Abundance of Heat Shock Proteins (hsp89, hsp60, and hsp27) in Malignant Cells of Hodgkin’s Disease

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ABSTRACT

Heat shock proteins (HSPs) or stress proteins are synthesized by cells in response to environmental stress. Expression of HSPs by cells may have important physiological or pathological implications. In this study, we carried out an immunohistochemical and biochemical examination of low (hsp27), intermediate (hsp60), and high (hsp89) molecular weight HSP expression in reactive lymph nodes and in lymph nodes of patients with various types of lymphomas. In normal or reactive lymphoid tissues, hsp89 is abundant in large “transformed” lymphoid cells and immunoblasts. Hsp60 is widely distributed in lymphoid tissues, whereas hsp27 is absent in all lymphoid cells and histiocytes. Among lymphomas, the Hodgkin’s Reed-Sternberg (H-RS) cells in Hodgkin’s disease (HD) had the greatest abundance of hsp89 and hsp60 and, in 20% of cases, hsp27, in contrast to a much weaker staining of anti-hsp89 and -hsp60 in the background reactive lymphoid cells. The large lymphoid cells in small lymphocytic lymphoma are also rich in hsp89, but not hsp60 and hsp27. In contrast, the malignant cells in anaplastic large cell lymphoma and most high-grade tumors, including immunoblastic lymphomas, expressed minimal amounts of hsp89 and hsp60 and virtually no hsp27. Thus, the cellular level of HSPs was neither correlated with the proliferative capacity nor with the aggressiveness of the lymphomas. Hsp89, hsp60, and hsp27, as well, serve critical roles in the chaperoning of cellular proteins (e.g., a $M_t$ 43,000 protein) in H-RS cells. The known interactions of HSPs with Rb, p53, peptide-MHC class II complexes, and cofactors of the glucocorticoid hormone receptor have further broadened the importance of HSPs in cell metabolism and in response to extracellular signals for proliferation, differentiation, or growth suppression (or apoptosis) of H-RS cells. Abundant HSP expression is seen only in HD, but not in other lymphomas. Such expression could have vital roles in the pathogenesis of HD.

INTRODUCTION

There are two major types of HSPs,1 one inducible and one (hsc) expressed constitutively, serving critical roles in the chaperoning of cellular proteins. The inducible HSPs are synthesized by cells in response to environmental stress such as elevated temperature or exposure to oxidants, viral infections, growth factors, or proteotoxic treatments (e.g., exposure to metals, metabolic inhibitors, and amino acid analogues; Refs. 1–7). The hsc proteins are expressed in a more constitutive manner compared with the inducible HSPs. Some HSPs are differentially expressed and/or regulated during the cell cycle, by hormones, and at different stages of development and differentiation (8). HSP/hsc and their amino acid sequences have been highly conserved during evolution (1–3, 7). The major mammalian HSPs and hsc include one $M_t$ 70,000 protein, two $M_t$ 60,000 protein, and one $M_t$ 27,000 a-crystallin-related protein (hsp27). Additional HSPs with lesser-known functions have also been reported, such as hsp75 (of the hsp90 family), hsp73 (of the hsp70 family), hsp56, hsp43, and others (8–10).

Functionally, HSP/hsc act as molecular chaperones that have a remarkable ability to recognize and selectively bind proteins in subcellular compartments. The purposes of this binding are: (a) to facilitate the folding and assembly of proteins by preventing inappropriate interactions with other proteins; (b) to facilitate the transfer of proteins across the intracellular membranes by maintaining them in a conformation competent for translocation between subcellular compartments; and (c) to prevent irreversible aggregation and to perform internal housekeeping (disassembly) of inappropriately folded or degraded proteins (8–11). Thus, these functions of HSPs are essential in every living cell and they are required for repairing damage resulting from stress. Recently, HSPs have been shown to contribute to the stability of several proteins (i.e., p53 and Rb) of tumor suppressor genes and to participate in the development of resistance to various cytotoxic drugs (11–14). Seemingly, HSPs could play important roles in the growth promotion and the suppression (apoptosis) and differentiation of cancer cells (15, 16).

