

Characterization of subpopulation lacking both B-cell and plasma cell markers in Waldenstrom macroglobulinemia cell line

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Cancer cells with tumorigenic potential are limited to a small population known as cancer-initiating cells (CICs). To date, CICs have not been identified in non-Hodgkin's lymphomas. Here, we investigated a candidate of CICs of an indolent non-Hodgkin's lymphoma, Waldenstrom macroglobulinemia (WM), using WM cell line MWCL-1. WM tumor expresses both B-cell and plasma cell markers, CD20 and CD138. When stained with anti-CD20 and anti-CD138 antibodies, MWCL-1 cells were classified into three subpopulations: CD20[−] CD138[−], CD20⁺ CD138[−], and CD20⁺ CD138⁺. When cultured, CD20[−] CD138[−] cells yielded all three subpopulations, but CD20⁺ cells did not yield CD20[−] CD138[−] cells. Higher reactive oxygen species (ROS) expelling and *in vitro* colony formation activities were detected in CD20[−] CD138[−] cells than in CD20⁺ CD138[−] and CD20⁺ CD138⁺ cells. When cultured in the absence of serum or with anti-cancer drug, CD20[−] CD138[−] cells were resistant to apoptosis. In contrast, CD20⁺ CD138⁺ cells were vulnerable to apoptosis in the same condition. In fact, the immunohistochemical analysis with clinical samples revealed that tumor cells in apoptosis were CD138-positive. The production of all three subpopulations, the efficient ROS expelling and *in vitro* colony-forming activities, and the resistance to apoptosis suggested that the CD20[−] CD138[−] cell might be a candidate of CICs in WM. *Laboratory Investigation* (2014) **94**, 79–88; doi:10.1038/labinvest.2013.129; published online 4 November 2013

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Tumors derive from a single clone, but consist of heterogeneous cell populations whose features and functions are diverse. Recently, it has been demonstrated that tumorigenic potential is limited to a small population known as cancer-initiating cells (CICs).^{1–10} CICs yield both CICs and non-CICs, whereas non-CICs rarely yield CICs.^{1,2,9,10} Relatively high number of colonies is yielded from CICs *in vitro*, as compared with non-CICs.^{9–11} CICs are one of the causes of tumor recurrence, because CICs efficiently escape apoptosis by effluxing antitumor drugs and degrading reactive oxygen species (ROS) that are related to radiation-induced apoptosis.^{9,10,12} For the complete cure of tumors, it is essential to eliminate CICs. However, the knowledge of CICs is limited in most tumors. Especially, CICs of indolent lymphoma have not been reported. This might be due to the relatively homogenous surface markers in lymphoma cells and the low rate of proliferation that make the assay of tumorigenic potential difficult.

Waldenstrom macroglobulinemia (WM) is an indolent non-Hodgkin's lymphoma that is classified into lymphoplasmacytic lymphoma (LPL), and overproduces monoclonal immunoglobulin M protein (IgM).^{13,14} In contrast to most indolent lymphoma, WM consists of tumors with relatively diverse surface markers. WM shows a spectrum of small B lymphocytes, plasma cells, and lymphoplasmacytoid cells.^{14,15} In addition to the expression of soluble IgM and the pan B-cell antigens such as CD20, WM tumor cells coexpress the markers of mature plasma cells such as CD138.^{14,16,17} Recently, WM cell line, called MWCL-1, has been established. MWCL-1 has a rigorous clonal relationship to the primary WM tumor, which retains similar immunophenotypic and biologic properties of the initial tumor clone.¹⁸ Because of the heterogeneous phenotypes of lymphoma cells and the establishment of cell line, WM is suitable for the study of CICs in lymphoma as compared with other types of indolent lymphoma. In the present study,

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using MWCL-1 cell line, we found that the population lacking both B-lymphocyte and plasma cell markers was the candidate of CICs of WM.

MATERIALS AND METHODS

Cell Line and Sorting

WM cell line, MWCL-1 was provided from Mayo Foundation for Medical Education and Research. MWCL-1 cells were cultured in IMDM + GlutaMAXTM-I (Gibco by Life Technologies, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS, HyClone Laboratories, Logan, USA). Cells were stained with CD20-Allophycocyanin (APC) (clone 2H7, BD Biosciences, San Jose, USA) and CD138-Phycoerythrin (PE) (clone MI15, BD Biosciences) antibodies, and CD20[−] CD138[−], CD20⁺ CD138[−], CD20⁺ CD138⁺ populations were sorted with FACS Aria II (BD Biosciences). When stained with CD20 and CD138, we removed the dead cells with propidium iodide and reacted cells in the presence of human FcR blocking reagent (Miltenyi Biotec, Auburn, USA). The proportion of dead cells was marginal (0.8%), and the dot blot pattern obtained with FcR blocking was comparable to the pattern without blocking (Supplementary Figures 1A and 1B). Then, in the following experiments using CD20 and CD138 antibodies, cells were stained without FcR blocking. In some experiments, cells were stained with CD20-APC, and sorted into CD20[−] and CD20⁺ cells. As negative controls, cells were left unstained under identical conditions. Isolated cells were cultured, and their immunophenotypes were examined with FACS Canto II (BD Biosciences). Data were analyzed by Cell Quest software (BD Biosciences).

Assay for Intracellular ROS Level

Cells were incubated with 10 μ M of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA, Invitrogen by Life Technologies, Carlsbad, USA) and hydrogen peroxide at 37 °C for 20 min. Then, CM-H₂DCFDA-expressing conditions were detected with FACS Canto II.

Immunocytochemistry

FoxO3a expression was immunocytochemically examined in MWCL-1 cells. Cells were cytospun, fixed with 10% formalin. After treatment with peroxidase blocking solution (DAKO, Glostrup, Denmark), cells were incubated with anti-FoxO3a antibody (Abcam, Cambridge, UK, dilution at 1:250). Then, cells were treated with ChemMate EnVision kit (DAKO). DAB (DAKO) was used as a chromogen.

