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A Novel B Cell Line Established from Ki-1-positive Diffuse Large Cell Lymphoma

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A novel cell line, designated KIS-1, was established from a patient with Ki-1-positive diffuse large cell lymphoma. Multiple phenotypic analysis of the KIS-1 cells was carried out with a total of 22 monoclonal antibodies defining hematopoietic cell subsets and lineages. The KIS-1 cells were positive for Ki-1, B4, HLA-DR, and 2D1 (common leucocyte) antigens, but were negative for the antigens reportedly specific for T cells, natural killer cells, granulocytes, monocytes, interdigitating reticulum cells and dendritic reticulum cells. The genomic analysis of the KIS-1 cells showed not only the rearrangement of JH and Jκ genes but also the probable rearrangement of Cλ genes. Moreover, the cells produced immunoglobulin λ chains. Thus, KIS-1 was considered to be of B-cell lineage. The lymphoma-cell derivation of KIS-1 was based on the following facts. The cytochemical, immunologic, cytogenetic properties and the results of the molecular genomic analysis in the KIS-1 cells were essentially the same as those of the original tumor cells, and the KIS-1 cells were negative for Epstein-Barr virus-associated nuclear antigen. KIS-1 is the only known B-cell line derived from Ki-1-positive diffuse large cell lymphoma, and should be useful for defining the biological implications of Ki-1 antigen.

Key words: Ki-1 antigen — B-cell line — Malignant lymphoma

Stein and co-workers produced a monoclonal antibody[1] (Ki-1) that reacted with Reed-Sternberg (RS) and Hodgkin (H) cells in Hodgkin's lymphoma tissue and with a small population of large cells preferentially localized in the T-zone of normal lymph nodes. Although Ki-1 was initially thought to detect RS and H cells specifically, further studies[2] showed that it reacted with the neoplastic cells of some non-Hodgkin diffuse large cell lymphomas. Such large cell lymphomas were proposed to constitute a single entity, as they had some characteristic histologic and phenotypic properties. Moreover, the phenotypic features of these large cell lymphoma cells shared by RS and H cells were stressed in the context of the cellular origin of RS and H cells. Obviously, further studies remain to be done for the precise understanding of Ki-1 antigen and Ki-1-positive lymphomas.

We report here cytochemical, immunological, chromosomal and molecular genomic analyses of a novel cell line, designated KIS-1, which is derived from a Ki-1-positive diffuse large cell lymphoma.

MATERIALS AND METHODS

Establishment of the Cell Line A 53-year-old man was admitted to our hospital with upper abdominal pain in September, 1985. At the age of 31, he had had a paritial gastrectomy for a duodenal ulcer. Thereafter, he was well until 1 month before admission, when he began to feel epigastric pain. An upper gastrointestinal series disclosed a narrowing of the lower esophagus and residual stomach. A presumptive diagnosis of gastric cancer was made, although histological examination of a forceps biopsy of the lesion did not confirm this. He was treated with total gastrectomy, and a diagnosis of diffuse large cell lymphoma (Fig. 1) was made following histological examination of operative specimens (stomach, pancreas, and lymph nodes). One month later, ascites and pleural effusion developed rapidly. Cytological examination of the cells in the ascites revealed that almost all (95%) of them were atypical. These cells were cultured at a concentration of 5×10^5 cells/ml in RPMI-1640

