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Stem Cells, Tissue Engineering and Hematopoietic Elements

Tumorigenic Potential of Mononucleated Small Cells of Hodgkin Lymphoma Cell Lines

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Tumor cells with tumorigenic potential are limited to a small cell population known as cancer stem cells (CSCs). CSCs yield both CSCs and non-CSCs, whereas non-CSCs do not yield CSCs. CSCs have not been identified in any malignant lymphomas. Hodgkin lymphoma (HL) is a mostly B-cell neoplasm that can be diagnosed by the presence of multinucleated (Reed-Sternberg; RS) cells admixed with Hodgkin cells with distinct nucleoli and various inflammatory cells. Here, the tumorigenic potential of cells with a single nucleus (S) and cells with multiple nuclei (M), which may be equivalent to Hodgkin and RS cells, respectively, was examined in HL cell lines L1236 and L428. Cultures of single S cells yielded both S and M cells, whereas M cell cultures yielded only M cells. When either cultured in methylcellulose or inoculated into NOD/SCID mice, the colony number and tumor size were both larger in S than in M cells. Concentrations of intracellular reactive oxygen species (ROS) were at low levels in a portion of S cells that abundantly expressed FoxO3a, a transcription factor that regulates ROS-degrading enzymes. In clinical samples of HL, FoxO3a was expressed in mononuclear Hodgkin cells but not in multinucleated RS cells. These findings suggest that smaller cells or Hodgkin cells that show low-ROS concentrations and high FoxO3a expression levels might be candidates for HL CSCs. ***(Am J Pathol 2010, 177:3081–3088; DOI: 10.2353/ajpath.2010.100089)***

Tumors consist of heterogenous cell populations derived from a single clone. Recently, it has been demonstrated that tumor cells with tumorigenic potential are limited to a small population, called cancer-initiating cells or cancer stem cells (CSCs), in several tumors, such as leukemia, breast, brain, and colon cancers.[1–8] CSCs yield both CSCs and non-CSCs, whereas non-CSCs do not yield CSCs.[8] CSCs efficiently efflux antitumor chemicals and degrade reactive oxygen species (ROS) that are related to radiation-induced apoptosis.[9] These characteristics enable CSCs to be resistant to antitumor drugs and radiation therapy. To date, CSCs have not been identified in any kinds of malignant lymphomas.

Cell origin of Hodgkin lymphoma (HL) had been controversial, but the studies based on molecular biology revealed that HL is mostly a neoplasia of B lymphocyte.[10,11] HL can be diagnosed by the presence of multinucleated Reed-Sternberg (RS) cells and mononuclear Hodgkin cells intermingled with small lymphocytes and various inflammatory cells. The RS cells are regarded as pathognomonic for HL. The RS and Hodgkin cells share cell markers, such as CD15 and CD30, but the proliferation potential in the RS cells was reported to be lower than that of Hodgkin cells.[12] To date, any markers that differentiate Hodgkin cells from RS cells have not been established. Here, a population showing CSC character was examined in HL cell lines L1236 and L428.[13,14] Tumorigenic potential and ROS-expelling ability were observed mainly in a portion of small-sized mononuclear Hodgkin cells. Large-sized RS-like cells appeared to be derived from a portion of small-sized Hodgkin cells. Hodgkin cells possess properties suggestive of CSCs in HL.

Materials and Methods

Cell Line

Hodgkin lymphoma cell lines L1236 and L428 were purchased from Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany). Cells were cultured in RPMI 1640 (Sigma, St Louis, MO) supplemented with 10% fetal calf serum (Nippon Bio-SupplyCenter, Tokyo, Japan).

Two Populations of Cells

A portion of cultured cells was cytospun on poly-L-lysine-coated glass slides at 1500 rpm for 3 minutes and stained with

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Table 1. Cell Size of S and M Cells in Hodgkin Lymphoma Cell Lines

	L1236	L428
S	29.6 ± 7.8 μm	21.0 ± 4.8 μm
M	49.5 ± 19.5 μm	32.8 ± 11.9 μm
P value between S and M	<0.001	<0.001

Giemsa solution. Cells with a single and multinucleus (more than two nuclei) were named as S and M cells, respectively. The mean size and SD of S and M cells are shown in Table 1. As observed by Wolf et al,[13] there was a limited number of large-sized cells (Figure 1A), mostly with multinucleus.

Limiting Dilution

Cells were counted and diluted to the concentration at one cell per 100 μl of the medium. Then, 100 μl was cultured in each well of 96-well plates to determine the replication capacity of a single cell. After 2 weeks, each well was checked under a phase contrast microscopy to determine the number and size of cells. At Giemsa staining, three types of wells were found; a well exclusively containing S cells, M cells, and mixed S and M cells. Because cells did not proliferate in several wells, the sum of percentage of S, mixed, and M wells was not 100. The proportion of multinucleated cells in S, mixed, and M wells is shown in Table 2.

