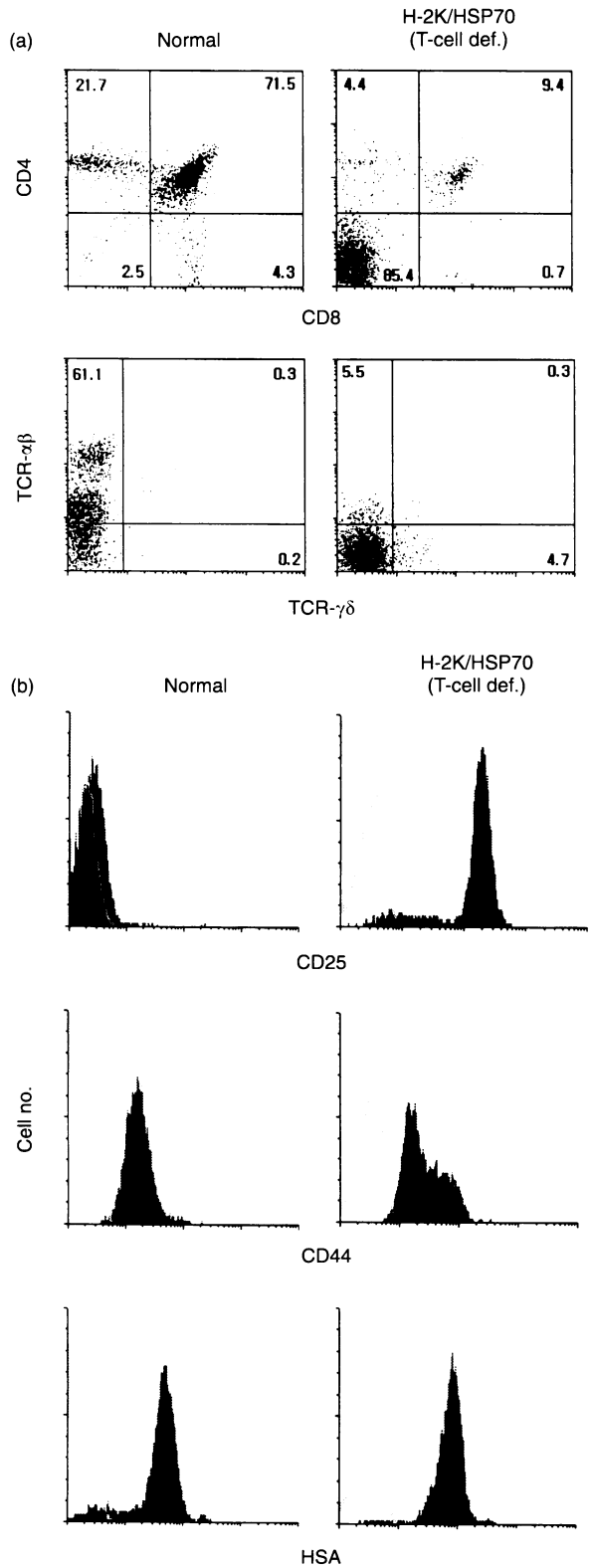

Figure 3. Flow-cytometric analysis of thymocytes isolated from a normal and a transgenic mouse. Results obtained from the two-color analysis (CD4/CD8 and TCR-αβ/TCR-γδ) are presented in dot plots (a) and results obtained from the single-colour analysis (CD25, CD44, and HSA) are presented in histograms (b), Each histogram (filled area) is superimposed on the background level of fluorescence (empty area). Data shown are representative of six independent experiments.

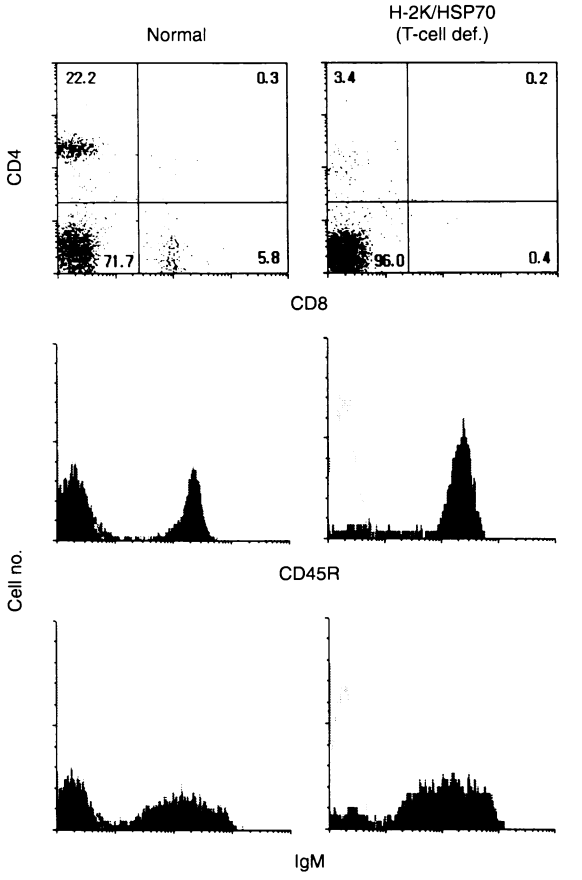

Figure 4. Flow-cytometric analysis of spleen cells isolated from a normal and a transgenic mouse. For the two-colour analysis (CD4/CD8), the results are presented in dot plots and for the single-colour analysis (CD45R and surface IgM), each histogram (filled area) is superimposed on the background level of fluorescence (empty area). Data shown are representative of six independent experiments.

