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Original Article

Expression of Heat Shock Protein 60 in Normal and Neoplastic Human Lymphoid Tissues

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A molecular chaperonin in mammals, heat shock protein 60 (HSP60) is constitutively expressed in the mitochondria at a low level and is rapidly up-regulated under stress. However, the role of HSP60 in the lymphoid tissues has not been well clarified. In the present study, expression of HSP60 was examined by flow cytometry and immunohistochemistry in normal peripheral blood mononuclear cells, reactive lymphoid tissues, and malignant lymphomas. HSP60 was found to be present constitutively at low levels in a fraction of resting T cells and most monocytes. The blastic change upon mitogen stimulation induced HSP60 at much higher levels in more T, B and NK cells. In normal lymphoid tissues, HSP60 was expressed preferentially in the cytoplasm of large-sized lymphoid cells and macrophages in the germinal centers and the interfollicular area.

In non-Hodgkin lymphomas strong expression of HSP60 was detected in most cases of diffuse large B-cell lymphoma, follicular lymphoma, and grade 3 and NK/T cell lymphoma. No immunostaining was observed in low grade B-cell lymphomas, including follicular lymphoma, grade 1 and B-lymphoblastic lymphomas. HSP60 immunoreactivity was variable in T-cell lymphomas. Intense expression of HSP60 was observed in Reed-Sternberg cells in all cases of Hodgkin lymphoma.
Key words HSP60, malignant lymphoma, immunohistochemistry, flow cytometry

INTRODUCTION

Heat-shock proteins (HSP) are a family of highly-conserved proteins found in all prokaryotic and eukaryotic cells. HSP synthesis increases in response to various environmental stresses, such as heat shock, inflammation, infection, irradiation, anoxia, and exposure to metals[1–9]. In addition, HSP may be constitutively expressed at low levels in normal cells[6]. Functionally, HSP act as molecular chaperones that bind non-native states of other proteins and assist them in assuming a functional conformation[10–14]; HSP facilitate folding and assembly or unfolding and disassembly as well as translocation of polypeptides, and they structurally stabilize newly synthesized polypeptides.

Different classes of HSP have been identified, the major classes being the HSP70 family, the HSP60 or GroEL chaperonin family, and the HSP90 family[10,11,14]. In contrast to the postulated unfolding and disassembly role of most forms of HSP70, the members of the HSP60 family, such as GroEL of *Escherichia coli* and the 65 Kd immunodominant antigen of *Mycobacterium spp* are thought to participate in the folding and assembly of oligomeric protein complexes[10–15]. The HSP60 family has been referred to as chaperonins from their ubiquitous occurrence in chloroplasts, mitochondria, and bacteria and functions that meet the criteria suggested for molecular chaperones[15]. The HSP60 proteins are abundant in the cell and are highly conserved between bacteria and man, with 50%–60% sequence homology[16,17]. Mammalian HSP60 is

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constitutively expressed in the mitochondria at a low level and rapidly up-regulated under stresses, such as heat shock, and on some occasions with changes in intracellular location including expression on the cell surface[18,19].

Over expression of HSP, including HSP60, has been demonstrated in several human tumor cells[20-22], with unusual expression patterns sometimes accompanied by cell membrane expression[23,24]. In the present study, we examined expression of HSP60 in normal peripheral blood lymphocytes, reactive lymphoid tissues, and malignant lymphomas. Strong expression of HSP60 demonstrated in Hodgkin's Reed-Sternberg cells was in accord with a previous report[22]. HSP60 immunoreactivity was also noted in some specific types of non-Hodgkin lymphomas.