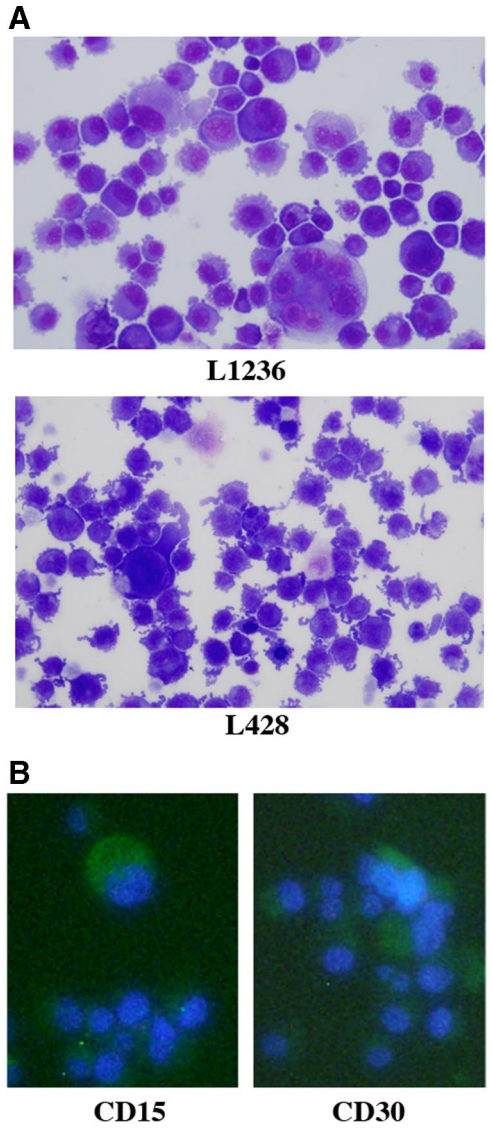

Figure 1. Typical morphology of HL cell lines L1236 and L428. **A:** Giemsa staining of original L1236 and L428 cells. **B:** Immunostaining for CD15 and CD30.

Table 2. Proportion of Multinucleated Cells in S, Mixed, and M Wells of the L1236 Cell Line

Type of Well	Proportion of Multinucleated Cells (%)	*P* Value versus S	*P* Value versus M
S	10.2 ± 1.5	NA	<0.001
mixed	19.0 ± 0.1	<0.01	<0.001
M	80.6 ± 2.7	<0.001	NA

NA, not applicable.

Immunofluorescence for CD15 and CD30

Cells were cytospun on poly-L-lysine-coated glass slides at 1500 rpm for 3 minutes, fixed with 10% formalin for 10 minutes, and treated with 0.25% Triton X-100 solution for 10 minutes. After blocking with 5% bovine serum albumin (Sigma), cells were incubated with anti-CD15 or anti-CD30 antibody (DAKO A/S, Glostrup, Denmark). Then, cells were treated with Alexa Fluor 488 anti-mouse IgG (Invitrogen, Carlsbad, CA). As the negative control, staining was carried out in the absence of primary antibody.

Immunocytochemistry for Cell Cycle Proteins (Ki-67 and Cyclin D2) and the Uptake of Bromodeoxyuridine (BrdU)

Cells were cytospun, fixed with 10% formalin for 10 minutes, and treated with 0.25% Triton X-100 solution for 10 minutes. After treatment with peroxidase blocking solution (DAKO), cells were incubated with anti–Ki-67 (DAKO) and anti–cyclin D2 (Abcam, Cambridge, UK) antibodies. Then, cells were treated with ChemMate EnVision kit (DAKO). DAB (DAKO) was used as a chromogen. As a negative control, staining was carried out in the absence of primary antibody.

The uptake of BrdU was examined using BrdU *in situ* detection kit (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's manual.

In Vitro *Colony Formation Assay*

The suspended 1000 cells were plated in culture dishes with 1 ml of methylcellulose-containing DMEM supplemented with 15% FCS. The number of colonies was counted on day14.