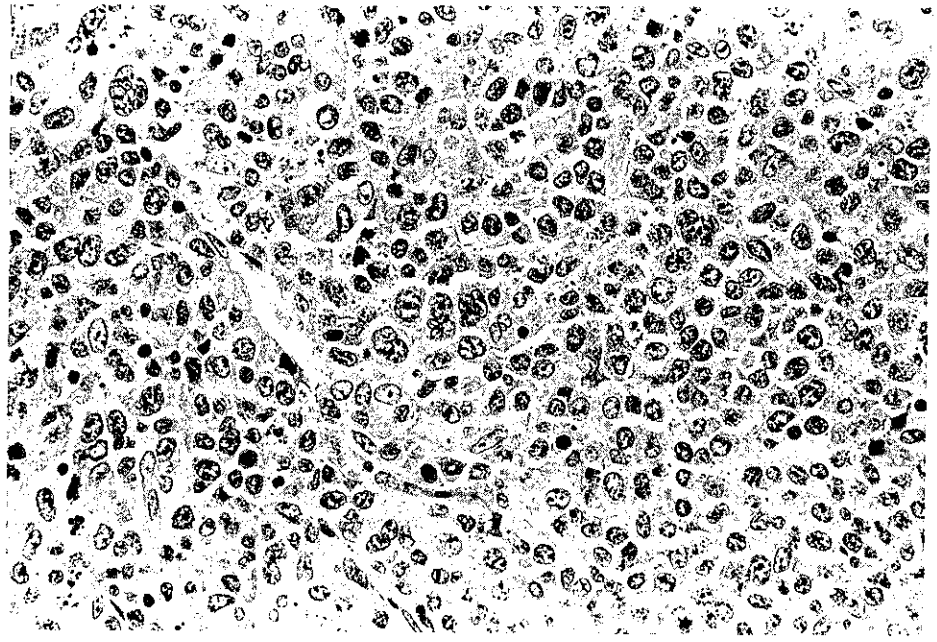

Fig. 1. Histological appearance of the lymph node biopsy specimen (H-E ×200).

supplemented with 10% fetal calf serum (FCS). Since then, they have been propagated in continuous culture for over 12 months. The cell line was designated as KIS-1. Three days after abdominal paracentesis, the patient died in spite of combination chemotherapy.

Detection of Epstein-Barr Virus An indirect immunofluorescence test[3] was used for the detection of Epstein-Barr virus-associated nuclear antigen (EBNA).

Chromosome Analysis Metaphase cells of KIS-1 and the parental ascites were prepared as previously described.[4] Chromosomes were analyzed by the trypsin-Giemsa banding method. Karyotypes were described according to the ISCN nomenclature (1985). An abnormality was defined as clonal when at least three cells from a given patient had an identical defect.

Cytochemical Methods In addition to routine staining with May-Grünwald-Giemsa, the following cytochemical tests were performed on cytocentrifuge preparations: peroxidase, acid phosphatase,[5] alkaline phosphatase,[6] α-naphthyl acetate esterase, and naphthol AS-D chloroacetate esterase.[7]

Electron Microscopy Cells were fixed in 2.5% glutaraldehyde in 0.1*M* cacodylate buffer, pH 7.4, at 4° for 2 hr. After washing, the cells were post-fixed in 1% OS_3O_4 in the same buffer at 4° for 2 hr, dehydrated in graded ethanols and embedded in an epoxy resin. Ultrathin sections were examined under an electron microscope. Counterstaining with uranyl acetate and lead citrate was used with these sections.

Studies on Immunologic Markers Immunoperoxidase methods: Paraffin sections were deparaffinized with xylene and a graded alcohol series, and rehydrated with phosphate-buffered saline (PBS; pH 7.6), as for routine processing. Frozen sections were fixed in acetone, at 4°, for 10 min prior to immunostaining. The staining procedures using heteroantisera against immunoglobulin and lysozyme were described in detail elsewhere.[8]

Immunofluorescence tests: Cell surface immunoglobulin (Ig) and cytoplasmic Ig were tested by direct immunofluorescence. Antigens defined by mouse monoclonal antibodies were detected by indirect immunofluorescence. Immunofluorescence was judged under an Olympus fluorescence microscope.

Secretion of immunoglobulins: The KIS-1 cells were cultured at the concentration of 1×10^5/ml in RPMI 1640 supplemented with 10% fetal serum for 24 hr. After centrifugation at 400*g* for 15 min, the supernatant fluid of the culture medium was concentrated ten times by freeze-drying and tested

for the presence of immunoglobulin κ, λ, α, γ, μ, and δ chains by double diffusion as described by Ouchterlony *et al.*

Molecular Genetic Analysis DNA extraction and Southern blotting: DNA was extracted as previously described.[9] DNA was digested with *Bam*HI or *Eco*RI, size-fractionated by agarose gel electrophoresis, and transferred onto nitrocellulose paper by the method of Southern.[10]

Preparations of labeled probe DNA: The 3.4 kb *Eco*RI-*Hin*dIII fragment of the joining region of the heavy chain gene, the 2.0 kb *Sac*I-*Sac*I fragment of the joining region of the κ chain gene, and the 1.7 kb *Bgl*II-*Hin*dIII fragment of the constant region of the λ chain gene were used as JH, Jκ, and Cλ probes, respectively. The 3.0 kb *Eco*RI-*Hin*dIII fragment of the constant region of the T-cell receptor β chain was also employed as a Cβ1 probe. Nick-translated, ^{32}P-labeled probes were hybridized to nitrocellulose filters in 5× Denhardt's solution, 0.9*M* NaCl, 50m*M* phosphate buffer pH 7.7, 5m*M* ethylenediaminetetraacetic acid (EDTA), 0.2% SDS and heat-denatured sonicated salmon sperm DNA (100 μg/ml) at 65°. After hybridization, the washed filters were autoradiographed.