Table 1. Expression of HSP60 in various malignant lymphomas

Type of lymphoma	Total No. of cases	No. of HSP60-positive cases(%)
B cell lymphoma	63	15(23.8)
Diffuse large	9	6(66.7)
Follicular grade 1	9	0(0.0)
grade 2	12	5(41.7)
grade 3	5	4(80.0)
Lymphoblastic	5	0(0.0)
BCLL/SLL	5	0(0.0)
Lymphoplasmacytic	2	0(0.0)
Mantle cell	6	0(0.0)
MALT	10	0(0.0)
T cell lymphoma	29	13(44.8)
Peripheral T(NOS)	8	2(25.0)
Angioimmunoblastic	3	2(66.7)
Lymphoblastic	9	2(22.2)
Adult T cell leukemia /lymphoma	9	7(77.8)
NK/T cell lymphoma	7	6(85.7)
Hodgkin lymphoma	10	10(100)
Nodular sclerosis	5	5(100)
Mixed cellularity	5	5(100)

MATERIALS AND METHODS

Cells

Peripheral blood mononuclear cells (PBMC) were obtained from the blood of 5 healthy Japanese persons by Ficoll-Hypaque density-gradient centrifugation using a commercial lymphocyte separation medium. PBMC were incubated in RPMI 1640 containing 10% fetal calf serum (FCS), 0.1% pokeweed mitogen (PWM) and 20 u/ml interleukin-2 (IL-2) for 72 h at 37°C in a CO_2 incubator and used as the source of lymphoblasts. Tonsillar lymphocytes were obtained from 3 patients with chronic tonsillitis. The resected tonsils were immersed in chilled RPMI 1640 medium containing 10% FCS. A single-cell suspension of lymphocytes was prepared by gentle dissection, dispersion, and passage through a fine nylon mesh. Dead epithelial cells and cell debris were removed by Ficoll density gradient centrifugation. Informed consent for the investigation was obtained from everyone examined.

Lymphoma and reactive lymphoid tissues

Formalin-fixed, paraffin-embedded tissues from 109 patients with malignant lymphomas were studied for expression of HSP60 in lymphoma cells. The cases examined included 99 with non-Hodgkin lymphomas of varied origin and 10 with Hodgkin lymphomas (Table 1). These lymphomas were diagnosed according to the WHO classification[25]. In addition, 8 samples of simple reactive lymphadenitis and 7 samples of chronic tonsillitis were used as reactive lymphoid tissues.

Immunohistochemistry

The indirect immunoperoxidase method was used for immunolocalization of HSP60 in tissue sections. Briefly, the dewaxed sections were treated with normal rabbit serum or 10% skim milk and then incubated overnight with anti-human HSP60 goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. After being washed with phosphate-buffered saline (PBS), the specimens were treated with horseradish peroxidase (HRP)-labeled secondary antibody for 30 min at room temperature and then incubated in a diaminobenzidine-H_2O_2 solution for color development.

To double immunostain the sections they were incubated with anti-pan-T cell (UCHL-1, CD45RO), -pan-B cell (L26, CD20), or -macrophage (CD68) mouse monoclonal antibodies (Mabs) (DAKO, Japan, Kyoto) for 45 min at room temperature and then treated with dextran polymer-conjugated secondary antibody labeled with peroxidase (Envision$^+$, DAKO

Japan) for 30 min. After color development with diaminobenzidine and counterstaining with methyl green, the slides were washed with 100 mM glycin-HCl buffer (PH 2.2) 3 times for 30 min. Then they were incubated with goat anti-human HSP60 antibody. Alkaline phosphatase-labeled anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody, and the reaction products were visualized with BCIP/NBT (DAKO Japan).

Flow cytometric analysis

Dual-color flow cytometric analysis was performed using anti-human HSP60 goat antibody (Santa Cruz Biotechnology) and phycoerythrin (PE)-conjugated anti-T (CD3), B (CD20), and NK (CD56) cells (Becton Dickinson, San Jose, CA, USA) or monocytes (CD14) (Beckman Coulter, Fullerton, CA, USA) mouse Mab. In the first step, 5×10^4 cells were incubated with PE-conjugated anti-CD3, CD20, CD56 or CD14 Mab for 30 min. In the second step, the samples were fixed in 2% paraformaldehyde solution for 12 min and made permeable with 0.1% saponin diluted in PBS for 5 min. The treated samples were incubated with goat anti-human HSP60 antibody for 35 min, followed by fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat IgG antibody for 30 min. All the procedures were performed on ice. The stained cells were analyzed with a FACScan flow cytometer (Beckton Dickinson). Negative control cells were stained with PE-conjugated mouse IgG and FITC-conjugated goat serum instead of each antibody.

