Establishment of Epstein-Barr Virus-negative Diffuse Large Cell Lymphoma Cell Line with an 8;22 Chromosomal Translocation

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ABSTRACT

A new human B-cell lymphoma cell line was established from a pleural effusion of a patient with a diffuse large cell lymphoma, originating from an ileocecal tumor. The cell line, designated KAL-1, has been passaged 280 times over a period of 22 months. This cell line was successfully maintained in a chemically defined serum-free medium; its doubling time is approximately 24 h. Immunologically, the cells were demonstrated to express IgM on the cell surface and to react with monoclonal antibodies to B-cell antigen including B1, B4, HLA-DR, and common acute lymphoblastic leukemic antigen but not with B2 and all the T-cell markers. Immunoglobulin gene analysis revealed rearrangements of both JH and Ca. These data indicate that this cell line represents the B-cell lineage at the immature B-cell stage. This cell line was negative for Epstein-Barr virus nuclear antigen and had no detectable Epstein-Barr virus genome in cellular DNA. Chromosome analyses revealed that the cells carried an 8;22 chromosome translocation, reminiscent of variant type Burkitt's lymphoma. However, there was no historical evidence for Burkitt's lymphoma. Molecular studies showed that KAL-1 had deregulated high constitutive expression of c-myc. This cell line was demonstrated to be highly tumorigenic when injected into athymic nude mice. This tumor model should provide clues about the molecular mechanism involved in the pathogenesis of B-cell malignancy and appears to be a useful in vivo model for the study of molecular events during B-cell differentiation and therapeutic investigations.

INTRODUCTION

Tumor development in vivo is a progressive, multistep process, which occurs through qualitatively different stages (1, 2). Recent cytogenetic and molecular analysis elucidated the point that chromosomal alterations should be a critical step in the pathogenesis of human cancer (3, 4). The well-studied example involved in such genetic changes is the case of Burkitt's lymphomas, characterized by specific reciprocal chromosomal translocations between the c-myc protooncogene on chromosome 8 and the immunoglobulin heavy chain locus on chromosome 14, t(8;14) or, in the case of variant translocation, either the κ or λ immunoglobulin light-chain loci on chromosome 2, t(2;8), and chromosome 22, t(8;22), respectively (5-7). The juxtaposition of c-myc to one of the immunoglobulin genes, as a consequence of these translocations, alters the expression of the c-myc gene, resulting in deregulation of cellular proliferation. Although such molecular genetic findings in Burkitt's lymphoma have provided valuable clues about the molecular mechanisms involved in the pathogenesis of human B-cell malignancy, the detailed mechanisms remain unknown (4, 8, 9).

Establishment of a well-characterized human B-cell lymphoma cell line is of paramount importance for studying such deregulated growth mechanisms. A number of B-lymphoma cell lines including Burkitt's type have been reported, and the majority of them are EBV-positive. To establish an EBV-negative B-lymphoma cell line from non-Burkitt's lymphomas is extremely difficult (10-12). Although EBV-negative cell lines have been established mostly from sporadic Burkitt's lymphoma, most of them bear an 8;14 translocation (13, 14). Only one report included detailed descriptions of characteristics of a variant type (EBV-negative) Burkitt's lymphoma cell line with a specific translocation t(8;22) (15). With this cell line, c-myc transcripts and the break point on chromosome 8 have been extensively analyzed (16).

Recently, we have established a new human EBV-negative lymphoma cell line, designated as KAL-1, from a malignant pleural effusion of a patient with diffuse large cell lymphoma. Interestingly, this cell line contained a specific chromosomal translocation t(8;22) and was shown to have a highly tumorigenic potential. We now describe in detail the characterization of the KAL-1 cell line and its relevance to variant type Burkitt's lymphoma.