RESULTS

Growth Characterization KIS-1 grew well in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). It had a doubling time of about 24 hr, and grew in suspension as either single cells or in small aggregates of 5–15 cells, not forming large clumps or becoming firmly attached to the bottom of the culture flasks. It has now been maintained for over 12 months.

Morphological Findings The KIS-1 cells usually had single, round or oval, cleaved or non-cleaved nuclei with multiple nucleoli when cytocentrifuge preparations were observed by light microscopy (Fig. 2A). Their nucleoli varied in size from small to large, but were not as conspicuous as those of Reed-Sternberg cells. Their cytoplasm stained moderately to deeply basophilic and contained only a few granules and vacuoles.

The ultrastructure of KIS-1 cells is shown in Fig. 2B. They measured approximately 11.0 μm in diameter. The surface of the cells was smooth, with only a few projections. The nuclei displayed an indented shape, with only slight condensation of peripheral chromatin and several prominent nucleoli. The cytoplasm was enriched in mitochondria. Several long strands of endoplasmic reticulum and a few granules were observed.

Immunofluorescence Test for EBNA The KIS-1 cells were tested in triplicate for EBNA. All observations were negative.

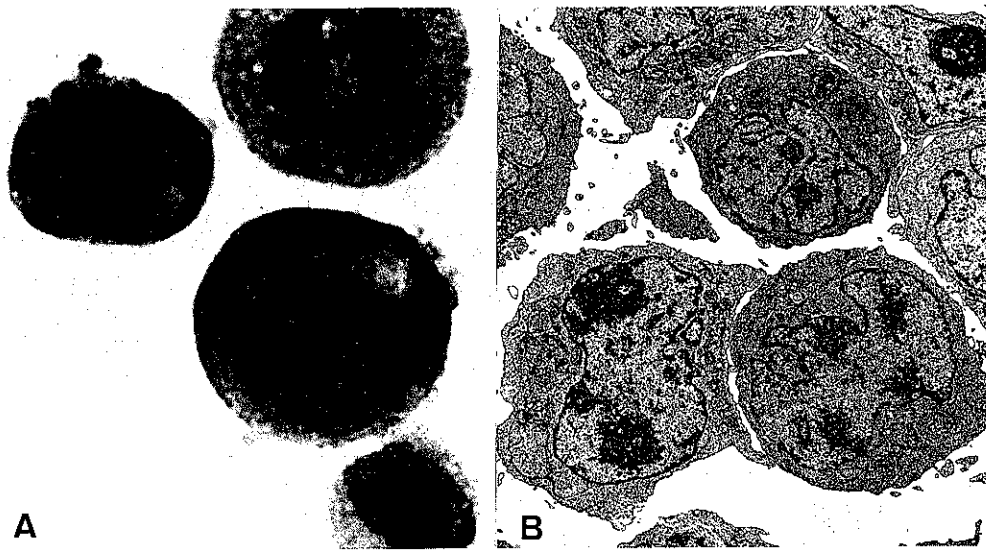

Fig. 2. A. Cytocentrifuge smear of KIS-1 cells (May-Grunwald-Giemsa ×1000). B. Electron microscopic observation of KIS-1 cells (×3400).