An understanding of the roles of HSP in the pathogenesis of cancer is a topic of great interest. Hsp27 is preferentially expressed, for example, in patients with acute lymphoblastic leukemia (pre-B-cell type), and the expression of different hsp27 isoforms may be related to the prognosis for this disease (17, 18). Hsp27 and hsp/hsc70, as well, are associated with doxorubicin resistance in certain cells (11, 14), and expression of hsp/hsc70 seems to correlate with the susceptibility of acute myelogenous leukemia cells to apoptosis in vitro (16). As to breast carcinomas, hsp27 overexpression is not associated with prognosis in lymph node-negative breast cancer patients, nor with the response to tamoxifen and the clinical course of disease in women with metastatic breast cancer (19, 20).

In the present study, we determined the distribution de novo of three HSPs (hsp89, hsp60, and hsp27) in normal lymph nodes as well as in lymphoid tissues involved by various types of lymphomas. We noted abundant expression of these HSPs, especially hsp89, in HD, but generally not in NHL. A chaperone function of hsp89 is confirmed by its binding to a $M_t$ 43,000 protein (actin) in long-term cultured H-RS cells. HSPs are likely to play vital roles in the pathogenesis of HD.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies to hsp27 and hsp60 were obtained from Santa Cruz Biotechnology (Houston, TX). Both antibodies were selected based on their reactivities in formalin-fixed and paraffin-embedded tissue sections. Antisera to hsp89 and p82 (used as controls) were produced in the laboratory of Dr. E. Appella at the National Cancer Institute (Bethesda, MD). Anti-hsp89 was obtained initially by immunization of rabbits with a purified $M_t$ 86,000 tumor-specific transplantation antigen from the murine MethA tumor (21). The antigen (p86) used for immunization was subsequently shown to be similar, if not identical, to the murine $M_t$ 89,000 HSP (22). This antisera specifically precipitates the hsp89 from mouse embryonic tissues (22) and the hsp89 in many human cell lines (as shown in this study). The
reactivity of anti-p82 has been described previously (23). Anti-v-fms (also used as control) was produced by immunization of rabbits with the recombinant v-fms polypeptide isolated from *Escherichia coli* bearing plasmid p57NB v-fms (provided by Dr. D. Sherr, St. Jude Research Hospital, Memphis, TN; Ref. 24). Anti-actin was purchased from Oncogene Science, Inc. (Cambridge, MA).

**Lymphomas and Lymphoid Tissues.** A total of 92 formalin-fixed, paraffin-embedded tissues from patients with malignant lymphomas of different origins were studied for expression of HSPs in lymphoma cells. These included 1–10 specimens from each of the histological types of malignant lymphomas (Table 1) and 25 specimens from HD. In addition, five lymph nodes and five tonsils used as normal or reactive tissue controls were included. Paraffin sections were prepared routinely and were dewaxed and rehydrated before staining, according to procedures described previously (25).

**Cell Lines, Tissue Culture, and PBMCs.** Several cultured lymphoma or leukemia cell lines were tested. These included T cell lines (MOLT-4 and HUT102), B cell lines (EB3, Raji, Daudi, SU-DHL-2, -4, -5, and -6), monocyte/histiocyte cell lines (U-937 and THP-1), a cell line from an anaplastic large cell lymphoma (SU-DHL-1), and two cell lines from HD (HDLM-1 and KM-H2). The cells either were obtained from the American Type Culture Collection (Manassas, VA) or had been provided for us by other investigators (26–28). All cultures were grown at 4 × 10^5–1 × 10^6 cells/ml in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS, 2 mM glutamine, 50 mM 2-mercaptoethanol, and 50 mg/ml gentamicin at 37°C in a humidified, 5% CO_2_ atmosphere. The medium was changed every 2–3 days. The viability of these cells was generally maintained at 95%. Cell viability was determined by the trypan blue dye exclusion test. In addition, normal PBMCs were isolated by means of gradient centrifugation and cultured in RPMI medium, as described above, for 1–2 days.

