Hematopathology / Cyclin A in Hodgkin Lymphoma Cells

Aberrant Expression of Cyclin A Correlates With Morphogenesis of Reed-Sternberg Cells in Hodgkin Lymphoma

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Abstract

Reed-Sternberg (RS) cells represent a histopathologic hallmark for Hodgkin lymphoma (HL). Viral proteins may induce aberrant expression of cyclin A and lead to multinucleation in virus-infected cells. We investigated whether Epstein-Barr virus (EBV) latent membrane protein-1 (LMP1) and cyclin A are involved in the morphogenesis of RS cells. We immunohistochemically analyzed “individual” tumor cells in 34 HLs for the subcellular expression of cyclin A and HL-related markers. In LMP1+ and LMP1− HLs, multinucleated RS cells aberrantly expressed cyclin A in cytoplasm, while the mononuclear Hodgkin cells expressed cyclin A predominantly in nuclei (P < .001). No differential expression of CD15, CD30, or CD99 in HL cells was found. In vitro, EBV-LMP1 increased cytoplasmic cyclin A expression and multinucleation in an HL cell line. Therefore, the aberrant expression of cyclin A is commonly associated with RS cell morphologic features in HL, probably through LMP1 signaling or other similar mechanisms in EBV− cases.

Hodgkin lymphoma (HL) is characterized morphologically by the presence of a spectrum of neoplastic cells: mononuclear Hodgkin (H) cells, multinucleated Reed-Sternberg (RS) cells, and mummified cells against an inflammatory background.1 Although RS-like cells may occur in diseases other than HL, such as infectious mononucleosis,2 RS cells remain the diagnostic hallmark for classical HL. H and RS cells and mummified cells are thought to be proliferative and apoptotic, respectively.2-4 However, the interrelationship among these variant HL cells remains enigmatic. It has been suggested that multinucleated RS cells are derived from mononuclear H cells through centrosome overduplication and disturbance of cytokinesis.5,6 Immunophenotypically, classical HL cells express CD15 and CD30 7; however, little is known about the cell biology and gene expression underlying the morphogenesis of RS cells.8,9

Studies have found that the accumulation of viral proteins in acutely or latently infected cells can show profound morphologic changes and multinucleation. Epstein-Barr virus (EBV)-encoded latent membrane protein-1 (LMP1) transfected into HL cell lines promoted RS cell formation.6 LMP1 expressed in B lymphoblastoid cell lines down-regulated CD99 expression, which subsequently resulted in the generation of typical multinucleated RS-like cells.5,10 More recently, LMP1 reprogrammed primary tonsillar germinal center B cells toward an RS-like phenotype.11 In addition, the Tax oncoprotein of human T-cell leukemia virus type 1 (HTLV-1) transformed infected cells into leukemic cells with multilobated nuclei (flower cells).12

Multinucleation and the formation of multilobated nuclei are closely related to cell cycle disturbance and centrosome amplification.13,14 In the host genes associated with
cell cycle progression, the aberrant expression of cyclin A has been linked to virus-associated genomic instability and oncogenesis in virus-infected cells.\textsuperscript{15-18} Aberrant cyclin A expression in cytoplasm also disturbs centrosome duplication and leads to multinucleation in hepatocytes that express hepatitis B virus pre-S2 mutant protein.\textsuperscript{16} These observations raise the possibility that EBV infection and LMP1 expression may potentially induce multinucleation through aberrant cyclin A expression. In this study, we therefore evaluated the expression patterns of cyclin A in individual RS cells, H cells, and mummified cells in HL and analyzed the correlation of cyclin A expression with EBV-LMP1. The expression patterns of 3 HL-related markers—CD15, CD30, and CD99—were also included for comparison. Furthermore, an in vitro experiment in which LMP1 was transfected into an HL cell line, L-428, was performed to clarify the role of LMP1 expression in the multinucleation formation of RS cells.

Materials and Methods

Study Subjects

We retrospectively reviewed the histories of patients with HL and enrolled 34 cases. They were consecutive cases, and all were diagnostic biopsies in untreated patients. The male/female ratio was 22:12, and the mean age was 36.7 years. All cases were classified according to the World Health Organization classification scheme.\textsuperscript{7} The distribution of each subtype was as follows. Nodular lymphocyte predominant HL accounted for 3% of the cases (n = 1), nodular sclerosis for 68% (n = 23), mixed cellularity for 15% (n = 5), lymphocyte-rich classic for 12% (n = 4), lymphocyte depletion for 0% (n = 0), and unclassified HL for 3% (n = 1). Our study protocol was approved by our institutional review board and was in accordance with the Helsinki Declaration of 1975, as revised in 1983. The tissue specimens were analyzed and fixed in 10% neutral formalin solution.