In Vitro Colony Formation Assay

A total of 5×10^4 and 1×10^5 suspended cells were plated in culture dishes with 1–2 ml methylcellulose-containing Dulbecco's Modified Eagle's Medium supplemented with 15% FCS. The number of colonies was counted on day 35 and 23, respectively.

Detection of Apoptotic and Senescent Cells

MWCL-1 cells were cultured with or without FCS for 6 h. In a separate experiment, MWCL-1 cells were cultured with 100 μ g/ml cis-diammine-dichloroplatinum (II) (CDDP, cisplatin) for 36 h. Apoptotic cells were detected with APOPCYTO Annexin V-Azami-Green Apoptosis Detection Kit (MBL, Nagoya, Japan) according to the manufacturer's instructions. Increase of senescence-associated expression of β -galactosidase (SA- β -Gal) activity is a known characteristic of senescent cells. The proportion of SA- β -Gal positive cells was counted using the senescence detection kit (BioVision, Milpitas, USA).

Immunohistochemistry

Cleaved-caspase 3 expression was immunohistochemically examined in clinical cases of LPL diagnosed in Osaka University Hospital from 2002 to 2013. Histological specimen was fixed in 10% formalin and routinely processed for paraffin-embedding. Paraffin-embedded samples were stored in a dark room of Department of Pathology, Osaka University at room temperature. Sections were cut at 4 μ m thickness. After antigen retrieval with Pascal pressurized heating chamber (DAKO), immunohistochemistry was performed using an automated staining system (Dako Autostainer, DAKO). Antibodies used for immunohistochemistry were CD20, CD3, CD138 (DAKO, dilution at 1:400, 1:50, 1:50, respectively), and cleaved-caspase 3 (Cell Signaling Technology, Danvers, USA, dilution at 1:150). Double staining of CD138 and cleaved-caspase 3 was performed using EnVisionTM G|2 Doublestain System, Rabbit/Mouse (DAB + /Permanent Red) (DAKO). CD138 was stained with brown color and cleaved-caspase 3 with red color. Double staining of CD20 and CD138 was also carried out, in which CD20 was stained in brown and CD138 in red. The study was approved by the institutional review board for clinical research at Osaka University Hospital (No. 12461).

Statistical Analysis

Statistical analyses were performed using *t*-tests. The values are shown as the mean \pm standard error (s.e.) of at least three experiments. The *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Immunophenotypic Transition of MWCL-1 Subpopulation

As in the case of WM, MWCL-1 is consisted of subpopulations with distinct immunophenotype, such as CD20⁺ or CD20[−] and CD138⁺ or CD138[−].¹⁸ First, immunophenotypic transition of CD20⁺ and CD20[−] cells was examined. Sorted CD20[−] cells yielded CD20⁺ subpopulation at 36 h after the initiation of culture (Figure 1a). The proportion of CD20⁺ cells gradually increased, and the CD20 expression profile was similar to the original at 132 h after the initiation of