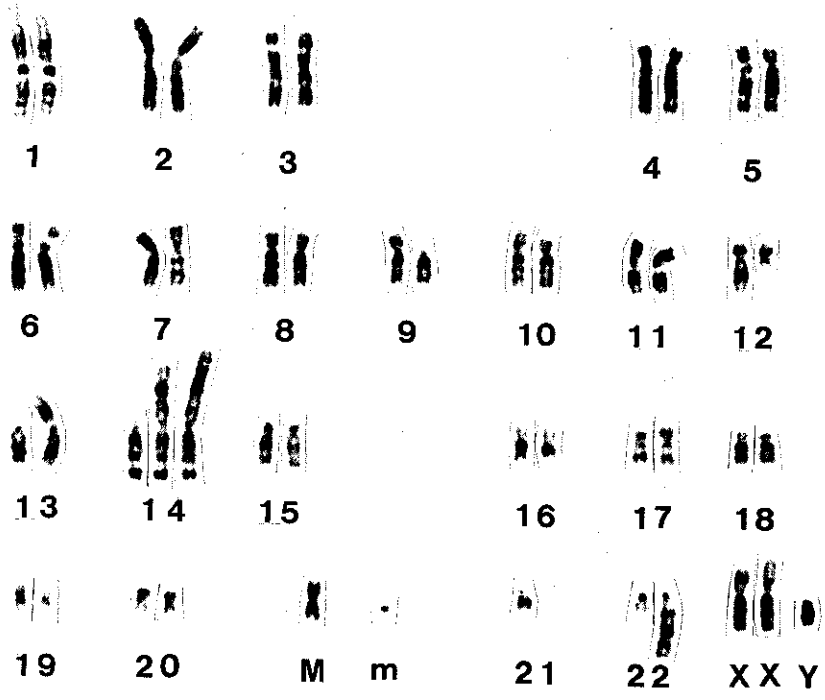

Fig. 3. G-banding karyotype of a KIS-1 cells: 49,XY, −1, −13, −14, −17, −21, −22, +der(x)t(x;?) (p11.4;?), +1p+(HSR), t(9;14) (p13;q32), del(12) (q13), +der(13)t(12;13) (q13;p12), +der(14)t(9;14;?) (?::14p12 → 14q32::9p13 → 9pter), +der(14)t(9;14;?) (?::14p12→14q32::9p13→9pter), del(16) (q22), +der(17)t(17;?) (p11.2;?), +der(22)t(1;22) (q11;q12), +mar, +min. This cell appears to have a random loss of one chromosome 21.

Cytogenetic Studies The established cell line was composed of hyperdiploid cells, with a modal number of 50 chromosomes. Karyotypes of KIS-1 cells were highly complex as shown in Fig. 3, but the clonal origin was ascertained by identifying common chromosome abnormalities: 1p+,t(9;14)(p13;q32), 12q+, 13p+, 14(p+&q+), 16q−, 17p+, 22q+, and 2 chromosomes of unknown origin. These chromosome abnormalities were also found in the ascitic parental cells.

Cytochemical Findings Enzyme cytochemistry failed to demonstrate peroxidase or naphthol AS-D chloroacetate esterase activities in the KIS-1 cells or in tumor cells in the lymph nodes. Alkaline phosphatase activities were also undetectable in the KIS-1 cells. Weak reactions for acid phosphatase and α-naphthyl acetate esterase were observed in the paranuclear regions of both KIS-1 cells and tumor cells in the lymph nodes.

Immunologic Marker Analysis By immunostaining fresh lymph-node biopsy tissue, tumor cells were found to be positive for Ki-1, HLA-DR, and 2D1 (common leucocyte) antigens, but were unreactive to monoclonal antibodies defining B-cells (B 1), T-cells (OKT3, OKT4, OKT6, OKT8, OKT11, Leu-1), NK cells (Leu7), and myeloid cells (OKM1, LeuM1, MCS2, My4, My9). However, immunohistochemical studies of formalin-fixed sections revealed that about 5% of the atypical cells in the lymph nodes were positive for monoclonal cytoplasmic immunoglobulin (λ chain), indicating that they are of B cell lineage. The immunologic phenotype of tumor cells in the ascites was identical with that of tumor cells in the lymph nodes. The KIS-1 line also showed almost identical immunologic features with those of the tumor cells both in the ascites and in the lymph nodes (Table I). The only difference was the

Table I. A Summary of Immunologic Markers

Antibody	CD	Specificity/Antigen	Results		
			LN	AC	KIS-1
B1	CD20	B cell	—	0 (%)	0 (%)
B4	CD19	B cell	NT	NT	46
OKIa1	—	HLA-DR antigen	+[a]	80[a]	100[a]
Leu1	CD5	T cell	—	0	0
OKT3	CD3	T cell	—	0	0
OKT4	CD4	Helper/inducer T cell	—	0	0
OKT6	CD1	Intrathymic T cell	—	0	0
OKT8	CD8	Suppressor/cytotoxic T cell	—	0	0
OKT11	CD2		—	0	0
OKM1	CD11	Monocyte; granulocyte	—	0	0
LeuM1	CD15	Monocyte; granulocyte	NT	0	0
MCS1	CD15	Granulocyte	—	0	0
MCS2	CD13	Monocyte; granulocyte	—	0	0
My4	CD14	Monocyte	—	0	0
Leu7	—	NK cell	—	0	0
R4/23	—	Dendritic reticulum cell	—	0	0
Ki-1	—		+[a]	98[a]	100[a]
J5	CD10	Common ALL antigen	—	0	0
2D1	—	Leucocyte	+[a]	96[a]	94[a]
Tac	CD25	IL-2 receptor	NT	42	0
Anti-lysozyme			—	0	0
Anti-human Ig					
S-Ig			NT	0	0
C-Ig			+[b]	+[b]	+[b]

LN=lymph node, AC=ascites, NT=not tested.
a) Most cells showed strong staining.
b) About 5% of the cells were positive for λ chain.

