Cytochemical, Immunologic, Chromosomal, and Molecular Genetic Analysis of a Novel Cell Line Derived From Hodgkin’s Disease

By Hiroshi Kamesaki, Shiroh Fukuhara, Eiji Tatsumi, Haruto Uchino, Hirohiko Yamabe, Hiroshi Miwa, Shigeru Shirakawa, Masakazu Hatanaka, and Tasuku Honjo

A novel cell line, KM-H2, was established from the pleural effusion of a patient with Hodgkin’s disease of mixed cellular type. Multiple phenotypic studies were carried out with this cell line. Acid phosphatase and nonspecific esterase activities were detected. Rosette formation with T lymphocytes and the receptors for C3b and Fc portion of IgG were positive. Among the antigens tested with a total of 22 monoclonal antibodies defining hematopoietic cell subsets or lineages, Ki-1, Leu-M1, MCS1, HLA-DR, and OKT9 antigens were found to be positive. The other antigens reportedly specific for T cells, B cells, natural killer (NK) cells, monocytes, interdigitating reticulum (IR) cells and dendritic reticulum cells were negative. These phenotypic features were identical to those of the Sternberg-Reed (SR) and Hodgkin (H) cells in the fresh materials reported by other researchers. Moreover, the KM-H2 cells and the parental pleural effusion cells shared several structural chromosome anomalies. These findings indicated that the KM-H2 cells are derived from the SR and H cells. Molecular genetic analysis of the KM-H2 cells disclosed that the human immunoglobulin J_κ gene was rearranged but not the JK gene, and that the human T cell receptor β chain gene was of the germline type. Based on these properties of the KM-H2 cells, Hodgkin’s disease may be derived from a cell lineage other than T cell or B cell.

Although the Sternberg-Reed (SR) and Hodgkin (H) cells are regarded as the tumor cells in Hodgkin’s disease, their nature and origin remain unclear. To clarify this question, a number of ultrastructural, histochemical, and immunologic studies have been performed on histologic sections or after separation from biopsy materials. As a result, several cell lineages of the hematopoietic system have been suggested as candidates for the origin of the SR and H cells: T lymphocytes, B lymphocytes, macrophages, granulocytes, and dendritic reticulum cells, as well as interdigitating reticulum (IR) cells.

Faced with the difficulties of characterizing the SR and H cells, many investigators have attempted to establish in vitro cell lines derived from the SR and H cells. However, this has been hampered because these cells usually constitute only a minor population and seem to grow poorly in the hitherto available culture systems. Several permanent cell lines have been reported, but most of these do not appear to have arisen from typical SR and H cells, with the exception of the L428 cell line.

We report here a novel cell line, KM-H2, established from the pleural effusion of a patient who was initially diagnosed as having Hodgkin’s disease of mixed cellular type. We also describe its cytochemical, immunologic, and molecular genetic properties, and discuss the cellular origin of Hodgkin’s disease.

MATERIALS AND METHODS

Establishment of the Cell Line

A 32-year-old man was admitted to our hospital in October 1986 with an enlarged left cervical lymph node. After biopsy of the lymph node, Hodgkin’s disease of the mixed cellular subtype was diagnosed (Fig 1A and B), and confirmed by two independent pathologists. After extensive examination, the case was identified as clinical stage IV and was successfully treated with combination chemotherapy consisting of prednisone, vinblastine, and cyclophosphamide. Five years later, an abdominal mass, ascites, and bilateral pleural effusion appeared. Cytological examination of the pleural effusion revealed that they were almost all atypical cells, including a number of Hodgkinoid cells (Fig 2A). The patient died 5 days after thoracentesis. At autopsy, mediastinal, paraaortic, and mesenteric lymph nodes were enlarged. Multiple nodules were observed in the lungs, liver, spleen, kidneys, pleura, and peritoneum. Microscopic examination of lymph nodes and nodules showed Hodgkin’s disease, lymphocyte depletion subtype (Fig 1C). The cells obtained by the thoracentesis were cultured at a concentration of 1 x 10^6 cells/mL in RPMI-1640 supplemented with 10% fetal calf serum (FCS), and 40% cell-free supernatant was obtained from the autologous pleural fluid. After 3 days, obvious cell growth was noted, with large floating clusters of cells. The cells were cultured in this condition for 2 weeks. Since then, they have been propagated in continuous culture for >5 years in RPMI 1640 with 10% FCS with a cell-doubling time of ~60 hours. The cell line was designated as KM-H2.