**Immunoperoxidase Staining.** The ABC method was used for immunolocalization of HSPs in tissue sections or cytosin smears, as described previously (25). Briefly, the sections or smears were treated with normal goat serum or horse serum and incubated with anti-HSP antibody at room temperature for 1 h. After being washed with PBS, the slides were reacted sequentially with biotin-labeled secondary antibody and ABC (Vector Laboratories, Inc., Burlingame, CA) and then developed in a diaminobenzidine-H_2O_2_ solution (25). The presence of hsp89 in cells was evaluated semiquantitatively with serially diluted antibodies. The two mouse monoclonal anti-HSP antibodies were used at 1 μg/ml.

**Detection of HSPs on Western Immunoblots and Specificity Confirmation.** Western immunoblot and radioimmunoprecipitation were used as supplementary tests for confirmation of the specificity of tissue or cell immunoperoxidase staining. A total of 2 × 10^6_ of Ficoll-purified PBMCs, cells in tissue culture, or cells from lymphoid tissues were washed twice with RPMI 1640 and then lysed on ice for 5 min in RIPA buffer [20 mM Tris-HCl (pH 7.2), 0.15 N NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM EDTA, 1 mM PMSF, and 0.2 mg/ml of aprotinin]. The lysate was clarified by centrifugation in a microcentrifuge for 10 min at 4°C. The amount of protein in the lysates was determined by the Bradford method (29). For immunoblotting, the cell lysates (15 μg/slot) were boiled in Laemmli sample buffer, electrophoresed on an 8% SDS polyacrylamide gel, and then electroblotted onto BA83 nitrocellulose (0.2 mm thick; Schleicher and Schuell, Keene, NH). For detection of transferred proteins, the nitrocellulose was stained with 0.5% Fast Green in 20% methanol, 5% acetic acid, and then destained.

Western immunoblots were performed for hsp89 and hsp60. For detection of hsp89, for example, the filter was blocked with normal goat serum (1:200 dilution) and 3% nonfat dry milk [prepared in TS buffer: 20 mM Tris-HCl (pH 7.6), 0.15 N NaCl, and 0.1 mM PMSF] for 30 min and then reacted sequentially with rabbit anti-hsp89 (3 h), biotin-labeled goat antirabbit IgG (30 min), and ABC (30 min). The filters were washed extensively with TS and 0.1% Triton X-100 between steps and finally developed in 4-chloro-1-naphthol (0.6 mg/ml) in TS with 20% methanol and 0.03% H_2O_2_. For quantitation, Western blotting of serial dilutions of lysozyme proteins and measurement by densitometry (Alpha Innotech IS1000 System) were performed.

**Labeling of Cells and Radioimmunoprecipitation.** Cells were metabolically labeled with [35S]methionine in methionine-free RPMI medium supplemented with 5% dialyzed FCS for 2 h at 37°C. The labeled cells were washed and lysed in RIPA buffer, as described earlier (30). For immunoprecipitation, 4 × 10^6_ cpm of cell lysate preclared with protein A-Sepharose (Pharmacia) was reacted with rabbit anti-hsp89 for 3 h and with protein A-Sepharose beads for an additional 30 min. After 3 washes with the RIPA buffer, the precipitated