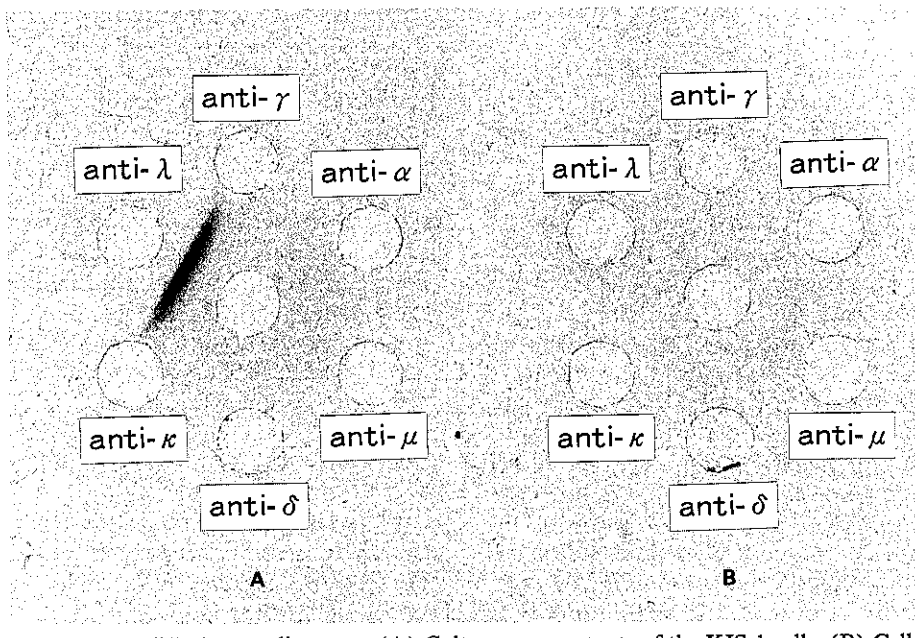

Fig. 4. Double diffusion gradient test. (A) Culture supernatants of the KIS-1 cells. (B) Cell-free culture medium.

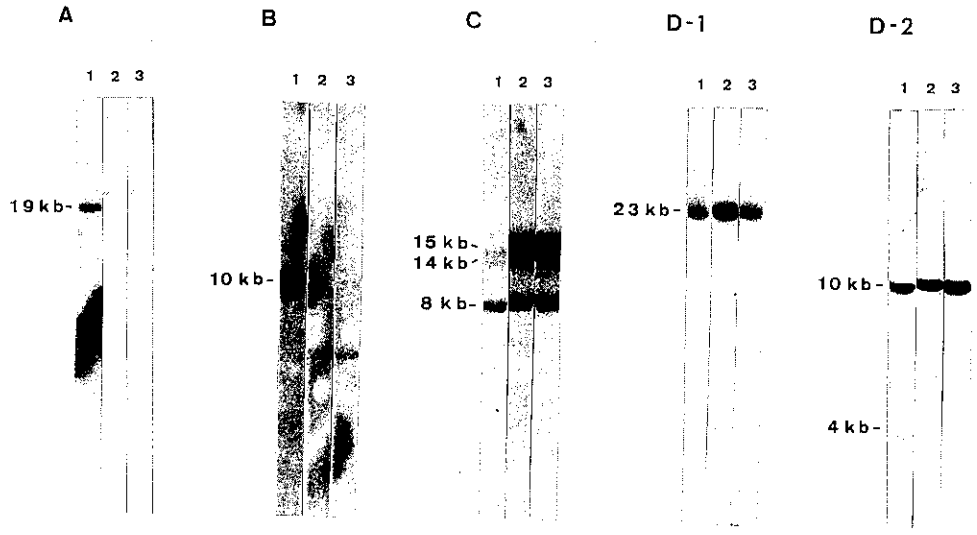

Fig. 5. Southern-blot analyses of KIS-1 cells with Ig JH (S), Ig Jκ (B), Ig Cλ (C), and T cell receptor Cβ1 (D-1, D-2) as probes. In Figs. A through D, left, middle, and right lanes are controls, ascitic tumor cells, and KIS-1 cells, respectively. In Figs. A, C, and D-2, DNA was digested with *Eco*RI. In Figs. B and D-1, DNA was cut with *Bam*HI. The control in Fig. A through C is MT-2 line. The control in Figs. D-1 and D-2 is BALL-1 line.

lack of Tac antigens on the KIS-1 cells. (Tac antigens were lost by the 14th day of culture, although their expression increased by 94% in the first 3 days of culture). Moreover, the KIS-1 cells secreted immunoglobulin λ chains into the culture medium (Fig. 4).