EBER In Situ Hybridization

EBV positivity was determined by using in situ hybridization to detect EBV-encoded RNA (EBER).\textsuperscript{19,20} The test for nucleotide integrity was performed by the use of the RNA positive control probe (Ventana Medical Systems, Tucson, AZ). The intended target was the poly A tail in messenger RNA found in nuclei.

Immunohistochemical Staining

Immunohistochemical staining was performed on deparaffinized tissue sections of formalin-fixed material. Detection was done with streptavidin-biotinylated peroxidase-conjugated reagents (LSAB+ kit; DAKO, Carpinteria, CA). The primary antibodies and working dilutions were as follows: LMP1 (1:20, monoclonal CS1-4; DAKO, Glostrup, Denmark), cyclin A (1:100, polyclonal C-19; Santa Cruz Biotechnology, Santa Cruz, CA), CD15 (1:20, C3D-1; DAKO, Glostrup, Denmark), CD30 (1:40, Ber-H2; DAKO, Glostrup, Denmark), and CD99 (1:100, 12E7; DAKO, Glostrup, Denmark). Appropriate tissues were used as positive control samples. For the antibodies showing differential expression on HL cells, the numbers of positive cells were counted, and subcellular localization was evaluated according to different tumor cell variants, ie, H, RS, and mummified cells. H cells are mononuclear tumor cells characterized by abundant cytoplasm, a large nucleus, and usually 1 prominent eosinophilic nucleolus; RS cells are multinucleated tumor cells that must have at least 2 nucleoli in 2 separate nuclear lobes; mummified cells have condensed cytoplasm and pyknotic nuclei.\textsuperscript{7}

Transfecting LMP1 Into an HL Cell Line

An EBV–HL cell line, L-428 (DSMZ, Braunschweig, Germany), was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT). A vector expressing B95.8 EBV-derived LMP1 was constructed in a pSG5 plasmid that encoded simian virus 40 promoter as previously described.\textsuperscript{21} Another vector, pEGFP-LMP1, and its control vector, pEGFP-C3, were gifts from Hao-Ping Liu, PhD, Chang Gung University, Taoyuan, Taiwan. The LMP1 expression construct was transfected using an electroporation machine (Microporator; Digital Bio Technology Suwon, Korea) with 2 μg of DNA at 1,100 V for 30 milliseconds. The L-428 cells were cultured and collected for further analysis 21 hours (day 1, D1), 42 hours (D2), 64 hours (D3), and 94 hours (D4) after they had been transfected.

Double Immunofluorescence for LMP1 and Cyclin A

After cytocentrifuging the cells and fixing them in acetone, the slides containing LMP1-transfected HL cells were washed with phosphate buffer solution (pH 7.4) and then incubated with primary antibodies against LMP1 (1:25, CS1-4; DAKO, Glostrup, Denmark) and cyclin A (1:50, C-19; Santa Cruz) for 2 hours at room temperature in the dark. After the cells had been washed with phosphate buffer solution, they were incubated with dye-labeled secondary antibodies. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (1:1,000; Invitrogen, Carlsbad, CA). The mononuclear and multinucleated cells were counted separately in the same fields on the whole slides from D1 to D4 and expressed as the ratio of multinucleated to mononuclear HL cells.

Flow Cytometric Analysis

Each sample of 2.6 × 10⁶ cells was transfected with pEGFP-LMP1 and pEGFP-C3 and then cultured in 6 mL of medium. Each sample of 1.5 mL was collected after 18, 24, 48,
and 72 hours. Flow cytometric analysis was performed for the multinucleated (large cell)/mononuclear (small cell) fraction on a flow cytometer (FACSCalibur with CellQuest software; Becton Dickinson, Franklin Lakes, NJ). Cell viability was determined with the trypan blue exclusion test.