| Table 1 Expression of HSPs in various lymphomas |
|------------------------|-------------------|-------------------|-------------------|
| Histological classification | Case no. | hsp89 | hsp60 | hsp27 |
| B-cell lymphomas (41) | | | | |
| Small lymphocytic | 5 | Immunoblasts and pseudo-germinal center cells | Very weak staining | Negative |
| Mantle cell | 2 | Rare large cells | Very weak staining | Negative |
| Follicular center | 5 | Negative | Very weak staining | Negative |
| Small cell | 5 | Negative | Very weak staining | Negative |
| Mixed small and large cell | 5 | Very weak staining of cells in follicles | Very weak staining | Negative |
| Large cell | 2 | Very weak staining of large tumor cells | Very weak staining | Negative |
| Diffuse large cell (including immunoblastic) | 10 | Very weak staining | Weak staining | Negative |
| Burkitt’s | 5 | Negative or very weak staining | Very weak staining | Negative |
| Precursor B-lymphoblastic | 3 | Negative or very weak staining | Very weak staining | Weak positive |
| Hairy-cell leukemia | 4 | Negative | Very weak staining | Negative |
| T- and NK-cell lymphomas (29) | | | | |
| Mycosis fungoides | 5 | Negative | Very weak staining | Negative |
| Peripheral T cell | 7 | Negative or very weak staining | Very weak staining | Negative |
| Angioimmunoblastic | 2 | Negative or very weak staining | Very weak staining | Negative |
| Angiocentric | 2 | Negative or very weak staining | Very weak staining | Negative |
| Intestinal T cell | 1 | Negative | Very weak staining | Negative |
| Anaplastic large cell | 5 | Very weak staining | Weak staining | Negative |
| Lymphoblastic | 7 | Negative | Very weak staining | Negative |
| HD (25) | | | | |
| Nodular sclerosis | 12 | Strongly positive in H-RS cells | Strongly positive in H-RS cells (reactive cells, weakly +) | Strongly positive in H-RS cells (reactive cells, weakly +) |
| Mixed cellularity | 7 | Strongly positive in H-RS cells | Strongly positive in H-RS cells (reactive cells, weakly +) | Strongly positive in H-RS cells (reactive cells, weakly +) |
| Lymphocyte-predominant (nodular L&H variant) | 2 | Positive in H-RS cells, variable in intensity | Positive in H-RS cells, variable in intensity (reactive cells, weakly +) | Negative |
| Lymphocyte-depletion | 4 | Moderately positive in H-RS cells | Moderately positive in H-RS cells (reactive cells, weakly +) | Weakly positive in H-RS cells |
| True histiocytic malignancies (7) | | | | |
| True histiocytic lymphoma | 3 | Weak staining of tumor histiocytes | Very weak staining | Negative |
| Malignant histiocytosis | 4 | Negative or very weak | Very weak staining | Negative |
proteins were eluted by boiling in SDS sample buffer and analyzed by SDS PAGE and autoradiography (31).

Sucrose Gradient Fractionation. To investigate the possibility that hsp89 in H-RS cells may complex with a unique species of protein, we used sucrose gradient centrifugation to fractionate the 35S-labeled cellular proteins according to their sedimentation velocity. A total of 5 × 10^7 cpm of 35S-labeled HDLM-1 cell lysate was layered onto a 10–40% glycerol gradient in RIPA buffer and centrifuged at 40,000 rpm for 16 h (30). Twenty-eight fractions were collected from the bottom of each gradient. The labeled proteins in each fraction were analyzed directly on gels or by immunoprecipitation with specific antisera, as described above. Furthermore, for characterization of the coprecipitated protein, the electroblotted membranes were blocked with 10% dried milk in TBS containing 0.1% Tween 20 for 1 h. The membranes were stained with anti-actin in TBS for 1 h, as described above.

RESULTS

Specificity of Anti-HSP Antibodies. The availability of a highly specific antibody is a prerequisite for the immunohistological identification of hsp89 antigen and other HSPs in tissue sections. Therefore, before tissue staining, we tested the specificity of anti-hsp89 by using radioimmunoprecipitation. As shown in Fig. 1, anti-hsp89 immunoprecipitated a single polypeptide (Mr 89,000) in the 35S-labeled lysate of SU-DHL-1 cells, whereas a control antibody (anti-p82) immunoprecipitated an Mr 82,000 antigen in the same cell extract. No significant background or cross-reactivity was observed with either antibody. Thus, anti-hsp89 seemed to be highly specific for the hsp89 antigen and suitable for tissue staining. The high specificity of anti-hsp89 and anti-hsp60 was also confirmed in the immunoblotting experiments, in which the antiserum detected a single Mr 89,000 and Mr 60,000 protein, respectively, in lysates of various cells. The specificity of anti-hsp27 has been reported previously (11).

