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Rapid Separation of Mononuclear Hodgkin from Multinuclear Reed-Sternberg Cells

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ABSTRACT

We describe a method to isolate small mononucleated Hodgkin (H) cells from multinucleated Reed Sternberg (RS) cells of Hodgkin lymphoma using the ScreenCell filter device. This filtration-based approach lends itself to future clinical applications in that it enables the separation of H and RS cells from lymph node biopsies, bone marrow aspirates, pleural effusions, and blood, including the isolation of monoclonal Hodgkin precursor cells from the blood.

KEY WORDS: Hodgkin • Reed Sternberg • filtration device • cell separation

INTRODUCTION

Mononucleated Hodgkin (H) and bi- to multinucleated Reed-Sternberg (RS) cells are the malignant cells of classical Hodgkin lymphoma (cHL). The total cellular lymph node mass contains less than 3% of these malignant cells and a majority of nonmalignant cells, mainly reactive T lymphocytes [1]. In analogy to the in vivo situation of diagnostic lymph nodes, where H cells are much more frequent than the rare RS cells, the cHL-derived cell lines L-428 [2], HDLM-2 [3], L-1236 [4], and U-HO1 [5] also are composed mostly of mononuclear cells, multinuclear RS cells being rare, varying from 1% to a maximum 10% of all cells in culture. Binucleated RS cells develop through endomitosis from mononuclear H cells [2-7]. H and RS cells show abnormally large pericentrin structures [8], centromere duplications [9], and a large number of numerical and structural chromosomal aberrations [10-13], indicating that genomic instability plays an important role in the development of this lymphoma.

Recently, the 3D structural and functional characterization of the transition from the mononuclear H to the diagnostic, multinuclear RS cells has been elucidated: our studies on 3D nuclear organization of cHL cell lines and cHL lymph node biopsies demonstrated severe telomere dysfunction and chromosome abnormalities, including structural aberrations and ploidy numbers [14,15]. In particular, 3D nuclear distribution of chromosomes territories, dynamic chromosomal rearrangements, and unequal telomere and chromosome distributions progressively increase from H cells to bi-, tri-, tetra, and multinucleated RS cells [16]. Chromosome painting identified ongoing breakage-bridge-fusion (BBF) cycles at the origin of complex chromosome rearrangements in multinucleated RS cells [16]. Thus, BBF cycles accumulate and increase the complexity of karyotype aberrations in every cycle, increasing cellular dysfunction until mitotic arrest [17-19].

Given the size differences between mononuclear H and multinuclear RS cells, we attempted to find a rapid and reliable method to separate H cells from RS cells, using in this pilot study a Hodgkin cell line, HDLM2. Different approaches can be applied to isolate viable RS cells from lymph node suspension and Hodgkin cell lines, including fluorescence activated cell sorting (FACS) [3,20], magnetic cell sorting (MACS) [21], and density gradient centrifugation [22]. In our experience, these methods are rather time consuming and the steps used to increase the purification of RS cell may result in substantial loss and/or death of the latter. Thus, we tested a different method that involves the use of the ScreenCell CC Filtration Device. We used this filter-based approach to separate H and RS cells based on size. This device was previously used as a rapid isolation technique

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for rare circulating tumor cells [23]. The isolated cells can be tested for their ability to grow in culture media and/or can be examined via immunocytochemistry, fluorescent in situ hybridization, and molecular assays [24]. This approach may, in the future, be applied to H and RS cells from Hodgkin patients using lymph nodes, pleural effusions, and blood.

MATERIALS AND METHODS

Cell Culture

The HDLM-2 cell line [3], originated from nodular sclerosis Hodgkin's lymphoma (DSMZ, Braunschweig, Germany), was grown in RPMI 1640 with 20% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin (Invitrogen/Gibco, Burlington, Ontario, Canada) and incubated at 37°C in a humidified atmosphere and 5% CO2. Cultures were split into new flasks and exchanged with fresh media changed twice weekly.

Cell Separation Using Screen Cell Culture Kit

The cell separation protocol in this study was carried out with minor modifications of the ScreenCell Culture kit instructions (ScreenCell, Paris, France). Cells were counted and diluted with serum-free media at a density of 10^6 cells/3 mL in a 15-mL sterile conical tube. After this step, 0.5 mL of Screen 3LC dilution buffer was added, mixed once by inverting the tube, and incubated 2 minutes at room temperature. After 2 minutes incubation, 2.5 mL of serum free media was added and mixed by inverting the tube. The ScreenCell CC Filtration Device (ScreenCell, Paris, France) was utilized to separate RS cells from H cells, and the filtration procedure was conducted as described by in Desitter et al [24].