Mice and Xenograft Transplantation

Six- to 8-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Charles River Laboratories Japan and kept under specific pathogen-free conditions. Before xenotransplantation, the mice were deeply anesthetized. All animal experiments were done according to the guideline of Osaka University Animal Center and approved by the institutional review board of committee of animal experiments. In the previous study,[13] 2×10^7 cells were injected for xenotransplantation. In this study, however, total number of M cells was prepared at 2×10^3, because of low proliferative potential of M cells. Then, 2×10^3 cells were used for xenotransplantation, and this was enough to form tumors by S cells. S and M were suspended in 0.2 ml of Matrigel (BD), and were injected subcutaneously into the frank of left and right side of the same animal, respectively. Tumor volume was estimated using the formula: $(\text{width})^2 \times (\text{length})/2$ according to the report by Meyer-Siegler et al.[15]

Assay for Intracellular ROS Level

Cells were incubated with 10 μmol/L of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen) and 100 μmol/L of H_2O_2 at 37°C for 20 minutes. Dead cells were stained with propidium iodide. ROS-high cells were stained in green and dead cells in red. At least 200 S and M cells were examined, and the proportion of ROS-high viable cells was counted.

Immunocytochemistry for FoxO3a and Catalase

FoxO3a and catalase expression was immunocytochemically examined with anti-FoxO3a antibody (Cell Signaling Technology Inc, Beverly, MA) and anti-catalase antibody (Sigma, St. Louis, MO). Cells were cytospun on poly-L-lysine-coated glass slides at 1500 rpm for 3 minutes, fixed with 10% formalin for 10 minutes and treated with 0.25% Triton X-100 solution for 10 minutes. After treatment with peroxidase blocking solution (DAKO), cells were incubated with anti-FoxO3a and anti-catalase antibodies diluted at ×250 and ×200, respectively. Then, cells were treated with ChemMate EnVision kit (DAKO). DAB (DAKO) was used as a chromogen. As the negative control, staining was carried out in the absence of primary antibody. At least 200 S and M cells were examined, and the proportion of FoxO3a expressing cells was counted.

Flow Cytometry and Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)

Cells were stained with CM-H_2DCFDA, and ROS-high and ROS-low cells were sorted with FACSAria (Becton Dickinson, Franklin Lakes, NJ). Total RNA was extracted from sorted cells using RNeasy RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, and cDNA was synthesized using oligo (dT) primers and SuperScript III reverse transcriptase (Invitrogen). The qRT-PCR was done with an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA) using Taqman probe/primer sets specific for human FoxO3a. GAPDH was used as a reference for gene amplification (Applied Biosystems).

Immunohistochemistry for FoxO3a

FoxO3a expression was immunocytochemically examined in clinical samples from 13 cases with Hodgkin lymphoma with anti-FoxO3a antibody. Histological specimens were fixed in 10% formalin and routinely processed for paraffin-embedding. Sections were cut at 4 μm thickness. After antigen retrieval with Pascal pressurized heating chamber (DAKO), the sections were incubated with anti-FoxO3a antibody diluted at ×250. Then, cells were treated with ChemMate EnVision kit (DAKO). DAB was used as a chromogen. As the negative control, staining was carried out in the absence of primary antibody. The study was approved by the ethical review board of Graduate School of Medicine, Osaka University. Two cases contained a limited number of RS cells. In these cases, approximately 20 RS cells and the surrounding Hodgkin cells were examined. In the remaining cases, at least 50 RS cells and the surrounding Hodgkin cells were examined.

Effect of Insulin and PI3K Inhibitor

To study the effect of insulin on the formation of M cells, insulin was added to culture medium of S cells at a final concentration of 0, 1, 3, 5, 10, and 50 μg/ml. To study the role of phosphatidylinositol-3 kinase (PI3K), PI3K inhibitor LY294002 (Calbiochem, Darmstadt, Germany) was added to cultures medium of S cells at a final concentration of 0, 1, 5, 10, 20, 50 μmol/L and incubated at 37°C for 40 minutes. After that, insulin was added at a final concentration of 0 and10 μg/ml. After 7 days, at least 1000 cells were cytospun, stained with Giemsa, and evaluated for the percentage of M cells. Expression level of FoxO3a was examined with immunocytochemistry.

Statistical Analysis

Statistical analyses were performed using Student's *t* tests. The values are shown as the mean ± SE of at least three experiments. The *P* values of less than 0.05 were considered to be statistically significant.

Results

Single Cell Culture of HL Cell Lines

HL cell lines contained two population of cells, S and M cells (Figure 1A). The mean size and SD of S and M cells were shown in Table 1. Both S and M cells expressed CD15 and