Distribution of hsp89, hsp60, and hsp27 in Normal Lymphoid Tissues. By using the ABC immunostaining method, we examined the presence of hsp89, hsp60, and hsp27 in human lymph nodes and tonsils. With low concentrations of the antiserum (dilutions of 1:2,000–1:6,000), intense hsp89 staining was detected in scattered immunoblasts. The staining intensity was medium to low in large germinal-center cells (centroblasts), whereas no staining was detected in histiocytes or in the small lymphocytes residing in the T-cell zone and mantle zone of the germinal centers (Fig. 2A). At a higher concentration of anti-hsp89 (diluted 1:400), however, the smaller lymphocytes also seemed to be weakly positive for hsp89.

Anti-hsp60 reacted weakly and in a nondiscriminatory manner with most lymphoid cells, histiocytes, and dendritic cells in lymphoid tissues. However, hsp27 was generally absent from lymphoid cells and histiocytes. Scattered endothelial cells and fibroblasts were intensely positive for hsp27. The staining patterns of hsp60 and hsp27 were not affected by the concentrations of antibodies used. Control
stainings with normal (nonimmunized) rabbit or mouse serum instead of anti-hsp were negative in all areas (data not shown).

**Distribution of HSPs in Various Lymphomas.** A total of 92 specimens of lymphomas of different types were tested for the expression of hsp89, hsp60, and hsp27, with the results for the HSP distribution shown in Table 1. These lymphomas had been diagnosed previously with a large panel of monoclonal antibodies. To assess the relative amount of hsp89 in individual tumor cells, we used a low concentration (1:2,000 dilution) of anti-hsp89 throughout. In general, the neoplastic cells of most lymphomas, including immunoblastic lymphoma and anaplastic large cell lymphoma, were negative for hsp89 at this antibody concentration. Large amounts of hsp89 were detected in scattered immunoblasts and in cells of the proliferation centers of small lymphocytic lymphomas (Fig. 2, B and C). However, the most striking observation was the intense hsp89 staining in H-RS cells in all 25 cases of HD (Fig. 2D). In these same specimens, the reactive lymphocytes and histiocytes generally reacted negatively or extremely weakly with anti-hsp89.

Abundant hsp60 was present in most H-RS cells. A weak-to-moderate staining intensity was noted, however, in reactive lymphoid cells and in histiocytes surrounding H-RS cells (Fig. 3C). Moderate or weak staining of hsp60 was also seen in all lymphoma tissues tested. Hsp27 expression could be seen in H-RS cells in approximately 20% of patients with HD (Fig. 3, A and B), but not in the NHLs tested. The expression of hsp27 did not correlate with the histological subtypes and clinical stages of HD.

Parallel control staining with normal rabbit serum or nonimmune mouse ascitic fluid instead of anti-HSPs did not reveal the same staining pattern in H-RS cells (data not shown). Therefore, the anti-HSP staining in the tumor cells of HD is neither due to endogenous peroxidase activity nor to a nonspecific reaction.

**Expression of hsp89 in Human Cell Lines.** Because hsp89 is abundant in H-RS cells in tissues, we examined whether such a high level of expression is also a characteristic of cultured H-RS cells. Cultured cells (H-RS cells and others, e.g., SU-DHL-1, U937, Raji, EB-3, etc.) were labeled with [35S]methionine, and each cell extract was precipitated with anti-hsp89. The results of the immunoprecipitation experiments indicated that most cells, except EB-3, expressed comparable amounts (determined by their radioactivities) of hsp89, irrespective of the original cell type (Fig. 4A). EB-3 cells contained approximately one-half of the amount of hsp89 when compared with that in others. In all cell lines, the amounts of hsp89 were estimated to be 0.1–0.3% of the newly synthesized proteins, based on the percentage of anti-hsp89-precipitable radioactivity.

**Comparison of hsp89 and hsp60 Levels in PBMCs, Normal Lymph Nodes, and Lymph Nodes Affected by HD.** The amounts of hsp89 in normal human lymph nodes and in the tumor tissue of HD were compared by immunoblotting. In the experiment illustrated in Fig. 4, C and D, equal amounts of protein in two normal lymph nodes and in tumor tissue from two patients with HD were analyzed. The average amount of hsp89 in the tumor tissue was approximately 1.5 times the average level of hsp89 in normal lymph nodes. However, the H-RS cells in the two HD specimens comprised, at most, 2–5% of the total number of cells. Therefore, we estimated that H-RS cells in tumor tissues might contain as much as 10–25 times the normal amount of hsp89 present, on the average, in normal lymphocytes on a per-cell basis.