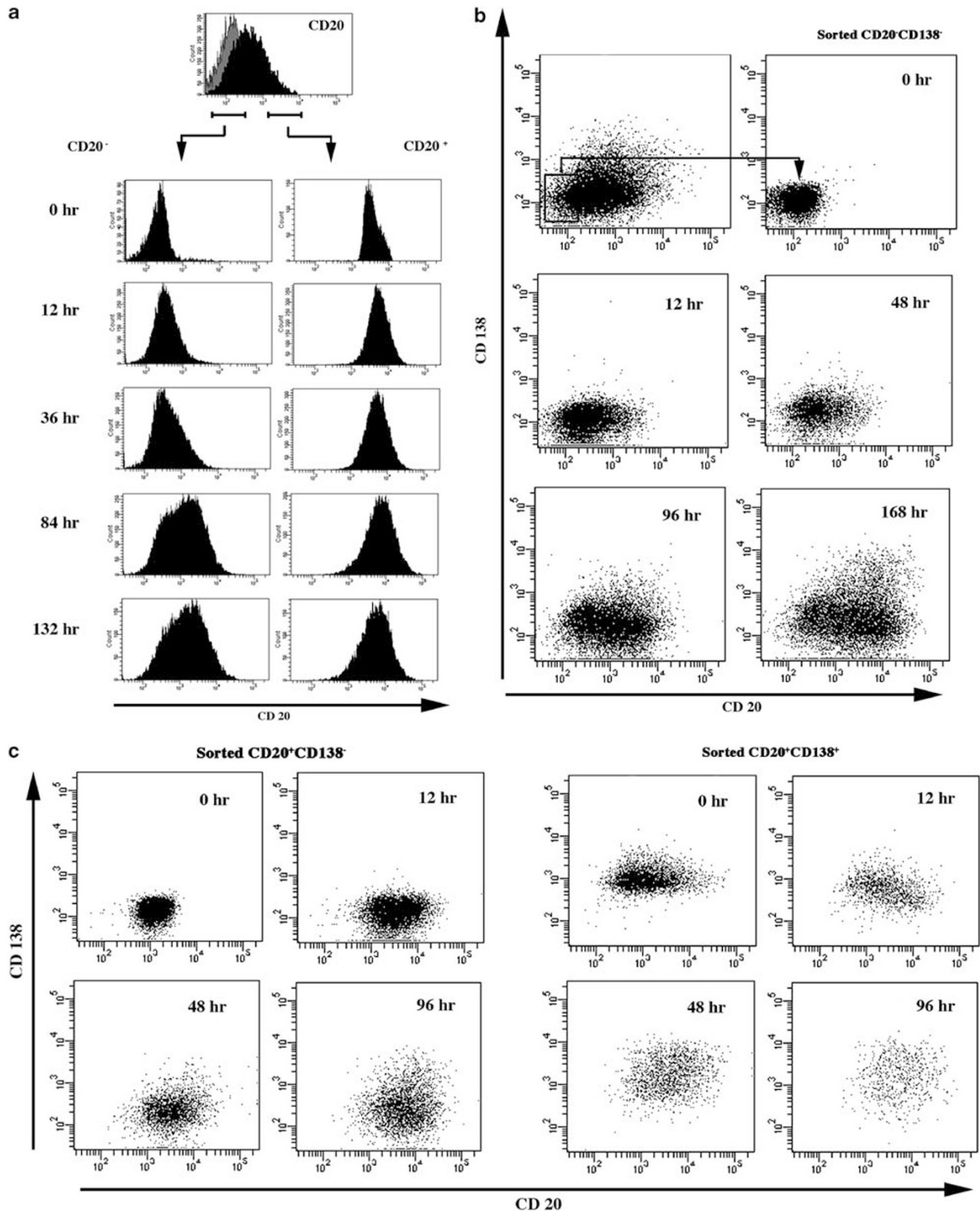


Figure 1 Immunophenotypic transition during the culture of sorted subpopulations. (a) MWCL-1 cells were stained with CD20. The sorted CD20⁺ and CD20⁻ cells were independently cultured, and the change of CD20 expression was examined for 132 h. (b) MWCL-1 cells were stained both CD20 and CD138. Most MWCL-1 cells were classified into three subpopulations: CD20⁻ CD138⁻, CD20⁺ CD138⁻, and CD20⁺ CD138⁺. The sorted CD20⁻ CD138⁻ cells were cultured, and the change of CD20 and CD138 expression was examined. (c) The sorted CD20⁺ CD138⁻ and CD20⁺ CD138⁺ cells were cultured, and CD20 and CD138 expression levels were examined.

culture (Figure 1a). In contrast, sorted CD20⁺ cells did not yield any CD20[−] subpopulation.

Next, MWCL-1 was simultaneously stained with both anti-CD20 and anti-CD138 antibodies. We detected four populations (CD20[−] CD138[−], CD20⁺ CD138[−], CD20[−] CD138⁺ and CD20⁺ CD138⁺), and the proportion of each populations was 14.4 ± 1.6 , 72.6 ± 2.2 , 1.4 ± 0.6 , and 11.7 ± 2.4 , respectively (Figure 1b). This proportion was similar to that observed in clinical samples of LPL (10.9 ± 1.2 , 82.3 ± 0.9 , 0.1 ± 0.1 , and 6.8 ± 0.4 , respectively, Supplementary Figure 2). As the number of CD20[−] CD138⁺ cells was extremely low (Figure 1b), the remaining three subpopulations (CD20[−] CD138[−], CD20⁺ CD138[−], and CD20⁺ CD138⁺) were sorted. When cultured for 168 h, cells derived from CD20[−] CD138[−] subpopulation showed similar immunophenotype to the original MWCL-1 (Figure 1b). The sorted CD20⁺ CD138[−] cells yielded CD20⁺ CD138⁺ but not CD20[−] CD138[−] subpopulation (Figure 1c). The sorted CD20⁺ CD138⁺ cells also yielded CD20⁺ CD138[−] subpopulation, but no CD20[−] CD138[−] subpopulation was detected (Figure 1c). These results indicated that CD20[−] CD138[−] subpopulation could yield all three subpopulations. Once CD20 was expressed, cells did not transit to the CD20-negative state. In contrast, CD138 expression level appeared to be interchangeable; CD20⁺ CD138[−] cells transited to CD20⁺ CD138⁺ and vice versa.

In addition to the bulk culture, a single cell culture of CD20[−] CD138[−] subpopulation was done. As in the case of bulk culture, cells derived from a single sorted CD20[−] CD138[−] cell showed similar immunophenotype to the original MWCL-1 (Supplementary Figure 3).

High ROS-Expelling Activity in CD20[−] CD138[−] Subpopulation

Cells were stimulated with hydrogen peroxide, and then ROS concentration was evaluated by staining with CM-H₂DCFDA after incubation for 20 min. During the incubation, cells with high ROS expelling activity become CM-H₂DCFDA-negative. Approximately half of CD20[−] cells became CM-H₂DCFDA-negative, whereas the proportion of CM-H₂DCFDA-negative cells was lower in CD20⁺ than CD20[−] cells ($44.5 \pm 0.1\%$ in CD20[−] vs $33.6 \pm 0.3\%$ in CD20⁺, Figure 2a). As most CD20[−] cells showed immunophenotype of CD20[−] CD138[−] (Figure 1b), these results suggested that ROS-expelling activity of CD20[−] CD138[−] cells was higher than CD20⁺ cells.

Distinct FoxO3a-Expression Pattern Between CD20[−] and CD20⁺ Subpopulations

It has been reported that ROS-expelling activity is correlated to the nuclear localization of the master transcription factor FoxO3a.^{19–21} When the sorted CD20[−] and CD20⁺ subpopulations was immunocytochemically stained with anti-FoxO3a, the localization of FoxO3a was different: FoxO3a was mostly localized in the nuclei of CD20[−] cells

and was diffusely present in the cytoplasm of CD20⁺ cells (Figure 2b).

ROS Expelling Activity and FoxO3a Expression in the Sorted Cells

We examined the ROS expelling activity and FoxO3a expression in the sorted CD20[−] CD138[−], CD20⁺ CD138[−], and CD20⁺ CD138⁺ subpopulations. As expected in the above-mentioned experiments, ROS expelling activity was the highest in CD20[−] CD138[−] subpopulation (Figure 2c). FoxO3a was localized in the nuclei in CD20[−] CD138[−] cells, but in the cytoplasm of CD20⁺ CD138[−] and CD20⁺ CD138⁺ cells (Figure 2c).

High *In Vitro* Colony Formation Activity in CD20[−] CD138[−] Subpopulation

The CD20[−] CD138[−], CD20⁺ CD138[−], and CD20⁺ CD138⁺ subpopulations were plated in methylcellulose-containing medium, and the number of colonies was counted. When a total of 5×10^4 cells were plated, colonies were obtained from CD20[−] CD138[−] subpopulation but not from CD20⁺ CD138[−] and CD20⁺ CD138⁺ subpopulations (Figure 3). When a total of 1×10^5 cells were plated, the number of colonies derived from CD20[−] CD138[−] subpopulation was significantly higher than that of CD20⁺ CD138⁺ and CD20⁺ CD138[−] subpopulations (Figure 3).