After filtration, H cells were collected from the collection tube and cultured in a 25-cm^2 flask containing RPMI 1640 with supplements as stated above. RS cells were grown on the filter by transferring the filter into an 8-well tissue culture plate containing the culture medium specified above. H and RS cells were cultured for 2 weeks, changing media every second day. H cells were split into a new flask when cells were approximately 80%-90% confluent. Three independent experiments of H-RS cell separation were performed.

Giemsa Stain and DAPI Stain of H and RS Cells

After filtration, flow-through H cells were spun down at 800g for 5 minutes at room temperature and resuspended in 1 mL of fresh serum-free media. Thirty microliters of this solution was gently placed on a slide and air-dried. H cells on a slide and RS cells on the filter were stained with 1% modified Giemsa solution (GS500, Sigma-Aldrich, Oakville, Ontario, Canada) in 0.15 mol/L K_2HPO_4/KH_2PO_4 phosphate buffer pH 8.0 for 5 minutes, rinsed with water, and air dried. For 2D nuclei imaging, the filter was counterstained with 1 µL/mL of 4′ 6-diamidino-2-phenylindole (DAPI) for 3 minutes and mounted with Vectashield (Vector Laboratories, Burlington, Ontario, Canada).

Fluorescent Immunocytochemistry (CD30)

Fluorescent immunohistochemistry with anti-CD30 antibody was performed on H and RS cells as follows: the unseparated HDLM2 cells before filtration and the isolated H cells from collection tubes were cytospun onto glass slides at 200g for 5 minutes. HDLM2 cells, isolated H cells on the slides, and RS cells on the filter were fixed with 3.7% formaldehyde for 10 minutes. After blocking with fetal bovine serum for 20 minutes at room temperature, cells were incubated with a monoclonal mouse antibody against human CD30 (Clone Ber-H2, DAKO A/S, Glostrup, Denmark) at a 1:100 dilution for 60 minutes at room temperature. Then, cells were stained with Cy3 goat anti-mouse IgG (A10521, Invitrogen/Gibco, Burlington, Ontario, Canada) at 1:200 dilution for 30 minutes at room temperature. After a final wash with 1× PBS (3 times, for 5 minutes each at room temperature), the slides and filter were counterstained with DAPI and mounted with Vectashield. As the negative control, staining was carried out in the absence of primary antibody.

Image Acquisition of CD30-Stained Cells

Images are acquired using a Zeiss AxioImager Z2 microscope (Carl Zeiss, Toronto, Canada), equipped with an AxioCam HR black and white camera and 63×/1.4 oil objective. A Cyanine 3(Cy3) filter was used for the detection of the Cy3-labeled anti-mouse IgG, and a DAPI filter for nuclear DNA detection with AXIOVISION 4.8 software (Carl Zeiss, Toronto, Canada).

Observation of H and RS Cells after Cell Separation

Isolated H and RS cells were observed and imaged every second day for 2 weeks by using EVOSf1 microscope (Advanced Microscopy Group, Mill Creek, WA). To examine whether the RS cells are capable of cell division(s), we studied the proliferative potential of RS cells were observed on days 1, 3, 5, 7, 9, 11, and 15, using H cells at the identical time points as internal controls.

RESULTS

Identification of H and RS Cells by Morphology and Fluorescent Immunocytochemistry after Cell Separation

To verify whether the ScreenCell live cell filtration device could separate small mononuclear H cells from RS cells and very large mononuclear H cells, Giemsa and nuclear DAPI staining were applied after filtration to identify the typical morphology of H cells from the collection tube and RS cells on the filter. The cell size of RS cells (>15 µm) exceeds that of the pore size of filters, which for live cell filters is 6.50 ± 0.33 µm [24], enabling only small H cells to be harvested in the collection tubes. All RS cells

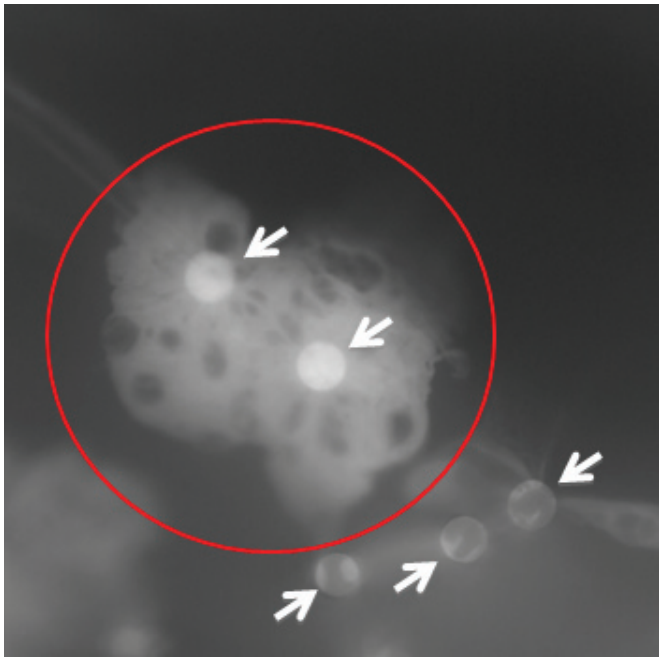

FIGURE 1. DAPI-stained RS cell on the filter after its separation from small mononucleated H cells. The circle highlights the RS cell. White arrows point to filter pores.

