Characterization of Anoikis-Resistant Cells in Mouse Colonic Epithelium

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(Received 5 January 2013/Accepted 13 April 2013/Published online in J-STAGE 30 April 2013)

ABSTRACT. Anoikis is a form of apoptosis triggered by inadequate or inappropriate cell matrix contacts. Anoikis resistance has been utilized to isolate adult stem or progenitor cell populations from various tissues. The aim of this study was to characterize the stem or progenitor cell markers expressed in anoikis-resistant cells isolated from mouse colonic epithelium. Mouse colonic epithelial cells were isolated by Ca2+-depletion, and anoikis-resistant cells were obtained by culturing the cells in ultra-low attachment dishes. Flow cytometry analysis showed that anoikis-resistant cells are positive for the epithelial cell marker CD326, but negative for the hematopoietic cell marker CD45, eliminating the possibility of hematopoietic cell contamination. The majority of anoikis-resistant cells were also positive for the stem or progenitor cell markers CD133 and DCLK1 by immunofluorescent analysis. Reverse transcriptase-polymerase chain reaction analysis revealed that anoikis-resistant cells express +4 position cell marker Hopx, but did not express the other reported stem or progenitor cell markers, Lgr5, Musash1, Bmi1, mTert and Olfm4. CD133 and DCLK1 double positive cells were observed both apical and basal crypts in mouse proximal colonic tissues. Together, anoikis-resistant cells in mouse colon epithelium were shown to be positive for CD133, DCLK1, Hopx and CD326, but negative for CD45, Lgr5, Musash1, Bmi1, mTert and Olfm4. This study has shown a possible approach to isolating stem cells from the intestinal epithelium.

KEY WORDS: anoikis, CD133, DCLK-1, epithelial cell, intestine.

MATERIALS AND METHODS

Animals: C57BL/6J mice were obtained from Japan SLC (Hamamatsu, Japan). All experiments and animal care procedures in this study were performed according to the Guide to Animal Use and Care of the Yamaguchi University and were approved by the ethics committee.

Antibodies: The following antibodies were used in the experiments described in this report: rabbit anti-DCLK1 (Abnova, Taipei, Taiwan), rabbit anti-CD133 (Novus, St. Louis, MO, U.S.A.), anti-mouse CD45 rat IgG2b FITC, anti-CD133 rat IgG1 FITC, rat monoclonal iso type control to IgG2b FITC, rat monoclonal isotype control to IgG1 FITC (eBioscience, San Diego, CA, U.S.A.), anti-CD326/EpCAM rat IgG2a, κ, rat monoclonal isotype control to IgG2a, κ, FITC (BioLegend, San Diego, CA, U.S.A.), Alexa Fluor488 conjugated anti-rat IgG, Alexa Fluor488 conjugated anti-rabbit IgG, Alexa Fluor594 conjugated anti-rabbit IgG, Alexa Fluor594 conjugated anti-rat IgG and Alexa Fluor 555 conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, U.S.A.).

Isolation of anoikis-resistant cells from mouse proximal colon: The chest cavity of an anesthetized mouse was opened, and the right atrium was cut. Physiological salt solution (PSS), containing heparin, was refluxed from the left ventricle to remove the blood from the body (Fig. 1). A segment of the proximal colon was detached from the mesenterium, and its luminal contents were removed by washing with PSS. The colon was opened, lengthwise and pinned on a silicon plate with the mucosal layer facing up. The colon strips were incubated with Ca2+/Mg2+-free Hank's balanced salt solution (HBSS) containing 1 mM EDTA (EDTA/HBSS) at 37°C, 3 times for 30 min. Detached cells were collected by centrifugation from the second and third incubation solutions. Cells were de-aggregated by treating with HEPES-buffered saline, containing 0.05% trypsin and 0.5 mM EDTA. After centrifugation, the cells were resuspended in the growth medium, Dulbecco's minimum essential medium (DMEM)/F12, containing an antibiotic and antymycotic solution (Invitrogen), INSULIN-TRANS-SEL-X (Invitrogen), mouse epithelial growth factor (EGF) (50 ng/ml; PeproTech, Rocky Hill, NJ, U.S.A.) and mouse leukemia inhibitory factor (LIF) (100 ng/ml; ProSpec-Tany TechnoGene, Ness-Ziona, Israel). Finally, cells were passed through a Cell Strainer (40 µm, BD Biosciences, San Jose, CA, U.S.A.) to obtain a single cell suspension. Single cells were used as “epithelial (Epi) cell” samples and cultured in ultra-low attachment dishes (HydroCell, CellSeed, Tokyo, Japan). Apoptotic cell debris was removed by centrifugation with StemFull tubes (Sumitomo Bakelite, Tokyo, Japan) after 3 days of culture, and the cells were cultured for an additional 3–5 days in HydroCell dishes and used as “anoikis-resistant (AR) cells” samples.