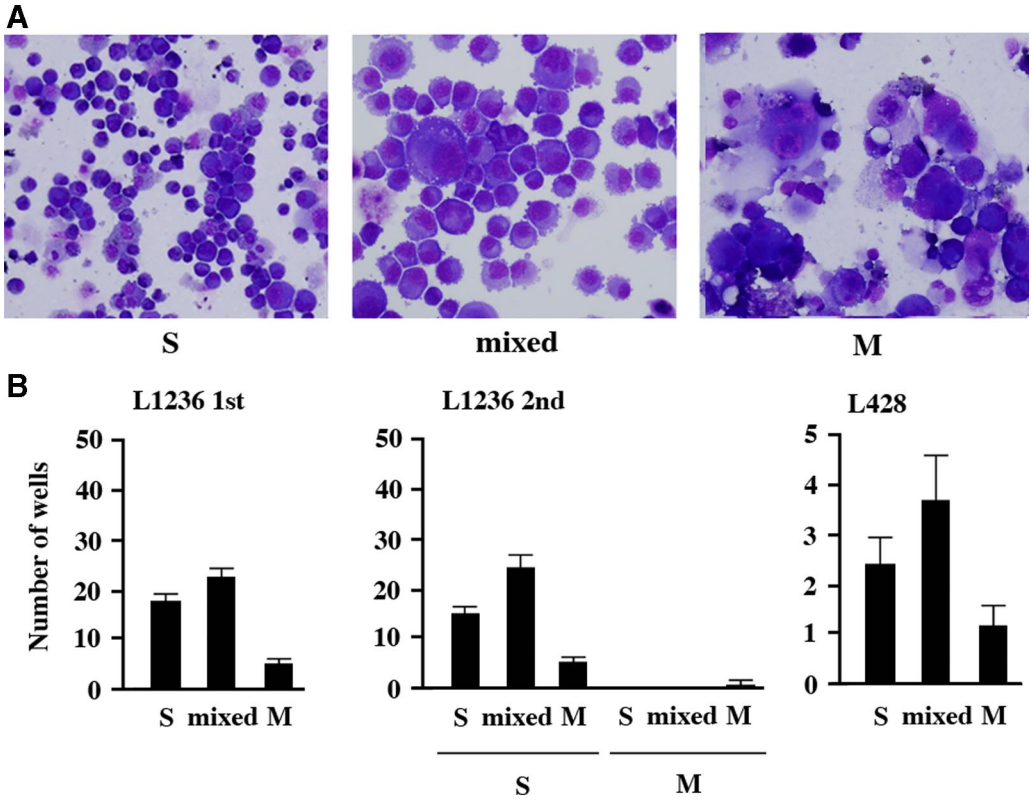

Figure 2. A limiting dilution of HL cell lines. **A:** When a single cell culture was done, wells containing exclusively S cells, mixed cell populations, or M cells were obtained. The representative fields of L1236 were shown. **B:** Number of wells containing S, mixed, and M cells per 100 wells is shown. A single cell culture from the original L1236 (**left**), from an S or M cell (**middle**), and a single cell culture from the original L428 culture (**right**). Because of the presence of wells without any cellular proliferation, the sum of the well number was not 100.

CD30 (Figure 1B). To evaluate the relation between S and M cells, a single cell culture of L1236 was performed with limiting dilution. After 14 days, the size of proliferating cells in each well was checked with Giemsa staining and was categorized as wells containing S cells, mixed S and M cells, or M cells (Figure 2A). The number of wells with S, mixed, and M cells per 100 wells was 18.1, 24.7, and 5.2 wells, respectively (mean of three independent experiments, Figure 2B). Next, a single cell culture was repeated with S and M cells. When a single cell culture of S cells was done, number of wells with S, mixed, and M cells was 15.6, 26.0, and 3.1 wells per 100 wells. When a single cell culture of M cells was done, number of wells with S, mixed, and M cells was 0, 0, and 1.0 wells per 100 wells (Figure 2B). These findings indicated that S cells yielded both S and M cells. The M cells possessed a limited proliferative potential and could not yield S cells. A limiting dilution was carried out with another HL cell line L428. As observed in L1236, the number of wells with M cells was lower than that of S and mixed cells (Figure 2B).

Proliferative Activity of S and M Cells

Proportion of Ki-67–positive cells was higher in S than in M cells of L1236 and L428 (Figure 3A). The proportion of