MATERIALS AND METHODS

Establishment and Maintenance of the Cell Line. The lymphoma cells were obtained from the pleural effusion of a 37-year-old man with diffuse large cell lymphoma, originating from an ileocecal tumor, according to the New Histologic Formulation (17). He was admitted to our hospital in July 1988 for evaluation of an abdominal mass. Excisional biopsy confirmed the diagnosis. He was treated with combination therapy, without response, and died in November 1988. Approximately 100 ml of heparinized effusion were immediately centrifuged and the pellet was washed in phosphate-buffered saline. Then the cells were separated by Ficoll-Hypaque (density, 1.077 g/liter) centrifugation and incubated in IMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated FCS (GIBCO Laboratories, Life Technologies Inc., Grand Island, NY) and 50 μg/ml of gentamicin. The cells were maintained in a 75-cm² tissue flask (Falcon 3024) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Clumps of cells were present after 4 weeks and the cells were subcultured by 1:5 dilution with fresh complete medium every 3 to 4 days. Once the cell line was established, the cells were also tested for growth in a commercially available serum-free medium (S-Clene SF-H; Sanko Junyaku, Tokyo, Japan).

Morphological and Cytological Staining. Cytospin preparations were used for May-Grünwald-Giemsa, periodic acid-Schiff, and acid-phosphatase stainings, performed by standard methods.

Electron Microscopic Examination. Cells were fixed with 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), dehydrated in ethanol, and embedded in Epon 812 (TAAB). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and viewed under a JEOL 100CX electron microscope.

EBNA Assay. EBNA was assayed according to the methods of Reedman and Klein (18). Using a EBV virus DNA probe (ENZO Diagnostics, NY), the integration of EBV genome into the KAL-1 cells was investigated by Southern blot analysis of purified cellular DNA subjected to digestion with the restriction enzyme EcoRI as described (19).

The abbreviations used are: EBV, Epstein-Barr virus; CALLA, common acute lymphoblastic leukemic antigen; IMEM, Iscove's minimum essential medium; FCS, fetal calf serum; EBNA, Epstein-Barr virus nuclear antigen.
RESULTS

Establishment of KAL-1 Cells. After 4 weeks in a quiescent state, the cells gradually began to proliferate in suspension, forming small clumps. Initial successful subculture was performed 6 weeks after initiation. Thereafter, the cells had been subcultured over 280 passages in 22 months, by diluting 1:5 with IMEM containing 10% heat-inactivated FCS. The cell line was designated KAL-1 and demonstrated to be free of Mycoplasma contamination. This cell line was successfully maintained in a chemically defined, serum-free medium, with a comparable growth rate as shown in serum-supplemented medium.

Morphological and Cytological Staining. Histological sections of the original ileocecal tumor from the patient revealed monomorphic lymphomatous proliferation with a high nuclear:cytoplasmic ratio, prominent nucleoli, and a high mitotic index, indicating a feature of malignant lymphoid tumor (Fig. 1A). However, the starry sky macrophages were not seen in all sections, and there was no evidence from the histopathological features that this malignant lymphoid tumor was derived from Burkitt’s lymphoma. Furthermore, the tumor sections obtained from the transplants into nude mice displayed the same morphological characteristics seen in the original tumor (Fig. 1A and B). The invasive cells from the pleural effusion revealed that abnormal cells had a morphology similar to that of the original tumor as shown in Fig. 1C. Cytocentrifuge preparations of KAL-1 cells were negative for stainings of peroxidase, acid phosphatase, and alkaline phosphatase but positive for PAS staining (data not shown). An electron micrograph of KAL-1 cells showed that the cells had irregular nuclei and a scanty cytoplasm with poor membrane organelles and rich polyribosomes. No viral particles were observed in both nuclei and cytoplasm (Fig. 1D).