Resistance to Apoptosis

After serum depletion, the apoptotic cells were examined in CD20[−] CD138[−], CD20⁺ CD138[−], and CD20⁺ CD138⁺ subpopulations. Even in the presence of serum, ~20% of CD20⁺ CD138⁺ cells were dead (Figure 4a). Serum depletion did not affect the proportion of apoptotic cells in CD20[−] CD138[−] subpopulation. In contrast, the proportion of apoptotic cells increased slightly in CD20⁺ CD138[−] subpopulation and significantly in CD20⁺ CD138⁺ subpopulation (Figures 4b and c).

Next, apoptosis was induced by the addition of anti-cancer drug, CDDP. When CDDP was added, the proportion of apoptotic cells was not changed in CD20[−] CD138[−] subpopulation. In contrast, apoptotic cells increased moderately in CD20⁺ CD138[−] and significantly in CD20⁺ CD138⁺ subpopulations (Figures 5a and b). These results indicated that the rank order of resistance to apoptosis was as follows: CD20[−] CD138[−] > CD20⁺ CD138[−] > CD20⁺ CD138⁺.

Cellular Senescence in CD20⁺ CD138⁺ Subpopulation

Cellular senescence was examined with SA- β -Gal staining. MWCL-1 cells were sorted into CD20[−] CD138[−], CD20⁺ CD138[−], and CD20⁺ CD138⁺ subpopulations, and stained with SA- β -Gal. Most cells in CD20[−] CD138[−] and CD20⁺ CD138[−] subpopulations were negative for SA- β -Gal. In contrast, approximately half of CD20⁺ CD138⁺ cells were SA- β -Gal-positive (Figures 6a and b).

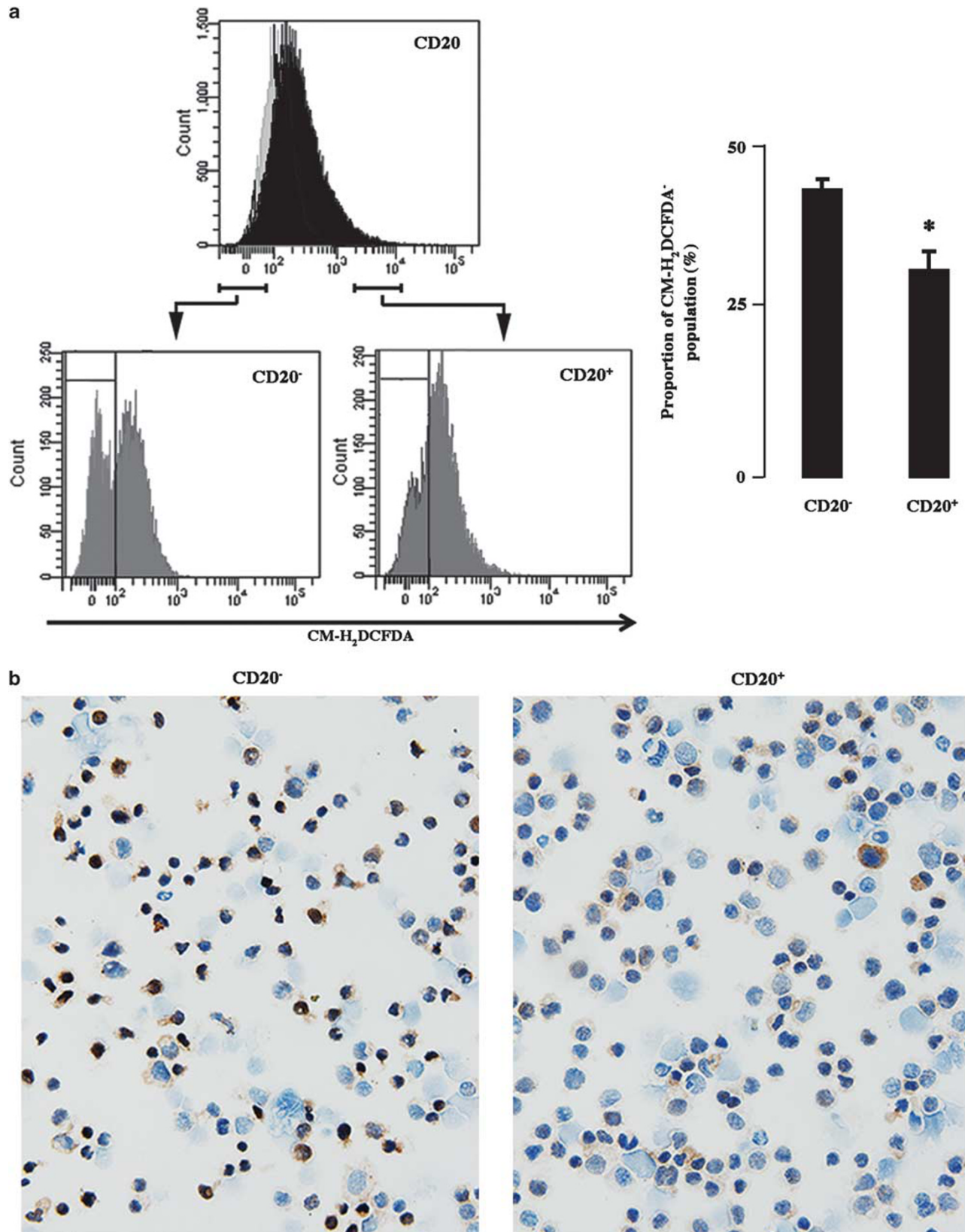


Figure 2 For caption see next page.