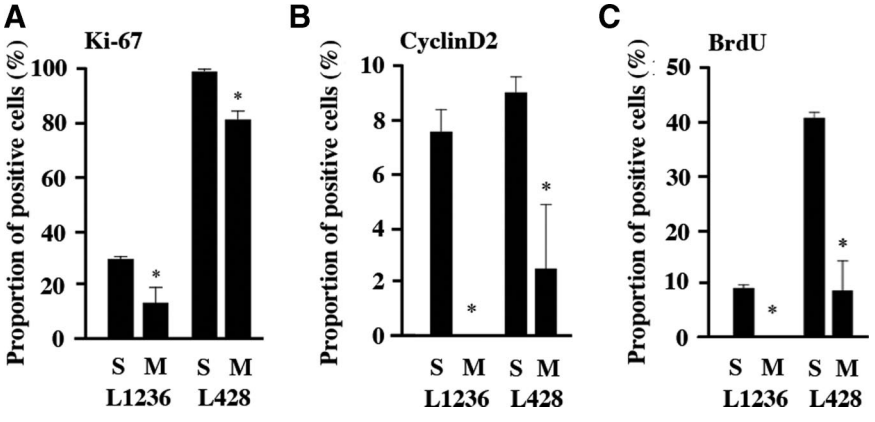

Figure 3. Analysis of proliferative potential. The proportion of Ki-67- (**A**) and cyclin D2- (**B**) positive cells and BrdU-uptaking cells (**C**) was compared between S and M cells of L1236 and L428. The values are the mean ± SE of three experiments. $^*P < 0.05$ by the Student's *t*-test.

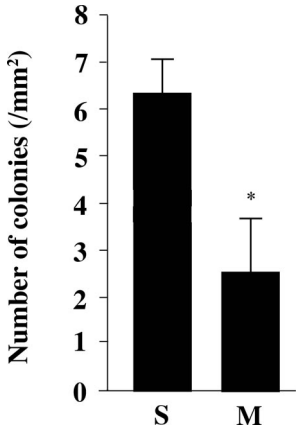

Figure 4. In vitro colony formation assay. The number of colonies by culture of S and M cells in methylcellulose are shown. The values are the mean ± SE of three experiments. $^*P < 0.05$ by the Student's *t*-test.

cyclin D2-positive cells (Figure 3B) and that of BrdU-uptaking cells (Figure 3C) were also higher in S than in M cells.

In Vitro *Colony Formation Assay and NOD/SCID Mouse Injection*

To assess colony formation ability of S and M cells, both types of cells were plated in methylcellulose-containing DMEM supplemented with 15% FCS. Number of colonies obtained from S cells was significantly higher than that from M cells (Figure 4).

To examine the tumorigenicity *in vivo*, S and M cells were injected into the left and right flank of NOD/SCID mice. At 2 weeks after the injection, tumor mass was found only in the injected site of S cells: the volume of tumor was 215.4 ± 122.7 mm^2, 813.6 ± 148.6 mm^2, and 1510.6 ± 430.3 mm^2, at day 18, 22, and 26, respectively (Figures 5, A–C). Tumor derived from S cells consisted of both S and M cells (Figure 5D). No tumor was found in the injected site of M cells throughout the observed period.

ROS Concentration in S and M Cells

CSCs are known to contain a lower level of ROS than non-CSCs. Therefore, the level of ROS was examined in L1236 cells after the addition of H_2O_2. Cells containing high ROS showed green fluorescence after the incubation with CM-H_2DCFDA. Although most M cells and a majority of S cells were stained in green, approximately 10% of S cells showed a marginal green fluorescence, indicating that the level of ROS was low in these S cells (Figure 6).

Expression of FoxO3a

It is known that ROS level and FoxO3a level are inversely correlated, because FoxO3a enhances the expression of ROS-degradation enzymes, such as superoxide dimustase and catalase. In fact, the expression level of FoxO3a was higher in ROS-low cells than in ROS-high cells (Figure 7A). Hematopoietic stem cells express FoxO3a at a high level, suggesting that CSCs of HL might show a high FoxO3a expression as well. As expected, the expression of FoxO3a was mostly confined to S cells, approximately 35% of S cells, suggesting that S cells contained a small population expressing FoxO3a at a high level and excluding ROS (Figure 7B). The expression level of catalase, whose expression was enhanced by FoxO3a, was also high in approximately 30% of S cells (Figure 7C). In