Molecular Genetic Analysis Figure 5 shows the results of Southern-blot analysis of the immunoglobulin genes and the T-cell receptor genes. The heavy chain genes of the ascitic tumor cells were rearranged. We could also demonstrate a single, faint band representing a rearranged Jκ gene, which led us to conclude that one κ allele was rearranged and the other deleted. Further, we were able to detect rearranged bands of λ chain genes, although we could not exclude the possibility of restriction fragment polymorphism. The T-cell receptor β chain gene showed a germ line configuration when tumor cell DNA was digested with *Eco*RI or *Bam*HI. As shown in Fig. 4, the electrophoretic pattern of the KIS-1 cells was identical with that of ascitic tumor cells in the analysis of both immunoglobulin genes and T-cell receptor genes.

Discussion

We have established a novel cell line, designated KIS-1, which has now been maintained in culture for over 12 months. As described in the results, the following findings indicate that KIS-1 cells were derived from Ki-1-positive large cell lymphoma: 1) KIS-1 cells had identical cytochemical and immunologic features with ascitic tumor cells, except for the absence of Tac antigen. 2) KIS-1 cells had chromosome anomalies in common with ascitic tumor cells. 3) In the analysis of the immunoglobulin and T-cell receptor β chain genes, KIS-1 cells showed an electrophoretic pattern identical to that of the ascitic tumor cells. 4) EBNA was not detectable in KIS-1 cells. 5) Ascitic tumor cells had immunologic characteristics identical with those of the cells in the lymph node. The absence of Tac antigens does not count against KIS-1 being derived from the tumor cells, since the expression of such functional molecules as IL-2 receptors can vary according to the cellular and environmental conditions.

Recent immunohistochemical studies[2] indicate that Ki-1-positive large cell lymphomas are mainly of T-cell origin, occasionally of B-cell origin, and seldom of histiocytic origin. In this patient, tumor cells are thought to be of B-cell lineage based on the following evidence. 1) About 5% of the cells in the lymph nodes and the ascites were positive for monoclonal cytoplasmic immunoglobulin (λ chain). 2) Tumor cells had rearranged heavy chain genes and rearranged κ genes (one allele deleted and the other rearranged). Moreover, they showed rearranged λ genes, although restriction fragment polymorphism could not be excluded. The KIS-1 line is also considered to be of B-cell lineage because it apparently originated from these tumor cells.

Stein *et al.*[2] recently suggested that Reed-Sternberg (RS) and Hodgkin (H) cells of Hodgkin's lymphoma were of T-cell or B-cell origin because they had similar phenotypic features to tumor cells of Ki-1-positive lymphoma. However, at least in this patient as well as several patients in a recent report,[11] the tumor cell phenotype was distinctly different from the RS and H cells of most types of Hodgkin's disease other than the lymphocyte predominance type, in the following ways. 1) Tumor cells were negative for LeuM1. 2) Tumor cells were positive for 2D1 (common leucocyte) antigen. Therefore, the relationship between tumor cells of Ki-1-positive lymphoma and RS and H cells needs further investigation and KIS-1 line may be useful for this purpose.

There are still several unsolved questions concerning Ki-1 antigens. 1) Although Ki-1 antigens can be expressed on B-cells and T-cells as well as RS and H cells, are these antigens the same molecules? 2) Although Ki-1 antigens are known to be induced by various stimuli, such as lectins and viruses, do they have any functional roles? 3) If Ki-1 antigens have some functional roles, how are they regulated by physiological substances? KIS-1 line is the only B-cell line derived from Ki-1 lymphoma, and may be useful in solving these questions, and other related problems. (Although several cell lines[12, 13] are known to express Ki-1 antigens, most of them are derived from other entities such as erythroleukemia, ALL, and Burkitt's lymphoma, and the others have not been proved to originate from Ki-1 lymphoma. Moreover, thier reactivity with Ki-1 antibody is lower than 30% and is weak, in contrast to KIS-1 line.)

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