Cell Marker Analysis. The results of immunophenotypic studies were summarized in Table 1. The cells from the patient’s pleural effusion, KAL-1 cells after 4 months of continuous passage, and the cells from the tumor implanted into nude mice were tested. The cells from each source were all strongly positive for B1 (CD20), B4 (CD19), CALLA (CD10), and HLA-DR, except for B2 (CD21) and all the T-cell lineage markers. Cyto- 

Estimates of growing tumors were plotted versus days after inoculation, and the tumor-doubling time in vivo was calculated from the resulting graph. Sections from the neoplastic tumor were fixed in 10% formalin, stained with hematoxylin-eosin, and examined by light microscopy.

Molecular Genetic Analysis. High molecular weight DNA was isolated from 2 x 10⁷ KAL-1 cells, digested with EcoRI, BamHI, or HindIII restriction endonuclease, and subjected to electrophoresis on a 0.8% agarose gel, 10 µg DNA/lane. The concentration of DNA was determined by absorbance at 260 nm. The running buffer was 40 mM Tris-acetate, pH 7.8–1.1 mM EDTA. The DNA was blotted onto nitrocellulose by Southern (23) and hybridized with a 32P-labeled DNA probe in a solution of 50% formamide-7 mM Tris, pH 7.4–4× standard saline-citrate at 42°C for 24 h. The following DNAs were used as probes: a 5.6-kilobase BamHI-HindIII fragment containing the immunoglobulin heavy chain joining (JH) region (24); a small 0.8-kilobase fragment of a Cβ region (25); a 1.8-kilobase Clal-EcoRI fragment containing the third exon of human c-myec (26); and a 650-base pair fragment of a chicken complementary DNA encoding histone H2B (27).

Total cell RNA was extracted from 1 x 10⁶ cells by lysis with guanidine isothiocyanate and ultrasound, followed by ethanol precipitation (28). Approximately 10 µg of the total RNA were electrophoresed through 1% formaldehyde/agarose gels and transferred to nitrocellulose for analysis with a human c-myec DNA probe described as above. Autoradiography was done using Kodak XAR-5 film at -70°C for 5 days.

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KAL-1 cell line produced detectable tumor formation 3 weeks after injection and the average tumor weight was 1.53 ± 0.53 (SD) and 1.76 ± 0.51 g for 2.0 × 10⁷ and 1.0 × 10⁸ cells/inoculation, respectively. Histological sections revealed identical features to the original ileocecal tumor as described above. Tumors attained an average weight of 44 g over a 7-week average survival time. The doubling time of in vivo tumor growth was estimated as approximately 60 h. Almost all the nude mice bearing large tumors died within 8 weeks.

Southern Blot Analysis. Cellular DNA from the KAL-1 cell line was analyzed for immunoglobulin gene rearrangement using the heavy chain probe (JH) and the light chain probe (CL), digested with BamHI and EcoRI, respectively. Fig. 4 demonstrated that immunoglobulin gene rearrangement for both JH and CL probes were detected. The detection of these immunoglobulin gene rearrangements would be consistent with the monoclonal population of neoplastic B-cells in the KAL-1 cell line. Furthermore, DNA samples from KAL-1 cells had no detectable rearrangements for T-cell receptor gene TCR Cβ and TCR Jγ using the restriction enzyme EcoRI (data not shown). In addition, integration of EBV DNA into cellular DNA of KAL-1 was not detected by Southern blot analysis using an EBV probe (data not shown).

Molecular Analysis of c-myc Gene in KAL-1 Cells. From the evidence that KAL-1 cells had a specific reciprocal translocation involving chromosomes 8 and 22, as noted in the variant type of Burkitt’s lymphoma, structural rearrangement and expression of the c-myc gene were studied. As shown in Fig. 5A, the rearrangement of c-myc was not detected on Southern blot analysis. Northern blot analysis indicates that a higher level of c-myc expression in KAL-1 cells were detected than that expressed in K-562 cells, which were demonstrated to have a high level of c-myc expression (29) (Fig. 5B). An approximately 4.4-fold relative integrated intensity of c-myc RNA was observed in KAL-1 cells compared with that in K-562 cells when quantitated by a scanning densitometer.