Western blot

Recombinant human HSP60 (Stressgen Biotechnologies, Victoria, BC, Canada), E. coli GroEL (Stressgen Biotechnologies) and *H. pylori* HSP60[26] were dissolved in sodium dodecyl sulphate (SDS) containing 5% 2-mercaptoethanol and separated by SDS/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA, USA). After blocking with PBS containing 5% skim milk for 1 h, the membranes were serially incubated with goat anti-human HSP60 for 50 min and then with HRP-labeled secondary antibody for 45 min. After washing with 0.1% PBS-Tween 20 buffer, the blots were detected using an ECL Western blotting detection system (Amersham Japan, Tokyo). All the procedures were performed at room temperature.

RESULTS

Specificity of anti-HSP60 antibody

As shown in Fig. 1, anti-human HSP60 goat antibody reacted with the recombinant human HSP60 and *E. coli* GroEL, but did not cross react with *H. pylori* HSP60.

Expression of HSP60 in normal resting lymphocytes, monocytes, and lymphoblasts

Flow cytometric analysis showed that a part of PBMC expressed HSP60 at low levels in all healthy persons, although the percentage of HSP-positive cells was variable, ranging from 8.12% to 24.21% (Table 2). In an unstimulated state most of these HSP60-positive cells were monocytes. A fraction of T cells also expressed HSP60 in 3 of 5 cases. Lymphoblasts induced by mitogen stimulation expressed HSP60 at higher levels and proportions than unstimulated, small lymphocytes (Figs. 2 and 3, Table 2). Augmentation of HSP60 expression was observed in not only T cells, but also B and NK cells (Fig. 3, Table 2). CD56-positive lymphocytes may include NK T cells. However, NK T cells represented only a small fraction of PBMC, and

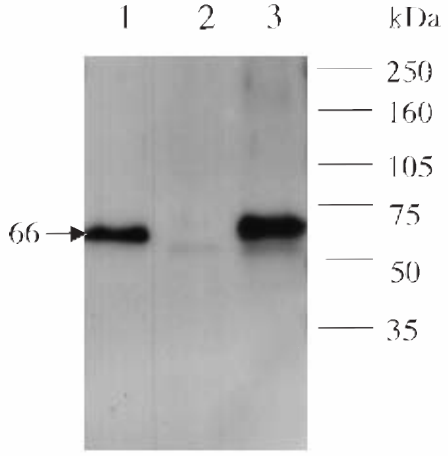

Fig. 1. Western blot analysis of HSP60 from various sources with polyclonal anti-human HSP60 antibody. Lane 1, *E. coli* GroEL; lane 2, *H. pylori* HSP60; lane 3, recombinant human HSP60.

Table 2. Expression of HSP60 in resting and mitogen-stimulated peripheral blood mononuclear cells

	HSP60-positive cells in each fraction(%)								
	Unstimulated PBMCs					Mitogen-stimulated PBL blast			
Case	Whole	T	B	NK	M	Whole	T	B	NK
1	8.12	0.46	0.20	0.06	7.08	70.09	36.49	10.29	13.11
2	9.29	0.66	0.48	0.04	6.55	52.95	22.47	4.72	3.94
3	12.29	5.39	0.03	0.01	3.63	51.29	14.58	3.94	1.99
4	22.35	3.45	0.31	0.31	19.05	69.91	20.29	1.35	1.47
5	24.21	7.13	2.34	1.73	12.26	70.97	30.04	6.47	4.09