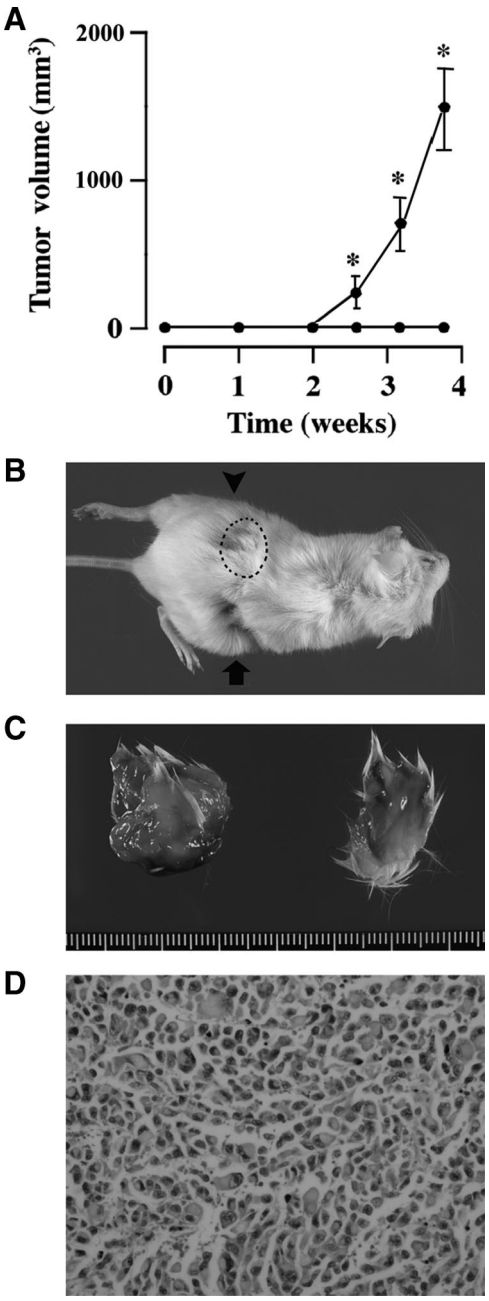

Figure 5. Xenotransplantation of S and M cells of L1236 into NOD/SCID mice. S and M cells were injected into the **left** (**arrowhead**) and **right** (**arrow**) flanks of NOD/SCID mice. **A:** The volume of tumors derived from S and M cells. The values are the mean ± SE of three experiments. $^*P < 0.05$ by the Student's *t*-test. **B:** Tumor mass was found in the injected site of S cells (**arrowhead**) but not of M cells (**arrow**). Tumor was found in **dotted area**. **C:** Resected tumor in the injected site of S cells (**left**). No tumor was found in the injected site of M (**right**). **D:** Resected tumor derived from S cells consisted of S and M cells (×100).

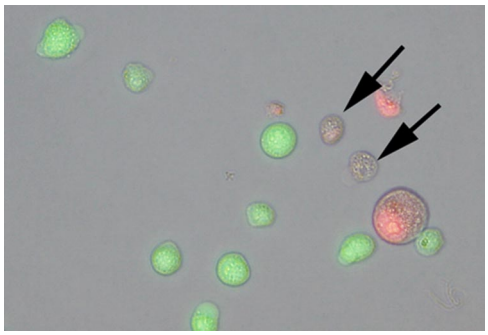

Figure 6. ROS concentration in S and M cells. ROS-high cells stained green and dead cells stained red. ROS-low viable cells are indicated by **arrows**.

the clinical samples of HL, mononuclear Hodgkin cells exclusively expressed FoxO3a, and multinucleated RS cells did not (Figure 7D).

Effect of Insulin on the Formation of M Cells

Insulin is known to cause the degradation of FoxO3a through PI3K pathway. To examine the role of FoxO3a for ROS degradation, insulin was added to the culture medium. The addition of insulin decreased the expression level of FoxO3a in S cells (Figure 8A). Approximately 10% of M cells expressed FoxO3a, but the addition of insulin decreased its proportion to 3% (Figure 8A). As compared to the culture without insulin, the proportion of M cells increased from 2% to 8% when insulin was added (Figure 8B). This increase of M cells was diminished by the addition of PI3K inhibitor LY294002 (Figure 8C). The addition of PI3K inhibitor alone without insulin did not affect the proportion of M cells (Figure 8C). These findings showed that the addition of insulin to S cells enhanced the formation of M cells via PI3K pathway.

Discussion

HL is mostly a B-cell neoplasm which can be diagnosed by the presence of multinucleated cells with distinct inclusion-like nucleoli (so-called diagnostic RS cells) admixed with Hodgkin cells with single nucleus in which large nucleolus is discernible, small lymphoid cells, and various inflammatory cells. Precise study based on the analysis of immunoglobulin gene rearrangement and fusion pattern of terminal repeats in Epstein-Barr virus (EBV) for microdissected single cells from HL lesion revealed the monoclonal nature, thus neoplastic B-cell in nature, of the RS cells and Hodgkin cell.[10,11] Here, the tumorigenic potential of these two types of cells was examined with HL cell line L1236 and L428. L1236 and L428 are composed of two types of cells, S cells that resembles to Hodgkin cells and M cells that resembles to RS cells. S and M cells share a common immunophenotype; both express CD30 and CD15, but not CD20.[13] EBV DNA is not detectable in both S and M cells, because L1236 and L428 cells are derived from the HL without EBV infection.[13,14]