Table 1 Immunophenotype in KAL-1 cells

<table>
<thead>
<tr>
<th>Markers</th>
<th>Pleural effusion</th>
<th>Cell line</th>
<th>Nude mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 (CD20)</td>
<td>60²</td>
<td>61</td>
<td>85</td>
</tr>
<tr>
<td>B2 (CD21)</td>
<td>ND*</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>B4 (CD19)</td>
<td>85</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>CD2</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CD3</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>CD5</td>
<td>1</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CD8</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>J-5 (CD10)</td>
<td>63</td>
<td>75</td>
<td>98</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>99</td>
<td>99</td>
<td>94</td>
</tr>
<tr>
<td>TdT</td>
<td>ND</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Anti-immunoglobulin</td>
<td>ND</td>
<td>75 lgM</td>
<td>80 lgM</td>
</tr>
<tr>
<td>Anti-α</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-λ</td>
<td>ND</td>
<td>77</td>
<td>89</td>
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</table>

* Percentage of positive cells.
* ND, not determined.
DISCUSSION

The KAL-1 cell line was newly established from a pleural effusion of the patient with a diffuse large cell lymphoma, which primarily originated from an ileocecal tumor and successfully cultured in serum-free medium. The cell line and the patient’s tumor cell had the same morphological features and immunological types, indicating that both were derived from the same origin. Extensive characterization of KAL-1 cell line revealed that this cell line represented the B cell lineage at the immature B cell stage because it had the cell surface marker of B1 (CD20), B4 (CD19), CALLA (CD10), HLA-DR, and the expression of surface IgM and the rearrangements for \( J_H \) and \( C_\lambda \) on Southern blot analysis. Furthermore, this cell line had no detectable rearrangements for TCR \( \beta \) and TCR \( \gamma \). The detection of immunoglobulin gene rearrangements also indicated that this cell line is of monoclonal origin.

Cytogenetically, KAL-1 cells had a diploid karyotype carrying the 8;22 chromosome translocation (q24;q11) as described in approximately 15% of Burkitt’s lymphomas (30, 31), whereas KAL-1 cells lack the expression of EBNA and B2 antigen (receptor for EBV) and have no detectable EBV genome on Southern blot analysis. It is known that most of endemic Burkitt’s lymphoma in Africa contained EBV DNA and that sporadic Burkitt’s lymphoma, which occurred rarely in Europe and the United States, often had no EBV DNA (32). The clinical differences between them have been reported (32-34), even though histological identity exists between endemic and sporadic Burkitt’s lymphoma (33-35). Histologically, Burkitt’s lymphoma consists of diffuse small noncleaved lymphoid cells. In the case reported here, however, both the original tumor and the s.c. tumor produced by implantation of KAL-1 cells into nude mice showed a diffuse large cell type histology without the starry sky characteristic, being histologically distinct from Burkitt’s lymphoma, despite having the cytogenetic property of Burkitt’s lymphoma. Although the normal cellular counterpart of Burkitt’s lymphoma has not been definitively identified, it is suggested that a translocation event could occur at different stages of B-cell maturation from pre-B-cells which are actively rearranging their immunoglobulin genes to immature B-cells which have recently began to express surface immunoglobulin, thus accounting for the variability in the histological types. Therefore, it is likely that diffuse large cell lymphoma with aggressive proliferative potential may carry a genetic alteration similar to that of the KAL-1 cell line. Further study would be
The specific chromosomal translocation in Burkitt's lymphoma has been shown to involve the c-myc locus on chromosome 8 and the immunoglobulin heavy chain locus on chromosome 14, or more rarely the \( \kappa \) or \( \lambda \) immunoglobulin light-chain loci on chromosomes 2 and 22, respectively (5–7). As a consequence of these translocations, c-myc becomes abnormally expressed. Molecular studies showed that KAL-1 had deregulated high constitutive expression of c-myc RNA. It is suggested that c-myc oncogene might be activated by its close proximity to a \( \lambda \) immunoglobulin light-chain locus on chromosome 22 since KAL-1 cells produced IgM with a \( \lambda \)-type light chain on the cell surface. However, Magrath et al. (36) reported that three Burkitt's-like lymphomas with acquired immunodeficiency syndrome synthesized the \( \kappa \) type despite the specific chromosome translocation (t(8;22)), suggesting that the activation of c-myc oncogene might occur independently of the immunoglobulin genes expressed on cell surface. Furthermore, they demonstrated that anti-immunoglobulin antibodies against the expressed cell surface immunoglobulin chains of some Burkitt's lymphomas bearing t(8;14) inhibit the proliferation of their cell growth and the expression of c-myc and \( \mu \)-heavy chain mRNA, indicating that the deregulated c-myc expression can be down-regulated by anti-immunoglobulin (37). They reported that such effects are not observed in the EBV-positive Burkitt's lymphoma cell lines with t(8;22), possibly because of being more mature than those with t(8;14). In this regard, we observed that anti-immunoglobulin toward the \( \lambda \) light chain did not inhibit the growth of KAL-1 cells, consistent with these findings (data not shown).