The amounts of hsp89 in cultured normal human PBMCs were also

![Fig. 3. Tissue staining with hsp27 (A and B) or anti-hsp60 (C). Note the abundant expression of hsp27 in H-RS cells (arrowhead) and apoptotic (arrow) cells are negative for hsp27 (B). C, intense hsp60 staining was seen in H-RS cells and histiocytes. Staining of hsp60 in lymphocytes was relatively weak as compared with that in H-RS cells.](image)

![Fig. 4. A (stained with anti-hsp89) and B (stained with anti-p82, for control): detection of hsp89 in cell lines EB3 (Lane 1), HDLM-1 (Lane 2), and PBMCs (Lane 3) from a normal individual. The EB3 cells express less hsp89 (A), but higher amounts of p82 (B), than do the HDLM-1 cells. The amount of hsp89 in HDLM-1 immunoprecipitate is approximately 6–8 times that in PBMCs. An increased amount of hsp89 was also obtained with another H-RS cell line, KM-H2, and other leukemia/lymphoma cell lines (data not shown). The abundant expression of hsp89 in cultured cells may reflect the fact that these cells were grown under artificial conditions. The level of hsp89 in cultured cells should not be used as indicating the distribution or quantitation of hsp in the original tumor cells in tissue. C and D, detection of hsp89 in normal lymph nodes (Lane 1) and in tumor tissue of HD (Lane 2, two cases each, C and D) by immunoblotting. In both HD cases, the number of H-RS cells in tissue did not exceed 5%, and yet the amount of hsp89 in lymph nodes from HD (Lane 2) was greater than that in normal lymph nodes (Lane 1).](image)
studied by immunoblotting. The level of hsp89 in normal PBMCs varied slightly among individuals and from culture to culture. However, the overall result indicated that the level of hsp89 expression in H-RS cell lines was approximately 10 times that in cultured PBMCs (Fig. 4, C and D). A similar result was obtained with hsp60 (data not shown).

**Association of hsp89 with a Mr 43,000 Protein in Cultured H-RS Cells.** Hsp89 has been shown to form complexes with other molecules, including actin, oncogene products, and steroid receptors, and to serve as a regulator of kinase or receptor function. Thus, the abundance of hsp89 in H-RS cells may suggest a high degree of complex formation in these cells. To investigate this possibility, we used sucrose gradient centrifugation to fractionate the 35S-labeled cellular proteins according to their sedimentation velocity, and we analyzed the distribution of hsp89 in each fraction by immunoprecipitation. As demonstrated in other studies (11, 13), this fractionation step is necessary for the detection of the complexed form of hsp89 because the majority of the cytosolic hsp89 molecules remain in the form of monomers or dimers, and because direct immunoprecipitation of the cell lysate with anti-hsp89 is not sufficient for identification of the complexed, fast-sedimenting form of hsp89.

The total 35S-labeled proteins of cultured H-RS (HDLM-1 or KM-H2) cells were fractionated on a 10–40% sucrose gradient, and individual fractions were subjected to immunoprecipitation with specific antibodies and to gel analysis (Fig. 5). Fig. 5B illustrates the distribution of total cellular proteins from HDLM-1 cells in each fraction of the gradient. The majority of soluble proteins were in the slow-sedimenting fractions 18–24. The fractionation of molecules on this gradient is apparent from the sizing effect, as demonstrated by the preferential distribution of larger proteins toward the bottom and smaller proteins toward the top portion of this gradient. Fig. 5, A, C, and D illustrate the results of immunoprecipitation with anti-hsp89 and with two control antibodies, anti-p82 and anti-v- fms, respectively. As expected, most of the hsp89 resided in fractions 18–24, where most of the soluble protein was found (Fig. 5A). However, a significant portion of the hsp89 also appeared in the faster-sedimenting fractions 4–16 (Fig. 5A). These rapidly migrating species of hsp89 may represent formation of oligomers or of complexes of hsp89 with other molecules. In fractions 8–10, a Mr 43,000 protein seemed to coprecipitate with hsp89, suggesting the presence of a heterocomplex between these two molecules. This coprecipitation seemed to be specific only for hsp89; the two control antibodies did not coprecipitate this Mr 43,000 protein (Fig. 5, C and D).