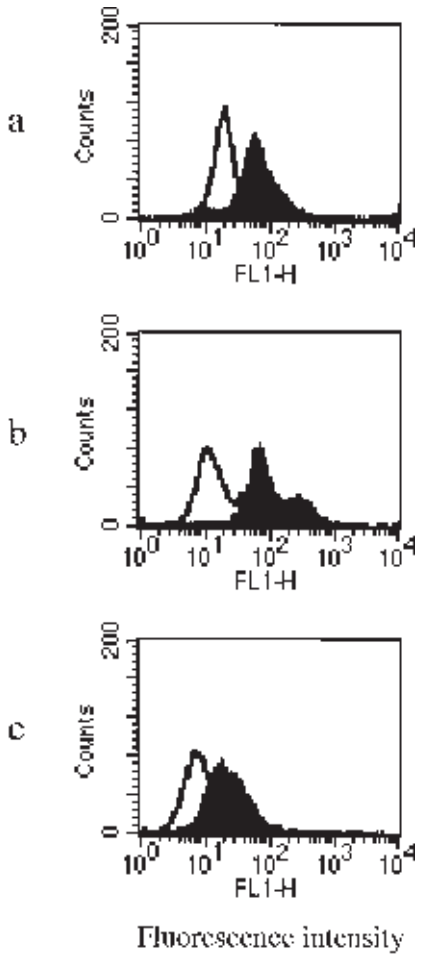

Fig. 2. Expression of HSP60 in unstimulated (a) and mitogen-stimulated (b) PBMC from Case 3 and tonsilar lymphocytes (c).

most of the CD56-positive lymphocytes were thought to be NK cells. Tonsillar lymphocytes were used as a model for tissue lymphocytes. The expression pattern of HSP60 in tonsillar lymphocytes was similar to that in PBMC (Fig. 2) ; HSP60 was expressed at a higher level in the large-sized cells than small lymphocytes.

Distribution of HSP60 in reactive lymphoid tissues

Expression of HSP60 was examined in human reactive lymph nodes and tonsils using the indirect immunoperoxidase method. Intense HSP60 staining was detected in the cytoplasm of large-sized lymphoid cells and macrophages located in germinal centers and in the interfollicular area (Fig. 4a). Staining was never observed in mantle zone lymphocytes and only rarely in small lymphocytes in other areas. Double immunostaining of HSP60 with CD45RO, CD20, or CD68 revealed that some T cells, B cells or macrophages expressed HSP60 (data not shown).

Expression of HSP60 in various lymphomas

A total of 109 lymphoma specimens of different types were examined for expression of HSP60 (Table 1, Fig. 4). Among the B-cell lymphomas, strong expression of HSP60 was detected in 6 of 9 cases of diffuse large B-cell lymphoma and in most cases of follicular lymphoma, grade 3. In about 40% of the cases of follicular lymphoma, grade 2, HSP60 was expressed in centroblastic large-sized neoplastic cells. No immunostaining was observed in low-grade B-cell lymphomas including follicular lymphoma, grade 1. Precursor B-lymphoblastic lymphoma cells were also negative for HSP60. In most cases of NK/T cell lymphomas, the neoplastic cells were also positive for HSP60. HSP60 immunoreactivity was variable in T-cell lymphomas. The most striking finding was intense cytoplasmic expression of HSP60 in Hodgkin and Reed-Sternberg (H-RS) cells among all cases of Hodgkin lymphoma.