In the present study, KAL-1 cells were shown to be highly tumorigenic in vivo in nude mice. The doubling time in vivo was approximately 60 h. Although it is suggested that both in vitro and in vivo malignant potential could be correlated with the expression of c-myc oncogene, tumorigenicity cannot be explained merely by the simple constitutive expression of the c-myc oncogene, which appears to be associated with the immortal proliferation of most if not all somatic cells (38). Lombardi et al. (39) proposed the hypothesis that both EBV infection and c-myc activation are sufficient for the tumorigenic conversion of human B-cells in vitro using the experiment of c-myc transfection into human EBV-infected lymphoblastoid cells. Schwartz et al. (40) demonstrated that ras and c-myc oncogenes can transform murine B-lymphocytes. Since the KAL-1 cell line does not carry the EBV genome, the highly tumorigenic potential of KAL-1 suggests the presence of another mechanism by which KAL-1, together with c-myc activation, may become tumorigenic. Recently, Wolf et al. (41) demonstrated that somatic cell hybrids between endemic Burkitt's lymphoma and nonmalignant EBV-immortalized lymphoblastoid cells become nontumorigenic despite the continued deregulated c-myc expression. They concluded that EBV infection and c-myc deregulation may not be sufficient for tumorigenicity. Several groups observed that partial trisomy for Iq including the duplication of the chromosome segment of Iq had more advantage for tumorigenicity (42–44). According to the cytogenetic studies, KAL-1 cells had a duplication of chromosome 1q(q21→q32). In this regard, the highly tumorigenic potential of KAL-1 cells should be associated with this chromosomal abnormality as well as the specific chromosomal translocation (t(8;22)). Although the detailed mechanisms of tumorigenicity are still unknown, the KAL-1 cell line should provide valuable clues about the molecular mechanisms involved in the pathogenesis of human malignancy. Moreover, this cell line appears to be a useful in vitro and in vivo model for the study of the molecular events during B-cell differentiation as well as the therapeutic efficacy of anticancer drugs.

**Table 2 Transplantability of KAL-1 cells into nude mice**

<table>
<thead>
<tr>
<th>No. of KAL-1 cells</th>
<th>Transplantability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 × 10⁶</td>
<td>2/2 (100)*</td>
</tr>
<tr>
<td>2.0 × 10⁶</td>
<td>3/5 (60)</td>
</tr>
<tr>
<td>3.5 × 10⁶</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>1.0 × 10⁷</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11/13 (85)</strong></td>
</tr>
</tbody>
</table>

* Results are expressed as number of mice with tumors/total number of mice inoculated with KAL-1 cells.
* Numbers in parentheses, percentage.

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