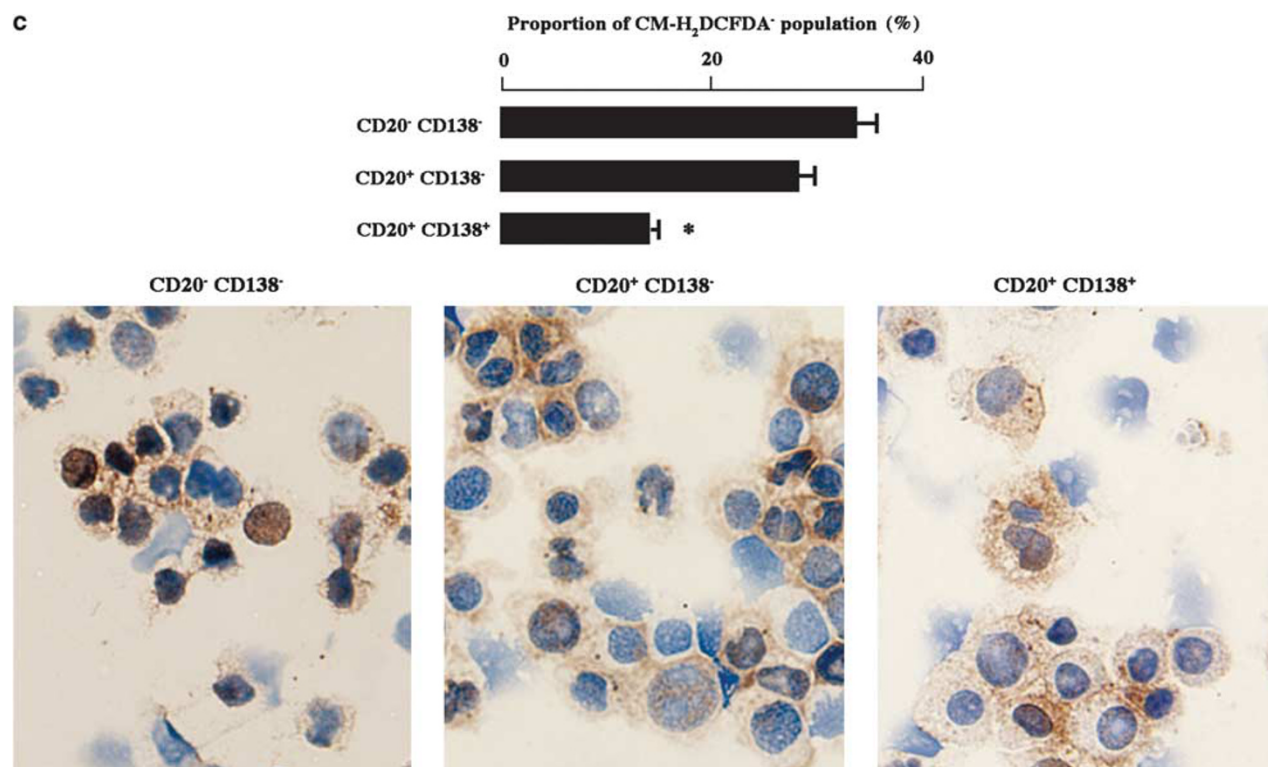


Figure 2 ROS expelling activity in CD20⁻ and CD20⁺ cells. (a) MWCL-1 cells were stained with CD20 (the gray area was correspond to negative control), and cultured with 100 mM of hydrogen peroxide and 10 μ M of CM-H₂DCFDA, a marker of ROS. At 20 min after the initiation of culture, the proportion of cells that became CM-H₂DCFDA-negative was higher in CD20⁻ than in CD20⁺ cells. Proportion of CM-H₂DCFDA-negative cells was shown by bars. **P* < 0.05 when compared with CD20⁺ cells. (b) Immunocytochemistry of CD20⁻ and CD20⁺ cells expressing FoxO3a. The proportion of FoxO3a-positive cells was higher in CD20⁻ than in CD20⁺ cells. FoxO3a was mostly localized in the nuclei of CD20⁻ cells, but diffusely present in the cytoplasm of CD20⁺ cells. (c) Proportion of CM-H₂DCFDA-negative cells in the sorted CD20⁻ CD138⁻, CD20⁺ CD138⁻, and CD20⁺ CD138⁺ cells treated with 50 mM of hydrogen peroxide. **P* < 0.05 when compared with CD20⁺ CD138⁺ cells. Immunocytochemistry revealed that FoxO3a was mostly localized in the nuclei of CD20⁻ CD138⁻ cells but in the cytoplasm of CD20⁺ CD138⁻ and CD20⁺ CD138⁺ cells.

CD20⁺ CD138⁺ Subpopulation in Clinical Samples of LPL

Finally, clinical samples of LPL were examined in the view of CD20 and CD138 expression. When stained with CD20 antibody, few CD20⁻ cells were detected, indicating that the number of CD20⁻ subpopulation was limited in clinical samples of LPL (Figure 7a). Then, lymphoma cells with CD138 expression were considered to be mostly CD20⁺ CD138⁺ subpopulation in clinical samples. Apoptotic cells are known to be cleaved-caspase 3-positive. Double staining of cleaved-caspase 3 and CD138 revealed that most CD138⁺ cells were stained with cleaved-caspase 3 (Figure 7b). This suggested that the proportion of cells in apoptosis was higher in CD20⁺ CD138⁺ subpopulation than in CD20⁺ CD138⁻ subpopulation, which was consistent with the result obtained in MWCL-1.