Detection of Epstein-Barr Virus

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

Chromosome Preparations

Chromosome analysis was performed on KM-H2 cells and the parental pleural effusion cells. Metaphase preparations were obtained after the incubation of 0.05 μg/mL of colcemid, and fixed with methanol acetic acid (3:1). Chromosomes were banded by the trypsin-Giemsa and the Quinacrine staining methods.

Cytochemical Methods

In addition to routine staining with May-Grünwald-Giemsa, the following cytochemical tests were performed on cytospin preparations: Peroxidase, acid phosphatase, alkaline phosphatase, α-naphthyl acetate esterase, and naphthol AS-D chloroacetate esterase.

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Fig 1.  (A) Mixed cell population lying between the interlacing fibroblasts. A classical Sternberg-Reed cell is apparent (H&E x 100).  (B) Higher magnification of a typical Sternberg-Reed cell (H&E x 400).  (C) Histological appearance at autopsy (H&E x 500).

Studies for Immunologic Markers

**Immunoperoxidase methods.** Paraffin sections were deparaffinized with xylene and graded alcohol and rehydrated with phosphate-buffered saline (PBS; pH 7.6), as in routine processing. Cytocentrifuged smears were fixed in acetone, at 4 °C, for 10 minutes prior to immunostaining. The staining procedures using anti-Leu M I or heteroantisera against lysozyme, α1-antitrypsin, and S100 protein have been described elsewhere in detail. The staining procedures with biotin-labeled peanut agglutinin were also performed as previously reported.

**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. Terminal deoxynucleotidyl transferase (TdT) was also stained by indirect immunofluorescence as previously described. The heteroantisera and monoclonal antibodies used in this study are summarized in Table 1. Immunofluorescence was judged under an Olympus fluorescence microscope.

**Rosette assays.** Cells bearing receptors for sheep erythrocytes, Fc portion of IgG and IgM, and complement were detected as reported previously. The T cell rosette assay was also performed as previously described.
Preparations of labeled probe DNA. The 3.4-kb EcoRI-HindIII fragment of the joining region of the heavy chain gene and the 2-kb SacI–Sac fragment of the joining region of the k chain gene were used as JH and Jk probes, respectively. The 3-kb EcoRI-HindIII fragment of the constant region of the T cell receptor β chain was also used as a Cβ probe. Nick-translated, 32P-labeled probes were hybridized to nitrocellulose filters in 5 × Denhardt's solution, 0.9 mol/L NaCl, 50 mmol/L of phosphate buffer (pH 7.7), and 5 mmol/L of EDTA, at 65 °C. After hybridization, the washed filters were exposed to film.

RESULTS

Morphological Findings

The KM-H2 cells appeared as clusters of large, nonadherent cells without villi in phase-contrast microscopy. In May-Grünwald-Giemsa-stained cytospin preparations, these cells had one to three round to oval nuclei with conspicuous nucleoli. Their cytoplasms were stained palely to moderately basophilic and sometimes contained vacuoles (Fig 2B). Their appearance was similar to that of the original pleural effusion cells.

Immunofluorescence Tests for EBNA

The KM-H2 cells were tested in triplicate for EBNA. All observations were negative.

Cytogenetic Findings

The established cell line was composed mainly of cells with 49 to 67 chromosomes, with a minor population having 44, 88, or >100 chromosomes. Karyotypes of KM-H2 cells were highly complex and unstable; however, they were compatible with those of the parental pleural effusion cells, which had near triploid and polyploid sets (Fig 3A). The banded analysis revealed various structural and numerical chromosome aberrations as shown in Fig 3B. The clonal origin of the KM-H2 cell line was ascertained by identifying common marker chromosomes: Sp+, 6p+, and 14p+. Ten years after the initial cultivation, the Sp+, 6p+, and 14p+ chromosomes were still preserved in all KM-H2 cells.

Cytochemical Findings

The original pleural effusion cells were negative for peroxidase reaction. Enzyme cytochemistry (Table 2) did not demonstrate any peroxidase, alkaline phosphatase, or naphthol AS-D chloroacetate esterase activity in the KM-H2 cells. A weak reaction for acid phosphatase and α-naphthyl acetate esterase was observed in the paranuclear regions of most cells. In a few cells, these reactions were distributed throughout the cytoplasm.