**Immunoblotting Assay**

HL cell lysates were lysed in sample buffer (3% sodium dodecyl sulfate, 1.6 mol/L urea, 4% β-mercaptoethanol), and differential subcellular fractions were separated into cytosol and nucleus using a protein extraction kit (ProteoExtract Subcellular Proteome Extraction Kit; EMD Biosciences, La Jolla, CA). Polyacrylamide gel electrophoresis and immunodetection of LMP1 were performed as described previously.22 Other antibodies for Western blotting included cyclin A (C-19; 1:500, SC-596; Santa Cruz), α-tubulin as a cytosol marker (Ab-2; 1:5,000, DM1A; NeoMarkers, Fremont, CA), and histone H1 as a nuclear marker (AE-4; 1:1,000; Millipore, Billerica, MA). The ratio of cyclin A in cytosolic and nuclear fractions was expressed as the amount of cyclin A in cytosol or the nucleus divided by the corresponding amount of cytosolic (α-tubulin) or nuclear (histone H1) markers, respectively, using an imaging analyzer (White Light Transilluminator; Bio-Rad Laboratories, Hercules, CA).

**Statistical Analysis**

Appropriate statistical tests, χ² test and paired and unpaired t tests, were used to examine the associations and correlations between variables using statistical software (SPSS, version 13.0; SPSS, Chicago, IL).

**Results**

**EBER and LMP1 Expression in HL**

All HL cases yielded valid results for EBV detection. The overall EBER+ rate was 53% (18/34), with 0% (0/1) for nodular lymphocyte predominant HL, 48% (11/23) for nodular sclerosis HL, 100% (5/5) for mixed cellularity HL, 25% (1/4) for lymphocyte-rich classic HL, and 100% (1/1) for unclassified HL.

Among the 18 EBER+ HL cases, 13 were immunoreactive for LMP1 stain (13/34 [38%]). We found 3 major LMP1 localization patterns—cell membrane, cytoplasm, and paranuclear/endoplasmic reticulum (ER)-Golgi area—as well as mixed patterns Image 18. LMP1 expression was frequently unstained in mummified cells (45/56 [80%]) and generally negative in background nonneoplastic lymphocytes.

**Staining Pattern of Cyclin A**

Cyclin A was expressed in more than 90% of tumor cells in each HL case, but subcellular localization varied in different tumor cell types of the same individual case. Five differential staining patterns could be found for cyclin A expression: nuclear, cytoplasmic, ER-Golgi area, and mixed (nuclear/cytoplasmic and nuclear/ER-Golgi area) localization Image 21. By evaluating individual variant HL cell cases, we found that nuclear cyclin A expression was more frequent in H cells than in RS cells Table 1 (54.4% vs 7.4%; P < .001; χ² test), whereas cytoplasmic and ER-Golgi expression were more frequent in RS cells than in H cells (36.6% and 50.3% vs 15.0% and 21.1%, respectively; P < .001). Mummified cells were more frequently unstained than RS and H cells (P < .001). Cyclin A expression in the background inflammatory cells was predominantly in the nuclei (Image 2A, arrow).

The pattern of differential subcellular localization of cyclin A in different HL cell cases was also found in LMP1– cases (n = 16) Table 2. As compared with H cells, RS cells more frequently expressed cyclin A in the cytoplasm (37.0% vs 21.0%; P < .001) and ER-Golgi zone (43.0% vs 33.0%; P < .001). In contrast, H cells more frequently expressed cyclin A in nuclei (23.0% vs 3.0%; P < .001). Mummified cells were also most frequently unstained (11/17 [65%]; P < .001). There was no statistical difference for the cytoplasmic expression of cyclin A in RS cells between LMP1+ and LMP1– groups (36.6% vs 37.0%; P = .877).

**Staining Patterns of CD15, CD30, and CD99**

There were no statistically significant differences in CD15 staining between H and RS cells (71.1% vs 61.9% for Golgi, 8.2% vs 8.5% for cytoplasm, 0.9% vs 0.8% for membrane, 1.9% vs 2.5% for ER-Golgi/cytoplasm, 0.7% vs 0.8% for ER-Golgi/membrane, and 17.2% vs 25.4% for unstained). Mummified cells were most frequently unstained (76.8%). There was no statistically significant difference in the CD30 staining pattern between H and RS cells (10% vs 9.4% for membrane, 69.2% vs 63.6% for cytoplasm, 17.3% vs 18.8% for ER-Golgi/cytoplasm, 3.4% vs 6.9% for ER-Golgi/membrane, and 0.1% vs 1.3% for unstained), although a significantly higher portion (27.5%) of mummified cells was unstained Image 3I (upper row). CD99 was absent in most H and RS cells (0.2% vs 2.0%) but present in 9.6% of the mummified cells (Image 3, lower row). The differential localization patterns in different tumor cell types, as noted for LMP1 and cyclin A, were not found for CD15, CD30, and CD99.