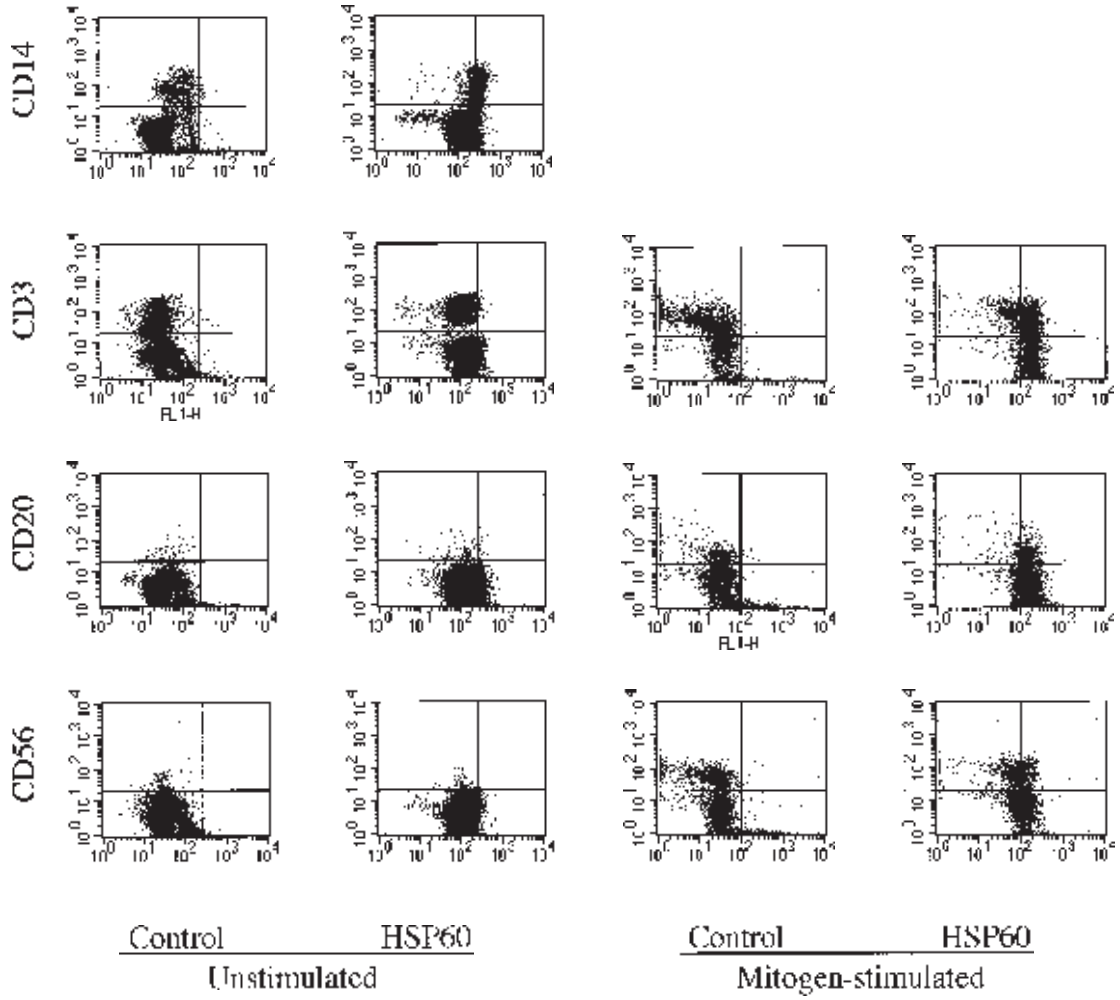

Fig. 3. Expression of HSP60 in unstimulated and mitogen-stimulated PBMC from Case 1. Control, reacted with control goat serum; HSP60, reacted with goat anti-human HSP60 antibody.