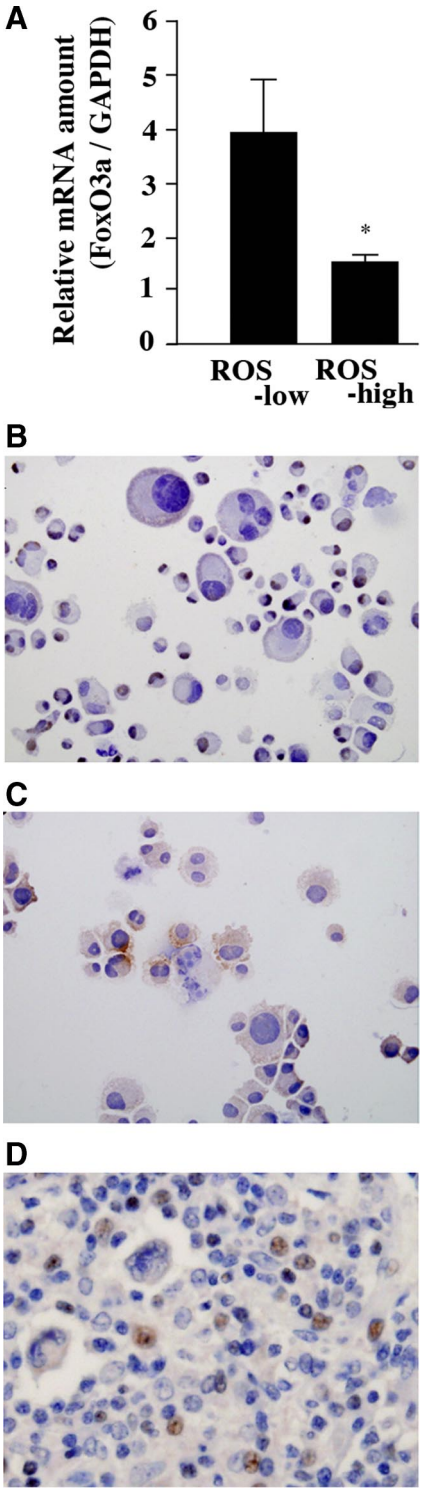

Figure 7. Expression of FoxO3a and catalase. **A:** Real-time quantitative RT-PCR for FoxO3a in ROS-low and -high L1236 cells. The values are the mean ± SE of three experiments. $^{*}P < 0.05$ by the Student's *t*-test. Immunocytochemistry of FoxO3a (**B**) and catalase (**C**) in L1236 cells (×400). **D:** Immunohistochemistry of FoxO3a in clinical samples of HL (×400).

When the single S cell was cultured, cell populations composed of S, M, and mixed of both were developed. In contrast, M cells hardly proliferated. Among 100 wells of a single cell culture of M cells, only one well yielded

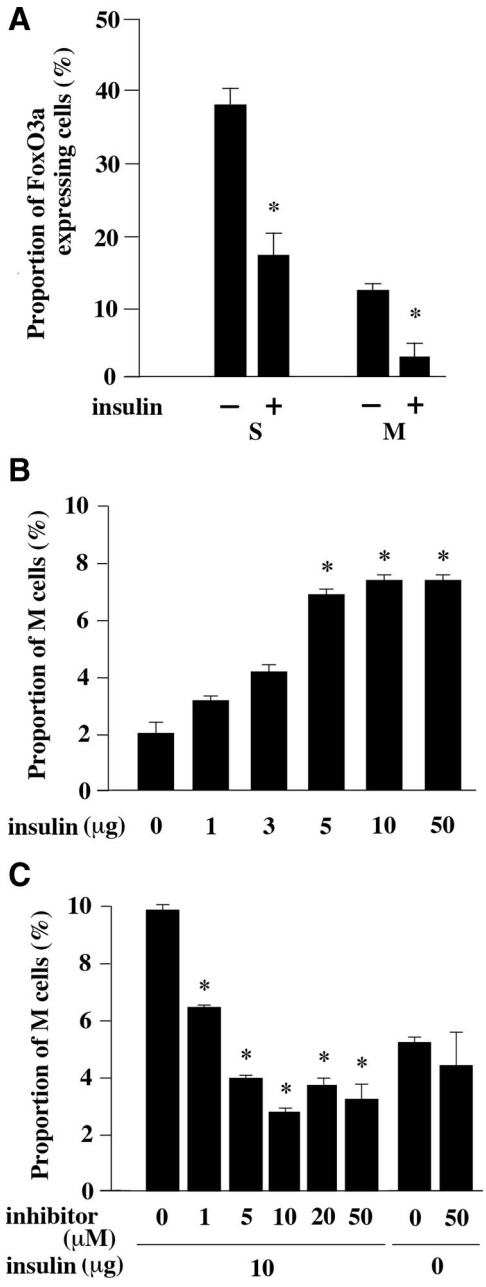

Figure 8. Effect of insulin on the formation of M cells. **A:** Change of proportion of FoxO3a-expressing L1236 cells by the addition of insulin. **B:** Proportion of M cells among L1236 cultured with insulin. **C:** LY294002 inhibited the formation of M cells. The values are the mean ± SE of three experiments. $^*P < 0.05$ by the Student's *t*-test.