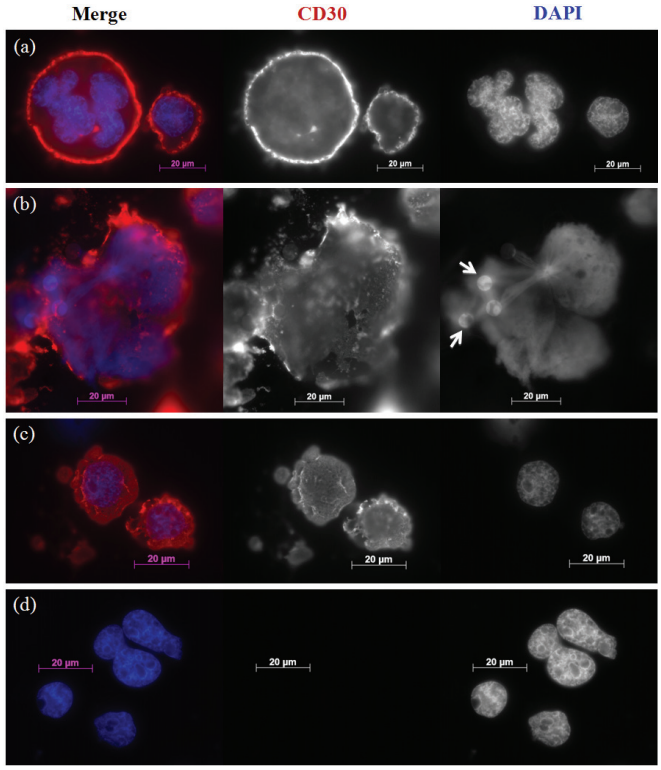

FIGURE 2. Fluorescent anti-CD30 staining in HDLM2 before (a) and after H-RS cell separation (b, c). (b) and (c) show RS cells on the filter and H cells from the collection tube, respectively. (d), negative control (secondary antibody only; see Materials and Methods). The white arrows point at filter pores.

detected were found on the filters (Figure 1). Fluorescent immunocytochemistry of anti-CD30 was utilized to stain H and RS cells before and after cell separation (Figure 2). Figure 2A shows H and RS cells before filtration, both exhibiting the expected CD30 staining. CD30-positive cells were identified on the filter (Figure 2B) and in the collection tube (Figure 2C). Collectively, these results confirm that the ScreenCell filtration device allows for the separation of RS cells from H cells.

Cell Culture Demonstrates that RS Cells are the End-Stage Tumor Cells

To document whether H cells are the precursor cells and generate RS cells as the end-stage tumor cells, we studied the growth potentials of viable isolated H and RS cells. After H-RS cell separation by the ScreenCell CC Filtration Device, the isolated H and RS cells were cultured, cell growth was monitored, and cells were analyzed for 2 weeks in 3 independent experiments. We found that the isolated H cells proliferated in culture and increased in numbers over the 2-week observation period (Figure 3). Individual RS cells appeared in the isolated H cell culture around day 9 (Figure 3b), and additional RS cells were observed on days 11, 13, and 15. The different sizes of RS cells in the isolated H cell culture are illustrated in Figures 3c and 3d. These results confirm that the mononucleated H cells are at the origin of multinucleated RS cells.

As expected, RS cells were unable to proliferate due to telomere disorganization and chromosome abnormalities that are characteristic for the transformation of H to RS cells [14,16]. During the 2-week time period, RS cells did not increase in numbers and around day 10 showed signs of cell shrinkage, possibly associated with cell death. RS cells of HDLM-2 clearly lacked proliferative potential.

DISCUSSION

This pilot study has shown that it is feasible to isolate H/RS cells using the ScreenCell CC Filtration Device. Using the live cell separation option, this method enabled us to culture both H and RS cells and show that small H cells were able to proliferate, while RS cells captured on the filter were unable to increase their cell numbers. This method does not require specific protein markers for isolation of H cells and RS cells since it is solely based on size. After the use of this filter device, precursor H and end-stage RS cells could be identified. Our results strongly suggest that the ScreenCell device may be applied to isolate primary H and RS cells from lymph node suspension, bone marrow aspirates, and eventually blood of Hodgkin patients. Pleural effusion is also a very important application. Indeed, recent