DISCUSSION

Few studies have been done on CICs of malignant lymphomas. In Hodgkin lymphoma, we have recently reported that a portion of small cells with a single nucleus resembling Hodgkin cells was a candidate of CICs, where high *in vitro*



Figure 3 *In vitro* colony formation. When a total of 5 × 10⁴ cells were plated in methylcellulose-containing medium, colonies were obtained from CD20⁻ CD138⁻ subpopulation but not from CD20⁺ CD138⁻ and CD20⁺ CD138⁺ subpopulations. When a total of 1 × 10⁵ cells were plated, the number of colonies obtained from CD20⁻ CD138⁻ subpopulation was significantly higher than that of CD20⁺ CD138⁺ and CD20⁺ CD138⁻ subpopulations. The number of colonies was counted on day 23 when a total of 1 × 10⁵ cells were plated, and on day 35 when a total of 5 × 10⁴ cells were plated. **P* < 0.05 when compared with CD20⁻ CD138⁻ cells.

colony formation, anti-apoptotic and ROS-expelling activities were detected.⁹ To date, CICs have not been identified in any kinds of non-Hodgkin's lymphomas. Here, we

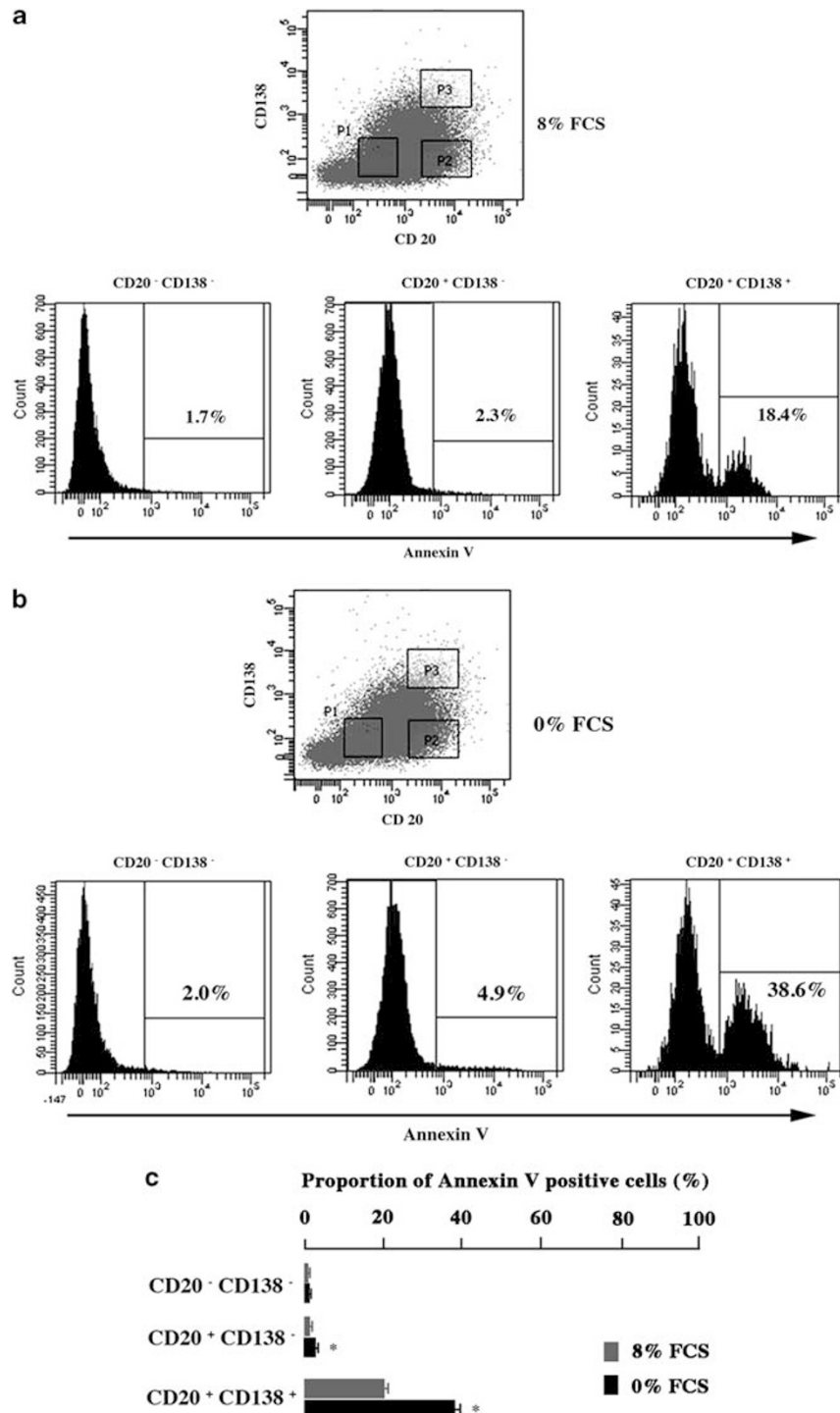


Figure 4 Resistance to apoptosis induced by serum starvation. MWCL-1 cells were cultured with (a) or without (b) FCS for 6 h, and then the apoptotic cells in the three subpopulations were detected with Annexin V staining. (c) Proportion of Annexin V-positive cells was shown. * $P < 0.05$ when compared with the presence of FCS.

investigated a candidate of CICs of WM using MWCL-1. WM tumor cells consist of small B lymphocytes, plasma cells, and lymphoplasmacytoid cells. In addition to the expression of the pan B-cell antigens such as CD20, WM tumor cells also coexpress the markers of mature plasma cells such as CD138.