**In Vitro LMP1 Expression Increased Multinucleated RS Cell Morphology in an HL Cell Line**

To investigate further whether EBV-LMP1 expression is involved in the induction of cytoplasmic cyclin A and multinucleation of HL cells, we studied the effects on cyclin...
A expression of transfecting a full-length LMP1 plasmid into the EBV-HL cell line L-428. We found shifts in the morphologic features of tumor cells in the culture. As measured by multinucleated/mononuclear ratios, the multinucleated forms of L-428 cells in the LMP1-expressing group were statistically significantly more frequent than in the control group (day 4, 15.5% vs 7.2%; \( P < .001 \)).

The fraction of larger or multinucleated cells assessed using flow cytometric cell sorting also increased over time from 11% (18 hours) to 15% (72 hours) in the LMP1-transfected L-428 cells. In contrast, multinucleated cells increased from 6% (18 hours) to 9% (72 hours) in the GFP-transfected control group.

**LMP1 Increased Cytoplasmic Expression of Cyclin A Over Time in L-428 Cells**

Double immunofluorescent staining showed that cyclin A colocalized with LMP1 in the cytoplasm and that the number of L-428 cells with cytoplasmic cyclin A expression increased from D2 (8%) to D4 (30%) Image 4A. Western blot analysis also showed that the cytoplasmic expression of cyclin A was significantly increased by LMP1 expression at 48 and 72 hours posttransfection Image 4B. Furthermore, L-428 cells with cytoplasmic cyclin A had significantly more multinucleated forms than those with nuclear cyclin A (40/70 [57%] vs 19/665 [2.9%]; \( P < .001 \)).
Image 21 Five staining patterns of cyclin A in Hodgkin lymphoma cells. 

A, Nucleus. A mononuclear Hodgkin cell shows nuclear staining. The nonneoplastic lymphocytes express cyclin A in nuclei (arrows) (×400).

B, Cytoplasm. Multinucleated Reed-Sternberg (RS) cells show diffuse cytoplasmic staining (×400).

C, Endoplasmic reticulum (ER)-Golgi area. An RS cell shows paranuclear dot-like staining (×400).

D, Nucleus/cytoplasm. A Hodgkin cell shows mixed nuclear and cytoplasmic staining (×400).

E, Nucleus/ER-Golgi area. An RS cell shows nuclear staining with ER-Golgi accentuation (×400).

F, A mummified cell is unstained (×400).
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Discussion

We showed, by specifically evaluating thousands of individual HL cells for the subcellular localization of 5 tested proteins, the differential expression of cyclin A in H cells, RS cells, and mummified cells of HL. Cyclin A tended to be expressed in the cytoplasm of RS cells, distinct from the consistently nuclear expression in H cells in LMP1+ and LMP1– cases. Other markers such as CD15, CD30, and CD99 showed no differential expression in different HL cells. Mummified cells showed absent or minimal expression of LMP1, cyclin A, and CD15, but they more frequently expressed CD99. In vitro studies further showed that LMP1 transfection increased the cytoplasmic expression of cyclin A and multinucleated RS cell formation. Therefore, the aberrant expression of cyclin A seems to represent a common mechanism for the morphogenesis of RS cells in EBV+ and EBV– HL cases. Other unidentified viral proteins or mechanisms similar to LMP1 signaling may be involved.

Viral proteins have been reported to induce multinucleation and the formation of multilobated nuclei of host cells through the induction of cytoplasmic cyclin A, such as the Tax oncoprotein of HTLV-1 and the pre-S2 mutant of hepatitis B virus. Cyclin A acts in the S phase of the cell cycle, where it is required to initiate DNA replication. Although cyclin A is localized predominantly in the nucleus, it shuttles between the nucleus and cytoplasm. There is mounting evidence that cytoplasmic cyclin A expression is associated with oncogenesis and abnormal nuclear morphogenesis. The differential expression of cyclin A in H and RS cells in the immunohistochemical studies drove us to test whether EBV-LMP1 has a role in the induction of cytoplasmic cyclin A expression. As expected, we demonstrated that EBV-LMP1 enhanced aberrantly cytoplasmic expression of cyclin A and multinucleated RS cell morphologic features in the L-428 cell line. These findings suggest that EBV-LMP1 signaling may be involved in the morphogenesis of RS cells in EBV+ HL.