catalytic subunit.[31] In rabbit reticulocyte lysate system, addition of purified HSP70 activates protein phosphatases that lead to a general decrease in protein phosphorylation.[35] These observations, in addition to our observations, suggest that HSP70 may interact with and inhibit the function of a signalling molecule(s) which is important for activation of early stage T cells and mature thymocytes. Identification of the molecule(s) which interact with HSP70 in the thymocytes will be beneficial in understanding of the molecular events that occur during T-cell differentiation and activation.

It is interesting that only T-cell, but not B-cell, differentiation is blocked in the HSP70 transgenic mice. So we tested other haematopoietic processes including erythropoiesis. In the bone marrow, the overall number of bone marrow cells was decreased by half and some minor alterations in the distributions of granulocytic- and monocytic-lineage cell populations were detected in the bone marrow and spleen cells (data not shown). We are currently investigating these cell populations to see whether these changes were caused by the expression of the HSP70 during haematopoietic processes or by the lack of functional T cells. Preliminary investigations on erythropoiesis such as reticulocyte count in the peripheral

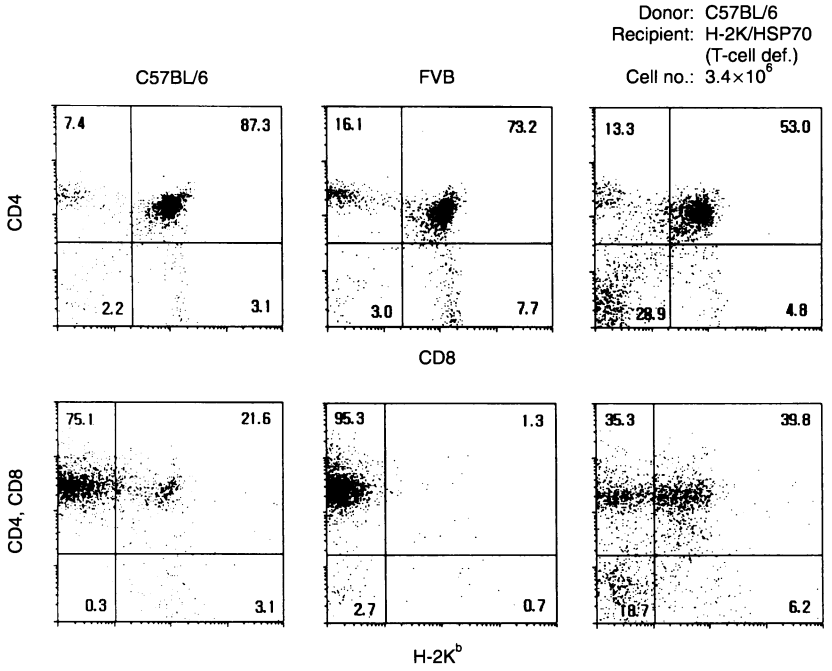

Figure 5. Flow cytometric analysis of the bone marrow chimera. Thymocytes isolated from a normal FVB, a normal C57BL/6J, and a chimeric mouse (a T-cell deficient transgenic mouse injected with C57BL/6J bone marrow cells) were stained as indicated. Number of total thymocytes is shown in the chimeric mouse. The expression of the normal H-2K molecule has been shown to be high in both pre-T cells and mature T cells. But the majority of immature T cells express low or no H-2K.[25–27]

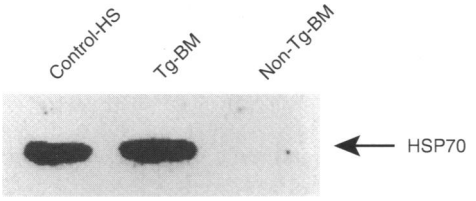

Figure 6. Western analysis of bone marrow cells isolated from a transgenic mouse and a non-transgenic litter mate. Proteins isolated from a heat-shocked human cell line was used as a control. An anti-HSP70 monoclonal antibody which can recognize only HSP70 was used. Data shown are representative of three independent experiments. ¤

blood failed to show any difference between the transgenic and control mice.

Our transgenic mice provide a unique model system in which we can access cellular events occurring during the differentiation of early stage T cells and the activation of mature T cells. Further experiments using this model system will widen our understanding of HSP70 and its function on a molecular level.

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