In fact, MWCL-1 cells express both CD20 and CD138. When stained with anti-CD20 and anti-CD138 antibodies, most MWCL-1 cells were classified into three subpopulations: CD20⁻ CD138⁻, CD20⁺ CD138⁻, and CD20⁺ CD138⁺. Then, these three subpopulations were sorted out and

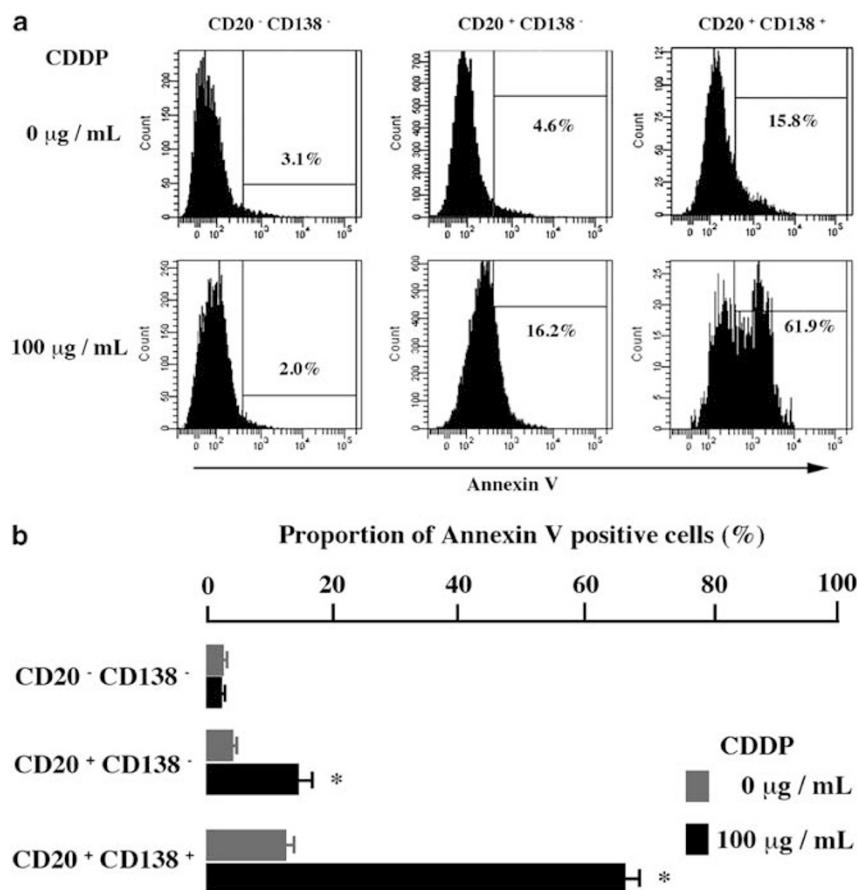


Figure 5 Resistance to apoptosis induced by anti-cancer drug. (a) MWCL-1 cells were cultured with or without 100 µg/ml CDDP for 36 h, and the apoptotic cells in the three subpopulations were detected with Annexin V staining. (b) Proportion of Annexin V-positive cells was shown. **P* < 0.05 when compared with the absence of CDDP.

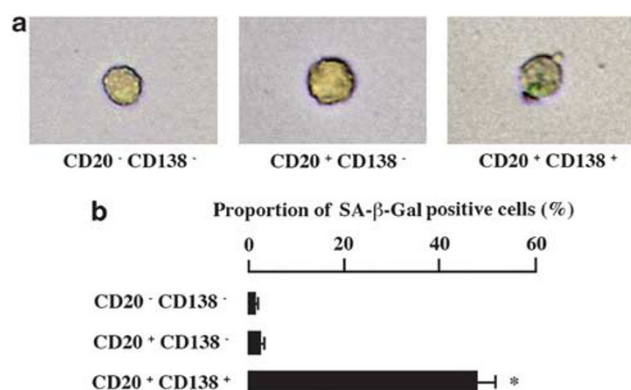


Figure 6 Cellular senescence of three subpopulations. (a) SA-β-Gal staining in CD20⁻ CD138⁻, CD20⁺ CD138⁻, and CD20⁺ CD138⁺ subpopulations revealed SA-β-Gal positive blue-stained cells were detected only in CD20⁺ CD138⁺ subpopulation. (b) Proportion of SA-β-Gal positive cells. **P* < 0.05 when compared with CD20⁻ CD138⁻ subpopulation.

examined. When cultured, CD20⁻ CD138⁻ cells yielded all three subpopulations, but CD20⁺ CD138⁻ and CD20⁺ CD138⁺ cells did not yield CD20⁻ CD138⁻ cells. CICs are

known to yield CICs and non-CICs, but non-CICs do not yield CICs. The result suggested that CD20⁻ CD138⁻ subpopulation was the candidate of CICs in WM.

Recently, CICs have been reported to keep the concentration of ROS at a low level.^{9,10,12} When MWCL-1 cells pulsed with hydrogen peroxide were stained with ROS marker, CM-H₂DCFDA, both ROS-high and ROS-low populations were detected. As compared with CD20⁺ cells, CD20⁻ cells possessed a higher proportion of ROS-low population. As most CD20⁻ cells in MWCL-1 showed immunophenotype of CD20⁻ CD138⁻, ROS-exPELLING activity of CD20⁻ CD138⁻ cells appeared to be higher than that of CD20⁺ cells. This was consistent with the conclusion that CD20⁻ CD138⁻ cells were a candidate of CICs in WM. ROS-exPELLING activity is correlated with the nuclear accumulation of master regulatory transcription factor FoxO3a.¹⁹⁻²¹ In fact, the immunocytochemical analysis revealed that the nuclear localization of FoxO3a was preferentially detected in CD20⁻ cells. FoxO3a is degraded in the cytoplasm when activated by AKT.²¹⁻²³ CD20 is related to the stimulation of B cell receptor signaling, which activates AKT.^{24,25} Owing to the lack of CD20, AKT might be unstimulated, resulting in the keep of FoxO3a expression and high ROS expelling activity in CD20⁻ CD138⁻ cells.