Cyclin A expression in HL, which has been immunohistochemically analyzed using N-terminus binding antibodies, is predominantly in the nucleus. However, by using a
C-terminus binding antibody, we showed that cyclin A was expressed also in cytoplasm and the ER-Golgi area, which was interestingly associated with RS cell morphologic features. The detailed mechanism for the intracellular redistribution of cyclin A in HL cells remains to be delineated. There are, however, some potential mechanisms that explain the cytoplasmic expression of cyclin A. SCAPER (S phase cyclin A–associated protein residing in the endoplasmic reticulum), a novel protein, interacts specifically with the cyclin A/Cdk2 complex, and SCAPER overexpression sequesters cyclin A in the cytoplasm and induces cells to accumulate in the M phase of the cell cycle.25 Alternatively, the accumulation of viral proteins in the infected cells induces alteration of calcium

**Table 3**

<table>
<thead>
<tr>
<th>Day (h)</th>
<th>L-428 LMP1</th>
<th>L-428 Vector</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (21)</td>
<td>5.5 (37/673)</td>
<td>8.0 (77/965)</td>
<td>.07</td>
</tr>
<tr>
<td>2 (42)</td>
<td>15.4 (67/435)</td>
<td>12.2 (120/985)</td>
<td>.15</td>
</tr>
<tr>
<td>3 (64)</td>
<td>12.0 (63/527)</td>
<td>7.5 (112/1,498)</td>
<td>.043</td>
</tr>
<tr>
<td>4 (94)</td>
<td>15.5 (45/290)</td>
<td>7.2 (77/1,065)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

LMP1, latent membrane protein-1.
* Data are given as percentage (number of multinucleated/mononuclear cells).
In EBV– HL cases, we observed a similar finding that RS cells frequently expressed cyclin A in cytoplasm, about 37% in RS cells in the LMP1+ and LMP1– groups. Therefore, RS cell morphologic features are commonly associated with cytoplasmic expression of cyclin A. The mechanism underlying aberrant expression of cyclin A in EBV– cases remains to be clarified. Other hitherto unidentified pathogens could not be completely excluded in EBV-undetected HL.29-31 Similar to the common activation of nuclear factor κB signaling in the tumorigenesis of EBV+ and EBV– HL,7,19 a mechanism

homeostasis via ER stress, which results in calpain-mediated cyclin A cleavage (H. C. Wang and I.-J. Su, unpublished observations, 2009). On the other hand, LMP1, a member of the tumor necrosis factor receptor family, activates several signaling pathways involved in the survival of HL cells.28 In this study, we found differential subcellular localization of LMP1 in variant HL cells. The mechanism underlying differential subcellular localization of LMP1 and its correlation with cytoplasmic cyclin A in HL cells is unknown, and studies are underway.

In EBV– HL cases, we observed a similar finding that RS cells frequently expressed cyclin A in cytoplasm, about 37% in RS cells in the LMP1+ and LMP1– groups. Therefore, RS cell morphologic features are commonly associated with cytoplasmic expression of cyclin A. The mechanism underlying aberrant expression of cyclin A in EBV– cases remains to be clarified. Other hitherto unidentified pathogens could not be completely excluded in EBV-undetected HL.29-31 Similar to the common activation of nuclear factor κB signaling in the tumorigenesis of EBV+ and EBV– HL,7,19 a mechanism
similar to LMP1 signaling may be involved in the aberrant expression of cyclin A in EBV− HL cases. Besides the LMP1 signaling, other mechanisms may operate in the morphogenesis of RS cells. LMP1 down-regulates CD99 in B cells, which leads to the generation of multinucleated RS-like cells.10 However, we found no differential expression of CD99 in H and RS cells in this study. It is interesting that mummified cells more frequently expressed CD99, which can activate apoptotic signaling in T cells.32 Thus, the activation of CD99 and/or down-regulation of the LMP1/cyclin A pathway may lead to apoptosis of HL cells and, hence, mummified cell formation. On the other hand, our finding that CD30 was uninhibited in apoptosis of HL cells, and, hence, mummified cell formation, other unidentified viral proteins or similar mechanisms involved in the LMP1/cyclin A pathway may have a role.

We demonstrated for the first time that the differential localization of cyclin A in different cell types is common to EBV+ and EBV− HL cases. In EBV+ cases, LMP1 seems to be pivotal in the cytoplasmic expression of cyclin A and multinucleation formation, whereas in EBV− cases, other unidentified viral proteins or similar mechanisms involved in the LMP1/cyclin A pathway may have a role.

References