detectable cell population composing exclusively M cells. These findings indicated that a single cell culture of S cells yielded both S and M cells. Whereas, M cells showed a limited proliferative potential and yielded M cells but not S cells. The proportion of Ki-67 or cyclin D2-positve, and BrdU-uptaking cells was generally higher in S cells than in M cells of both L1236 and L428: approximately 30% and 10% in Ki-67, 7% and 0% in cyclin D2, and 10% and 0% in BrdU in L1236; 100% and 80% in Ki-67, 9% and 2% in cyclin D2, and 40% and 10% in BrdU in L428, respectively. (The former value is S and the latter is M.) These findings showed the limited proliferative potential of M cells. CSCs are known to yield both CSC and non-CSC, whereas non-CSCs yield only non-CSCs.[8] Therefore cells with CSC character appeared to be present among S cells. CSCs possess a strong tumorigenic ability when cultured in semisolid condition or transplanted into NOD/SCID mice. In methycellulose culture and inoculation into NOD/SCID mice, the number of colonies and the size of tumors were larger in S cells than in M cells. These findings supported the above-mentioned notion that CSCs were present among S cells.

Wolf et al[13] reported the xenotransplantation of 2×10^7 of L1236 cells into Scid mice: tumor was formed at 6 weeks after transplantation, reached the size of 0.5 to 1 cm in diameter, then regressed completely. Whereas the tumor was not spontaneously regressed even after reaching the diameter of approximately 2 cm at up to 4 weeks in present study. This might be partly explained that the injected cells were not the original L1236 but the selected S cell population. In the current study, 2×10^3 S cells were enough to form tumors at 2 week after transplantation, suggesting that the S cells selected from mixed population possessed more tumorigenic potential than nonselected L1236 cells. Higher proportion of Ki-67, cyclin D2–positive, and BrdU-uptaking cells among S cells than M cells found in the present study explained more tumorigenic potential of S cells than M cells.

Next, we further characterized the S cells. Recently, CSCs have been reported to possess the ability to keep the concentration of ROS at a low level.[9] Then, H_2O_2 was added to L1236, and the concentration of ROS in each cell type was measured with CM-H_2DCFDA. M cells and most of S cells showed high ROS concentration in the presence of H_2O_2, but the concentration was at a low level in a portion of S cells. FoxO3a is a transcription factor regulating the expression of ROS-degrading enzymes, such as catalase.[16–19] A portion of S cells exclusively expressed FoxO3a and catalase, whereas M cells did not. In the clinical samples of HL, FoxO3a was expressed mainly in the mononuclear Hodgkin cells but not in the multinucleated RS cells. These findings suggested that approximately 10% of S cells keeping ROS concentration at a low level and showing high FoxO3a and catalase expression might be a candidate for CSCs of HL.

Recently, Jones reported the circulating clonotypic B cell population with small sizes and high expression of aldehyde dehydrogenase and CD27 in HL patients.[20] This population efficiently formed colonies *in vitro*. These findings were similar to those found in the S cells population reported in our study. Further studies will clarify the relation between these two populations.

Wolf et al[13] described that L1236 may not be a representative HL cell line due to numerous chromosomal aberrations and atypical karyotype for HL. Then, another HL cell line L428, which shows fewer chromosomal aberrations than L1236, was examined in the present study, and the similar results were obtained.[21,22] Although the limited proliferative potential of M cells might be due to the culture system, we considered that S cells possessed tumorigenic potential as compared to M cells based on the *in vivo* xenotransplantation experiment, the distinct

higher expression level of Ki-67 and cyclin D2, and BrdU uptake in S than in M cells.

It is reported that activation of PI3K pathway suppresses the FoxO3a expression.[23] It is known that many reagents, including insulin, activate PI3K.[23,24] If FoxO3a plays a role for maintenance of CSCs in HL, the addition of insulin might result in decrease of number of tumorigenic S cells and increase of nontumorigenic M cells. As expected, the addition of insulin increased the proportion of M cells, and this was inhibited by LY294002, an inhibitor for PI3K pathway. Yilmaz et al and Zhang et al reported that the hematopoitetic stem cells are diminished when phosphatase and tensin homolog (PTEN), an antagonist against PI3K, is knocked-out.[25,26] It is suggested that the activation of PI3K pathway appeared to decrease the stem cell activity not only in hematopoietic system but also in HL.

Taken together, among morphologically distinctive HL neoplastic cells, a portion of single-nucleated cells was tumorigenic and possess CSC-like characters.

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