The Mr 43,000 protein seemed to differ from a Mr 44,000 protein that was present in the anti-hsp89 immunoprecipitate of fractions 20–24. This conclusion is based on three observations: (a) the precipitation of p44 in fractions 16–24 seemed to be nonspecific, because all three antisera reacted with their respective antigens, but all reacted with the associated p44 in these fractions (Fig. 5, A, C, D); (b) when the anti-hsp89 immunoprecipitates of fractions 8 and 20 were run side by side on the same gel, the molecular weights of the two proteins consistently seemed to be different (Mr 44,000 and Mr 43,000; Fig. 5E); and (c) the presence of the Mr 43,000 protein in fractions 8 and 10 was not due to nonspecific diffusion or spill-over from other portions of the gradient because this coprecipitating event was not evident in the neighboring fractions 12–16. The Mr 43,000 protein region seemed to contain actin as shown by a positive staining with anti-actin using electroblotted membrane.

Similar results were obtained for KM-H2 cells. It is probably important to determine whether such a complex is present in H-RS cells in tissues. However, such a study cannot be done because of the extreme paucity of H-RS cells (<5%) in most lymph nodes involved by HD.

**DISCUSSION**

In this study, we demonstrated the distribution of a number of HSPs (including hsp89, hsp60, and hsp27 de novo) in normal and reactive lymph nodes, as well as in lymphoid tissues, from patients with various types of lymphomas. At the cellular level, high levels of hsp89 were found to exist in the large germinal-center cells (large noncleaved cells and centroblasts) and immunoblasts of normal lymph nodes. Other types of lymphoid cells may contain small quantities of hsp89 below the threshold of the detection method. Abundant expression of hsp89 was noted in H-RS cells as well as in large lymphoid cells in small lymphocytic lymphoma. The low level of expression of hsp89 in follicular lymphoma and immunoblastic lymphoma is, however, somewhat unexpected. The hsp60 is constitutively expressed by...
most normal cells, and its presence in most, if not all, neoplastic lymphoid cells is expected. The hsp27 was absent from normal lymphoid cells as well as most lymphoma cells, except for a subpopulation of H-RS cells. The readily detectable hsp89 and hsp60 in H-RS cells are attributed, in part, to the large size of the cells and the abundance of cytoplasm. More likely, the increased staining intensity of hsp89 and hsp60 may be a result of an increased synthesis or stability of these proteins in H-RS cells. We estimated that the levels of hsp89 and hsp60 expression in H-RS cells are at least 10 times those in normal lymphocytes on a per-cell basis.

The mechanisms responsible for the abundant HSP expression in H-RS cells have yet to be determined. In tumor cells, abnormal HSP synthesis may be attributed, in part, to the changes in heat tolerance (febrile condition) that accompany malignant transformation (32–34). The pattern of tissue distribution of hsp89 in cells, however, is not necessarily reconcilable with the suggested role of this protein in protecting cells against hyperthermia or heat shock. For example, the testis contains a high level of hsp89, and yet it is one of the most heat-sensitive tissues in the body (2). Enhanced HSP (e.g., hsp89) synthesis in cells may also be related to their unique microenvironments and to differences in their cellular metabolism (5, 6). The increased expression of HSPs in H-RS cells could be mediated in part by cytokines, either directly or indirectly. Expression of HSPs (especially hsp/hsc70) can be enhanced when cells are stimulated with IL-1, IL-2, or TNF-α (35, 36). These cytokines and many others are not deficient in the microenvironment of H-RS cells, and they could significantly affect HSP expression in H-RS cells (37, 38).