Immunofluorescent staining of cells: Cells were attached and fixed on TACAS slides (MBL, Nagoya, Japan), according to the manufacturer’s instructions. After treating with antibodies against CD133 (1:200) and DCLK1 (1:200) overnight at 4°C, Alexa Fluor-conjugated secondary antibodies (1:1000) were applied at 1 hr temperature, prior to fluorescent images being captured using an Eclipse TE2000-S system (Nikon, Tokyo, Japan).

Fluorescence-assisted cell sorting (FACS) analysis: Cells (2 × 10^6) were treated with 0.2 µg of anti-mouse CD16/32 (eBioscience) in 20 µl of FACS buffer (phosphate buffered saline (PBS), pH 7.4, containing 2% fetal bovine serum and 0.01% sodium azide) for 10 min to block Fc binding. Primary antibodies (0.2 µg) specific for the indicated protein or isotype control, in 20 µl of FACS buffer, were added for 30 min on ice. The fluorescence intensity of 3,000 cells was analyzed using a CyFlow space flow cytometer (Partec, Munster, Germany).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: RT-PCR was performed as described elsewhere [21]. Briefly, total RNA was extracted from cells by using TRIzol (Invitrogen), as per the manufacturer’s protocol. The concentration of the RNA was adjusted to 0.5 µg/µl, and RT-PCR was performed using PrimeScript RT-PCR kits (Takara, Otsu, Japan), following the manufacturer’s protocol. The specific primers used for mouse Msi1, Lgr5, Bmi1, Hopx, Olfn4, mTert and GAPDH were: Msi1 forward
CCTTGATTGCCACAGCCTTC, reverse ATAAAAGC-GACCAGGGAGAG; Lgr5 forward TAAAGACGACGCAACAGTG, reverse GAAGATCGACGATTCGGAAG; Bmi1 forward TCCCCACTTAATGTGTGTCC, reverse TTCTCCTCGTCCTATCG; Hopx forward CACCAC-GCTGTGCTCTCATCG, reverse CAAAACAGCCTGGTAAGCC, Olfin4 forward GCCACTTTCCAATTTCAC, reverse GAGCCTCTTCTCATACAC, mTert forward ACCCAGGATGTACTTTGTTAAGG, reverse AGCAAA-CAGCGTGTCTCCATGTC and GAPDH forward AG-GCCGGTGCTGAGTATGTC, reverse TGCCTGCTTCAC-CACCTTCT.

Immunohistochemistry of mouse proximal colon: Short segments of the mouse proximal colon were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4) for 2 hr at 4°C. After washing with PBS, tissues were soaked overnight in 30% sucrose in PBS at 4°C for cryoprotection, embedded in OCT compound (Miles Laboratories, Elkhart, IN, U.S.A.) and quickly frozen. Cryostat sections were cut at a thickness of 10 µm with a Leica CM3050 cryostat (Leica Microsystems, Wetzlar, Germany) and thaw-mounted on poly-1-lysine-coated glass slides. After washing with PBS, sections were preincubated for 20 min with 5% normal donkey serum and diluted with PBS containing 1% bovine serum albumin (BSA/PBS), before incubation with antibodies against CD133 (1:100 in BSA/PBS) and DCLK1 (1:500) overnight at 4°C. After washing with PBS, sections were incubated with Alexa Fluor 488-conjugated donkey anti-rat IgG (for CD133) and Alexa Fluor 555-conjugated donkey anti-rabbit IgG (for DCLK1) diluted 1:1,000 in PBS. Sections were then washed with PBS, counterstained with 4′, 6′-diamino-2-phenylindole (DAPI; 1:1,000 in PBS) and mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, U.S.A.). Control tissues were prepared by omitting the primary antibodies from the incubation solutions; specific immunoreactivity was not observed. Immunofluorescence images were examined with a Leica TCS-SP2 confocal microscope (Leica Microsystems) with excitation wavelengths of 350, 488 and 543 nm. Images were collected and analyzed using Leica Confocal Software (Leica Microsystems).