DISCUSSION

In this study, we demonstrated the distribution of HSP60 in PBMC, reactive lymphoid tissues, and various types of lymphomas. HSP60 was found to be present constitutively at low levels in the monocytes of all the healthy persons examined and also in a fraction of T cells in some persons. This implies that HSP60 may play some essential roles in maintaining cell function in these cells. The HSP60-positive healthy persons may have been under subclinical stress including microbial or viral infection. Mitogen-induced lymphoblasts expressed HSP60 at a much higher level than small lymphocytes, which did not show a blastic change upon mitogen stimulation. This result was comparable to previous reports on HSP70 and HSP90[27,28]

In normal lymphoid tissues, HSP60 was preferentially expressed in large-sized lymphocytes, such as centroblasts and immunoblasts and some macrophages, which contradicted a previous study that showed ubiquitous weak expression of HSP60 in lymphoid cells, histiocytes, and dendritic cells[22]. This discrepancy may be due to differences in the antibodies used and the staining methods. We used the polyclonal anti-HSP60 antibody and the indirect immunoperoxidase method. In contrast, Hsu and Hsu used a monoclonal antibody and the ABC method[22].

In malignant lymphomas, intense HSP60 immunostaining was invariably detected in the

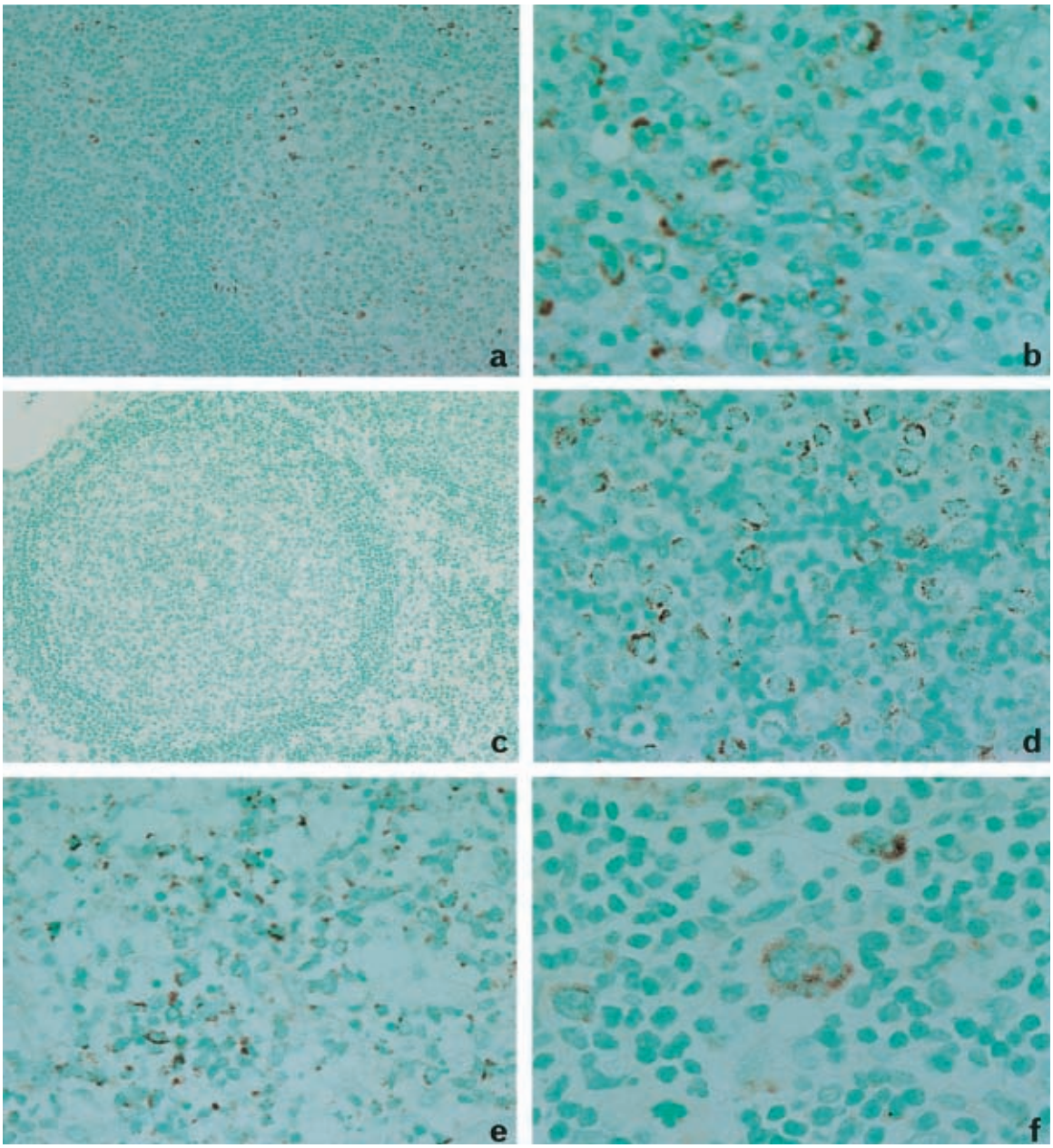

Fig. 4. Expression of HSP60 in reactive lymphoid tissues and malignant lymphomas. (a), chronic tonsillitis, x 100; (b), diffuse large B-cell lymphoma, x 400; (c), follicular lymphoma grade 1, x 50; (d), follicular lymphoma grade 3, x 400; (e), NK/T cell lymphoma, x 200; (f), Hodgkin lymphoma, mixed cellularity, x 400

H-RS cells of Hodgkin lymphomas, as shown in the earlier report[22]. Although the mechanism responsible for the abundance of HSP60 in H-RS cells remains to be determined, interaction with their cellular microenvironment may be partly responsible for the induction of HSP60. H-RS cells, which are surrounded by an excess of $CD4^+$ T lymphocytes, express many members of the tumor necrosis factor receptor (TNFR) superfamily, including major histocompatibility complex class II antigens, B7/BB1, CD30, CD40, CD54, CD95, and TNFR, and they secrete various cytokines, including IL-1, IL-5, IL-6, IL-9, M-CSF, TNF, and have receptors for several cytokines[29].