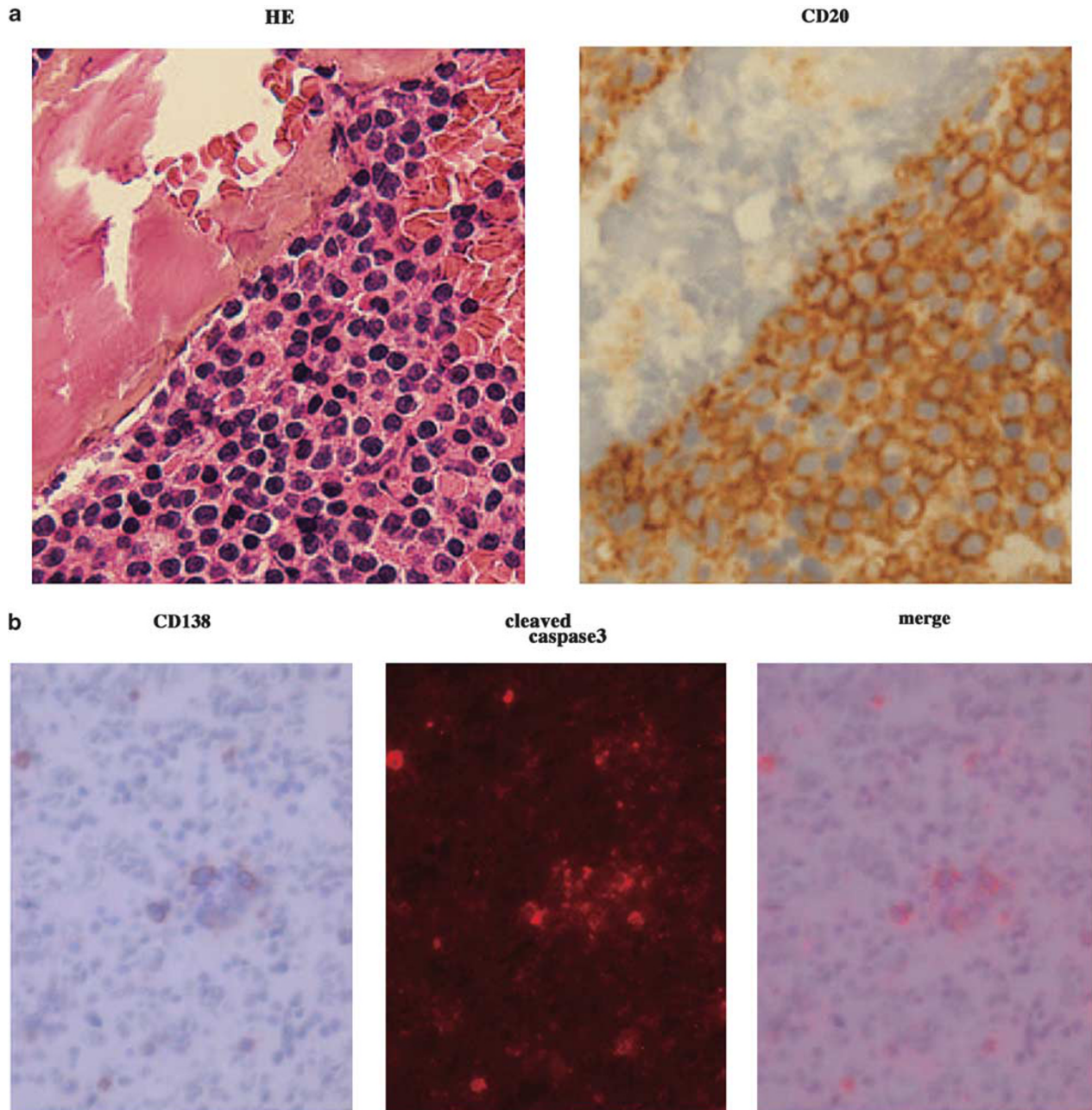


Figure 7 Immunohistochemistry in clinical samples of lymphoplasmacytic lymphoma. **(a)** Representative image of HE and CD20 staining in a serial section ($\times 400$). Almost all tumor cells were CD20-positive. **(b)** Double staining of CD138 and cleaved-caspase 3. CD138-positive cells were shown in brown, and cleaved-caspase 3-positive cells in red. Merged image was also shown. Most CD138⁺ cells were stained with cleaved-caspase 3.

CICs possess high colony formation ability when cultured in semisolid condition.^{9–11} In methylcellulose culture, the number of colony obtained from CD20⁺ CD138⁺ cells was the highest than that of CD20⁺ CD138⁺ and CD20⁺ CD138⁺ cells. In addition, the escape from apoptosis is a prerequisite for CICs.²⁶ Then, we further investigated the resistance of apoptosis induced by starvation and anti-cancer drug CDDP. As expected, the rank order of resistance to apoptosis was as follows: CD20⁺ CD138⁺ > CD20⁺ CD138⁺. These results supported the

current conclusion that CD20⁺ CD138⁺ cell was a candidate for CICs of WM.

In contrast to CD20⁺ CD138⁺ cells, CD20⁺ CD138⁺ cells were vulnerable to apoptosis. Studies with clinical samples of LPL revealed that the apoptotic cells were mostly CD20⁺ CD138⁺, which was consistent with the result obtained in MWCL-1. CD20⁺ CD138⁺ cells showed the highest SA- β -Gal activity, a known characteristic of senescent cells. Therefore, the therapy driving CD20⁺ CD138⁺ cells to CD20⁺ CD138⁺ status will be helpful to cure LPL.

As compared with the examined three populations, the number of CD20[−] CD138⁺ cells was extremely low. The senescent cells were observed in CD20⁺ CD138⁺ sub-population, suggesting that CD20⁺ CD138⁺ may be in the most differentiated status. The differentiation process might start at CD20[−] CD138[−] cells, which appeared to change to CD20⁺ CD138[−] and then to CD20⁺ CD138⁺ cells. Although the biological meaning of CD20[−] CD138⁺ cells was obscure, the main stream of phenotype change did not appear to go through CD20[−] CD138⁺ status. In the current study, CD20⁺ CD138[−] and CD20⁺ CD138⁺ cells were interchangeable, suggesting that the process from CD20⁺ CD138⁺ to CD20⁺ CD138[−] might be dedifferentiation.

Immunohistochemically, most tumor cells were CD20-positive in clinical samples of LPL, and CD20[−] CD138[−] cells were hardly detected. Recently, it has been reported that the high proportion of CICs is correlated to poor prognosis in several tumors.²⁷ To evaluate the prognosis of LPL, useful markers expressing in CD20[−] CD138[−] cells will be required.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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