Immunologic Marker Analysis

By immunostaining of the lymph node biopsy from the patient, the SR and H cells were stained distinctly for Leu-M1 and faintly for peanut agglutinin. They were negative for cytoplasmic lysozyme, α1-antitrypsin and S100 protein. Moreover, the KM-H2 cells revealed the same staining pattern as these SR and H cells in situ (Table 3).
As demonstrated in Table 4, the reactivity of the KM-H2 line was almost identical to that of the SR and H cells in the fresh biopsy material reported by others.48,49 They expressed Ki-1, Leu-M1, HLA-DR antigens and receptors for T cells, transferrin, C3b, and Fc portion of IgG, but lacked markers characterizing B cells (surface Ig, cytoplasmic Ig, B1), T cells (receptors for sheep erythrocytes, Leu-1, OKT3, OKT4, OKT6, OKT8), monocytes (OKM1, MCS2, My4, My7, lysozyme, α1-antitrypsin), dendritic reticulum cells (R4/23), IR cells (OKT6, S100 protein), and NK cells (Leu-7, Leu-11, OKM1). The expression of MCS1 antigens is also compatible with the above results because granulocyte-specific antigens, such as TU9, 3C4, and VIM-D5, are detected in freshly obtained SR and H cells.6

Phagocytic Activity

The KM-H2 line did not exhibit phagocytosis of C3b-coated zymosan beads or IgG-coated erythrocytes or India ink particles.

Heterotransplantation in Nude Mice

Subcutaneous inoculation of the KM-H2 line failed to induce tumors in five attempts.

Molecular Genetic Analysis

Figure 4 shows the results of Southern-blot analysis of the immunoglobulin genes and the T cell receptor genes. The JH segment of BamHI-digested DNA from the KM-H2 cells was observed as a single band at 10-kb, indicating the presence of a heavy chain gene rearrangement; the Jk segment digested by BamHI was detected at 10 kb, however, and was considered as a germline of the κ chain gene. Gene rearrangement of the T cell receptor β chain did not occur in the DNA digested with EcoR1 or BamHI.

DISCUSSION

In an effort to determine the origin of the SR and H cells in Hodgkin’s disease, many investigators have attempted the establishment of permanent cell lines from tissues and body fluids of patients with Hodgkin’s disease, but whether the cell lines established derived from SR or H cells seems to be open to question. In fact, several authors48,50 appear to have erroneously identified the overgrowth of EBV-transformed lymphoblastoid cells as SR and H cells because of their morphologic similarity. Moreover, Harris and colleagues51 have recently proved that several lines are not related to Hodgkin’s disease but are non-human contaminants.

Three other cell lines deserve further discussion. One is the
SU/RH-HD-1 cell line described by Olsson and co-workers. The neoplastic nature of this line is apparent from the presence of chromosomal aberrations and the absence of the EBNA reaction. Its human origin is also indicated by the presence of the “alu” family in its DNA. However, the fact that this line does not react with Ki-1 or Leu-M1, which is reported to stain SR and H cells from all cases of Hodgkin’s disease, obscures its relation to the SR and H cells.

The other cell line, established by Poppema and colleagues, is the DEV cell line. Their extensive studies clearly indicate this line’s neoplastic nature and its close relation to the SR and H cells in the patient’s lymph node, but the atypical immunologic phenotype of the original SR and H cells, such as staining with B1 or anti-α2 chain antibody, seems to restrict its importance as a representative of the SR and H cells.

Another cell line, L428, has been reported by Schaad and co-workers. It was grown from the pleural effusion of a patient with advanced Hodgkin’s disease of nodular sclerosing type. Its malignant nature is apparent from multiple structural and numerical chromosome abnormalities associated with a monoclonal pattern of multiple marker chromosomes. In addition, its cytochemical and immunologic features were identical to those of the SR and H cells in the fresh biopsy materials. Therefore, this line appears to be the only one hitherto proved to be derived from the SR and H cells.

Table 2. Cytochemical Findings

<table>
<thead>
<tr>
<th></th>
<th>KM-H2 cells</th>
<th>SR and H cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>α-NAE</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>CAE</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

α-NAE, α-naphthyl acetate esterase; CAE, naphthol AS-D chloroacetate esterase.

*Data on SR and H cells were taken from previous reports.

Table 3. Immunohistochemical Findings

<table>
<thead>
<tr>
<th></th>
<th>KM-H2 cells</th>
<th>SR and H cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Leu-M1</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S100 protein</td>
<td>—</td>
<td>—</td>
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</table>

PNA, peanut agglutinin.
*SR and H cells in the patient’s lymph node.
Table 4. A Summary of Immunologic Properties