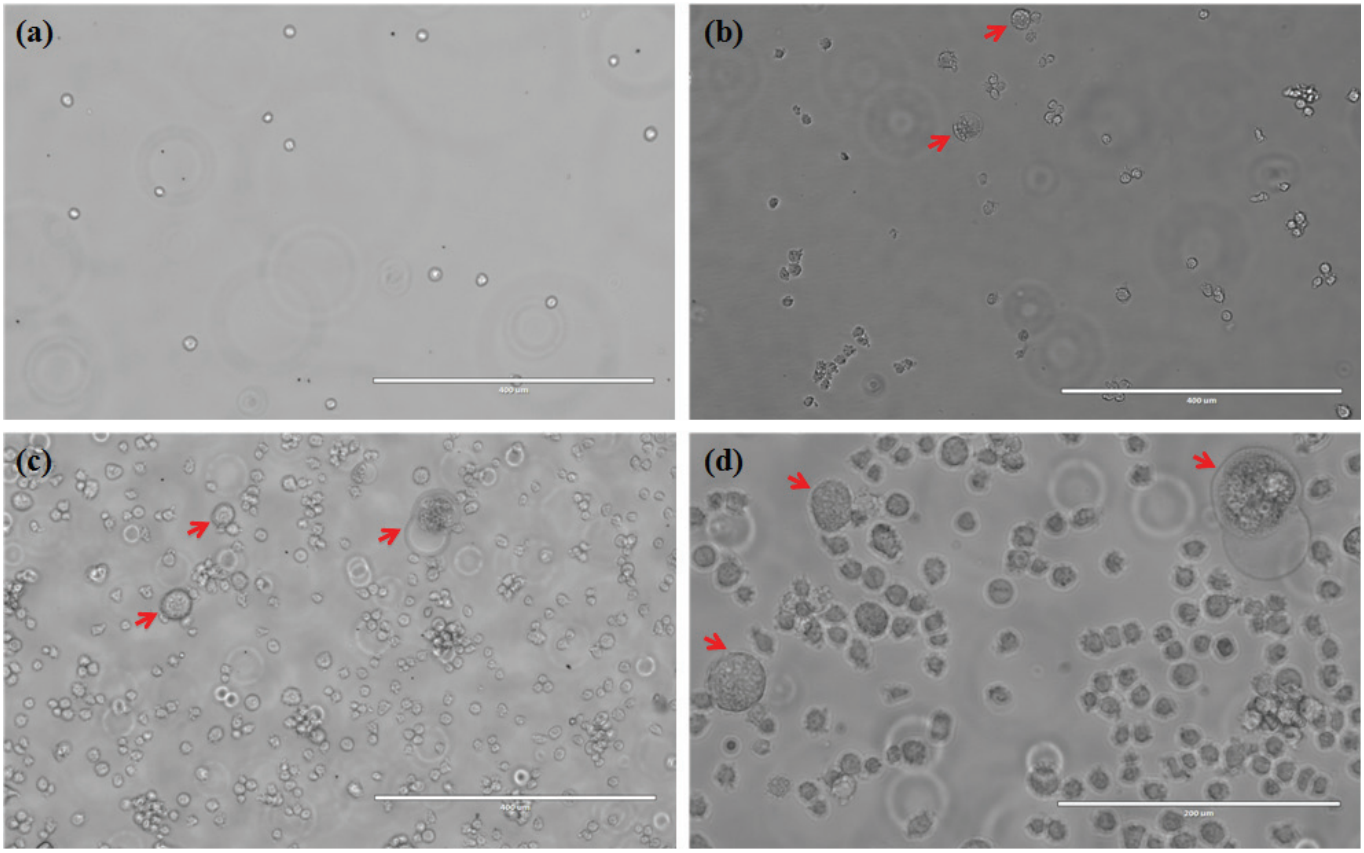

FIGURE 3. Filter-isolated H cells were grown in culture for two weeks. In A, B, and C, cells were observed on day 3, 9, and 15, respectively. Day 9 (B) was the first day that RS cells were found in the H cell culture. Red arrows point to RS cells. In (C) and (D), cells were imaged in the same field but using different magnifications [×10 for (C) and ×20 for (D)].

studies found pleural effusion in over 20% of patients with cHL [25,26]. The etiology of pleural effusion is frequently considered to be lymphatic obstruction but may also be due to direct pleural involvement of malignant cells [1,25-27]. RS cells may be located in pleura and interfere with pleural lymphatic drainage. Therefore, this filtration device may be helpful in the examination of pleural fluid for the identification of RS cells. Moreover, flow-through H cells may be tested for their doubling potential and proliferation rates in culture since we know that HL patients have small mononuclear B cells that are precursors of HL [28]. In conclusion, we identified an approach that enables the isolation of H and RS cells from Hodgkin cultures. Further studies are being developed to apply this method to Hodgkin's patients as it allows for the rapid and sensitive detection of RS and H cells from various sites including blood, lymph node, bone marrow aspirates, and pleural effusions.

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