On the other hand, $CD4^+$ T lymphocytes express several adhesion molecules and secrete variable cytokines. It has been reported that HSP60 is induced in monocytic leukemia cell lines by treatment with interferon-γ and TNFα[30]. Expression of HSP70 is also enhanced when cells are stimulated with IL-1, IL-2, or TNF-α[31,32]. Thus, autocrine and paracrine stimulation of H-RS cells with cytokines could enhance HSP60 expression.

In non-Hodgkin lymphomas, over expression of HSP60 was generally associated with the large-cell morphology of lymphoma cells, but not necessarily correlated with the grading of lymphomas. HSP60 was over expressed in diffuse, large B-cell lymphomas, high grade follicular lymphomas, and NK/T cell lymphomas. This result was again different from the previous report[22]. Hsu and Hsu found very weak staining in all types of non-Hodgkin lymphomas tested[22]. This nonselective, very weak immunoreactivity of HSP60 in all non-neoplastic and neoplastic lymphoid tissues, except for H-RS cells, is unreliable compared with the present result which showed a discriminative and distinct immunostaining pattern.

At the cellular level, HSP60 was present in the cytoplasm in both normal and neoplastic cells. No immunostaining was detected at the cell surface. In nonlymphoid tissues, HSP60 is known to be present in cells with abundant mitochondria[33]. The immunoreactivity of HSP60 showed a granular pattern suggesting mitochondrial distribution. Thus, HSP60 immunoreactivity in lymphoma cells may simply reflect the quantity of mitochondria.

Some cytokines are involved in the induction of HSP60. Cytokines in the microenvironment may also partly affect the level of HSP60 in lymphoma cells. However, the precise mechanism and significance of HSP60 over expression in some non-Hodgkin lymphomas have yet to be clarified. Recently, HSP have been shown to contribute to the stability of tumor suppressor gene products, such as p53 and Rb[34,35], and to participate in the development of resistance to various cytotoxic drugs[36,37]. HSP may also play some role in the apoptosis of tumor cells[21,37,38]. Thus, the cellular level of HSP could be correlated with the proliferative capacity, apoptosis, and drug sensitivity of lymphoma cells.

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