<table>
<thead>
<tr>
<th>Property</th>
<th>KM-H2 Cells Positive (%)</th>
<th>SR and H cells*</th>
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<tbody>
<tr>
<td>Sheep erythrocyte receptor</td>
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<td>−</td>
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<tr>
<td>Fcy receptor</td>
<td>18</td>
<td>±</td>
</tr>
<tr>
<td>Fcγ receptor</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>C3b receptor</td>
<td>8</td>
<td>±</td>
</tr>
<tr>
<td>Receptor for T cells</td>
<td>56</td>
<td>+</td>
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<tr>
<td>Surface immunoglobulin</td>
<td>0</td>
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<tr>
<td>Cytoplasmic immunoglobulin</td>
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<td>(±)?</td>
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<tr>
<td>OKla1</td>
<td>100 (strong)</td>
<td>+</td>
</tr>
<tr>
<td>B1</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>Leu-1</td>
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<tr>
<td>OKT3</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>OKT4</td>
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</tr>
<tr>
<td>OKT6</td>
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</tr>
<tr>
<td>OKT8</td>
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<td>−</td>
</tr>
<tr>
<td>OKT9</td>
<td>91 (Strong)</td>
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</tr>
<tr>
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<td>Leu-M1†</td>
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<td>+</td>
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<tr>
<td>MCS1</td>
<td>48</td>
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<td>0</td>
<td>−</td>
</tr>
<tr>
<td>R4/23</td>
<td>0 (Weakly positive?)</td>
<td>−</td>
</tr>
<tr>
<td>Ki-1</td>
<td>96</td>
<td>+</td>
</tr>
<tr>
<td>J5</td>
<td>0</td>
<td>Not known</td>
</tr>
<tr>
<td>2D1</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>Peanut agglutinin†</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Lysozyme†</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α1-antitrypsin†</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>S100 protein†</td>
<td>−</td>
<td>−</td>
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*Data for SR and H cells are taken from previous reports.45-48
†Tested by the immunoperoxidase method.

Our KM-H2 cell line was established from the pleural fluid of a patient whose diagnosis was histologically confirmed by two independent pathologists. This diagnosis was supported by the finding that the "SR and H cells" in the patient's lymph node were distinctly positive for Leu-M1.14 This line's neoplastic nature was indicated by the presence of structural and numerical chromosome aberrations and by the absence of the EBNA reaction, although tumor formation after subcutaneous inoculation of KM-H2 cells was not observed. Moreover, the KM-H2 cells showed the same immunostaining pattern as the SR and H cells in the patient's lymph node and shared nine positive properties and a number of negative properties with the SR and H cells in the fresh biopsy materials reported by other authors44-48 (Tables 2 and 4). The cells also retained some of the chromosomal abnormalities observed in the pleural effusion cells. Thus, it seems reasonable to conclude that the KM-H2 cell line originated from the SR and H cells of Hodgkin's disease.

Recent advances in molecular biology have made it clear that somatic recombinations of the immunoglobulin genes occur in B cell ontogeny. Korsmeyer and co-workers33,54 proposed a model of genetic maturation in human B cell lineage and revealed that analysis of the immunoglobulin gene rearrangement was useful to determine the origin of leukemias and lymphomas.55 Recently, several authors56,57 have also reported that the T cell antigen receptor genes have a genomic structure similar to that of the immunoglobulin genes and that somatic recombinations of these genes take place in T cell differentiation. Because T cell receptor gene rearrangement is expected to occur only in T cell lineage, the analysis of this gene appears useful to identify the cellular origin of hematopoietic tumors.58,59 Therefore, we used these molecular genetic approaches to analyze the cellular origin of the KM-H2 line, which is still obscure in spite of extensive cytochemical and immunologic studies.
NOVEL CELL LINE DERIVED FROM HD

The J4 region of the immunoglobulin genes in this line was rearranged, but not the Jk region or the constant region of the T cell receptor β chain genes. The germline configuration of the T cell receptor β chain genes, as well as the lack of the T cell antigens, virtually excludes the possibility of its T cell origin. However, the presence of only J4 recombination is not sufficient to conclude that it is of B cell lineage, since such a genomic pattern is also observed in some myelogenous leukemia cells.50,61

Indeed, the following observations favor its origin from the nonlymphoid cell lineage rather than from the B cell lineage, in contrast to recent reports52,62 that support the B cell origin for the nodular sclerosing subtype of Hodgkin’s disease: (a) The expression of common ALL antigens or TdT, which is usually observed at this stage of B cell differentiation, could not be detected in the KM-H2 cells; (b) neither cytoplasmic Ig nor B1 antigens could be induced by the presence of β-phorbol-12-myristate-13 acetate (TPA) (unpublished observations); (c) Leu-M1 and MCS1 antigens were expressed, and (d) a reaction for acid phosphatase and α-naphthyl acetate esterase was observed in most cells. Therefore, our results suggest the heterogenous cellular origin of Hodgkin’s disease, especially among the histological subtypes, although further studies are needed.

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