Hsp89 plays important structural and functional roles within cells. Hsp89 shows a transient association with several products of oncogenes (p60src, p140prf, and p94yes), and it inactivates the tyrosine kinase activity of these transforming proteins (30, 39–42). These interactions are necessary for correct insertion of the oncogenic protein kinases into the plasma membrane. Hsp89 forms inactive complexes with steroid-hormone receptors, and its dissociation from these complexes is accompanied by activation of the receptors (41–43). It binds reversibly to tubulin and F-actin modulated by calcium-calmodulin (44, 45), and it might be implicated in the transport of proteins inside the cell. Clearly, the reversible association of hsp89 with actin (43 kDa), oncogene products, and steroid receptors indicates a function in the regulation of important biological events within the cells. Furthermore, hsp89 may act as a tumor-specific transplantation antigen in various cells (21, 22).

The formation of a complex of hsp89 with steroid receptors or with known oncogene products was not detected in the cultured H-RS (i.e., HDLM-1) cells. It must be emphasized that the M 43,000 protein may not be the sole protein associated with hsp89; the dynamic chaperone function of hsp89, and of other HSPs as well, may be more complicated and diverse in H-RS cells de novo in tissues. The possibility exists that hsp89 interacts with other proteins in H-RS cells in tissues. The expression of hsp27 in H-RS cells and pre-B acute lymphoblastic leukemia cells is also of interest (17, 18). In normal tissues, hsp27 functions as a p29 estrogen receptor-associated protein, and, thus, it is not unexpected to note that hsp27 is expressed mainly in estrogen target organs (8). Hsp27 may cooperate with hsp89/90 and hsp/hsc70 in chaperoning steroid receptors (8). Because both hsp27 and hsp89/90 are also associated with actin, they may play a role in steroid receptor transportation from the cytoplasm to the nucleus by an actin-based mechanism. Hsp27 expression is also associated with resistance to doxorubicin, colchicine, and vinblastine in certain cells (14); some of these drugs have been widely used for treatment of patients with HD. It is worthwhile to study whether hsp27 participates along with the larger HSPs in the development of drug resistance and plays a role in disease relapse or progression in patients with HD.

H-RS cells are likely to express several constitutive HSPs, including hsp/hsc70 and hsp84. H-RS cells express MHC class II antigen and are potent antigen-presenting cells (46). The production of peptide-MHC class II complexes is an intricate, intracellular process that may require the function of molecular chaperones. Hsp/hsc70 and related proteins (e.g., PBP72/74) could play a role early in this pathway (antigen scavenging and peptide transport; Ref. 47). Hsp84 can form a complex with a temperature-sensitive p53 mutant protein (13) and, perhaps, the wild-type p53 protein, if the latter adopts a conformation recognized by a monoclonal anti-p53 antibody designated as PAb240 (13). The wild-type p53 protein with a PAb240+ conformation is frequently detected in H-RS cells (46). Hsp84, along with other factors, may contribute to the accumulation or increased stability of p53 protein in H-RS cells (48, 49). A p53-binding protein, MDM2, is also increased in amount in H-RS cells (50).

In conclusion, our results indicate that the cellular level of hsp89 and two other HSPs (hsp60 and hsp27) may not be correlated with the proliferative capacity of tumor cells. Expression of hsp89, hsp60, and occasionally hsp27 is abundant in H-RS cells, but generally not in NHL cells; this suggests a unique role of these HSPs in the pathogenesis of HD. The expression of abundant HSPs may be related to the febrile state of the patient or to presentation of as yet undetermined stimuli (e.g., cytokines or virus) to the H-RS cells. Moreover, the interactions between members of the constitutive HSPs with Rb, p53, peptide-MHC class II complexes, and cofactors of the glucocorticoid hormone receptor have further broadened the importance of HSPs in general cell metabolism and in response to extracellular signals for proliferation, differentiation, or growth suppression (or apoptosis). Such activities could also have vital roles in the pathogenesis of HD.

ACKNOWLEDGMENTS

We thank Dr. D. Sherr (St. Jude Research Hospital, Memphis, TN) for providing the v-fms probe and Drs. E. Appella and L. W. Low (National Cancer Institute, Bethesda, MD) for providing the antibody as well as valuable suggestions for the preparation of this manuscript.

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Abundance of Heat Shock Proteins (hsp89, hsp60, and hsp27) in Malignant Cells of Hodgkin’s Disease

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