RESULTS

Anoikis-resistant cells are CD133-positive epithelial cells: Primary Epi cells were isolated by the Ca²⁺-depletion method, and AR cells were obtained by culturing in ultralow attachment dishes for 6–8 days (Fig. 1; See Material and Methods for details). Anoikis was induced in the majority of the Epi cells within 1–2 days, and the cell debris was removed by centrifugation. AR cell viability was confirmed by trypan blue exclusion (data not shown). Epi cells and AR cells were immunostained with the stem/progenitor cell marker CD133 (Fig. 2). A small number of Epi cells were
CD133-positive. On the other hand, the majority of AR cells (98.3 ± 0.72%) were positive for CD133, even though the brightness of the staining was heterogeneous. Expression of CD133 in AR cells was also confirmed by FACS analysis (Fig. 3A). To eliminate the possibility of contamination by hematopoietic cells, surface expression of the epithelial cell marker CD326/EpCAM and hematopoietic marker CD45 was also analyzed by FACS analysis (Fig. 3B, C). Mouse splenocytes, isolated from same strain, were used as positive controls for CD45 expression (Fig. 3D). These data confirmed that the AR cells were of the epithelial lineage.

Stem/progenitor cell marker expression of anoikis-resistant cells: For further characterization, AR cells were double immunostained for CD133 and DCLK1 (Fig. 4). Although heterogeneous expression patterns were observed, the majority of the AR cells (93.7 ± 2.34%) were positive for both DCLK1 and CD133. Of note, all of the observed DCLK1-positive cells were also positive for CD133. Stains without the primary antibodies were used as negative controls. To study the expression of other stem/progenitor markers, the total RNA was isolated from Epi and AR cells, and mRNA expression was analyzed by RT-PCR (Fig. 5). AR cells express Hopx, an atypical homeobox protein which has been suggested as +4 cell marker [29]. On the other hands, AR cells did not express Msi1, Lgr5, Bmi-1, Olfm4 or mTert. The cDNA from Epi cells was used as a positive control.

Localization of DCLK1/CD133 double positive cells in mouse colonic epithelium: Finally, the localization of DCLK1/CD133 double positive cells was observed in the mouse colon (Fig. 6). Both DCLK1 and CD133 expressions were restricted to a small proportion of the cells. Interestingly, DCLK1/CD133 double positive cells were detected not only in basal crypts but also in apical crypts. These data suggest that DCLK1 marks at least 2 types of epithelial cell lineages in the mouse colon.
Although extensive studies have tried to identify and isolate intestinal stem cells, the localization of these cells remains under discussion and the isolation method has not been fully established. The AR characteristic of stem and progenitor cells has been utilized to isolate the adult stem cell population from various tissues [2, 18]. The aim of the present study was to characterize AR cells in the intestinal epithelium. The AR cells, isolated by the described method, were positive for the epithelial cell marker CD326 and negative for the hematopoietic cell marker CD45, eliminating the possibility of hematopoietic cell contamination. As expected, the majority of the mouse colonic epithelium AR cells were positive for the stem or progenitor cell marker CD133. Further characterization revealed that mouse colonic AR cells express DCLK1 and Hopx, which have been reported as a +4 position cell marker [16, 29], but negative for Lgr5, Msi1 and Bmi1.

DCLK1 is a microtubule-associated protein kinase that is expressed throughout the embryonic central and peripheral nervous systems [14]. Through a dynein-dependent mechanism, DCLK1 regulates the formation of bipolar mitotic spindles and the proper transition from prometaphase to metaphase during neurogenesis [26]. It also mediates fiber tract decussation and neuronal migration during brain development [13]. Although DCLK1 has been considered to be a marker of putative quiescent stem cells [9, 16, 17], recent findings have revealed that it marks a subset of colorectal stem cells, and also a rare subset of epithelial cells called tuft cells [7, 8]. In the present experiment, an immunohistochemical investigation of the mouse colon revealed that DCLK1/CD133 double positive cells are located not only in basal...
crypt, but also in apical crypt. It is highly possible DCLK1/CD133 double positive cells located in apical crypt are tuft cells. These observations raise the question of whether or not AR cells are tuft cells. Interestingly, DCLK1-positive tuft cells express stem or progenitor cell markers, such as Lgr5, Bmi1, Msi1 and CD133 [12], even though they are terminally differentiated. Because the AR cells in this study were negative for Lgr5, Bmi1 and Msi1, they are unlikely to be tuft cells.

Unexpectedly, AR cells do not express either Lgr5 or Bmi1. There are several possibilities to explain this. First, the expression of these markers may have been down-regulated during floating culture. Lgr5 is a receptor for R-spondin and enhances the canonical Wnt signal by associating with Wnt receptor complexes [3]. Conversely, Lgr5 expression is up-regulated by the Wnt signal [32]. Bmi1, one of the Polycomb group repressors, plays a crucial role in cell fate decisions. Bmi-1 expression is positively regulated by the transcription factor c-Myc, and serum stimulation up-regulates the association of c-Myc with the Bmi-1 promoter region [10]. Because the culture medium used in the present experiments did not contain Wnt agonists or serum, the cells may have lost Lgr5- and Bmi1-expression during the floating culture. Another possibility is that the cells that express these markers were removed by floating culture.

Two types of stem or progenitor cells, the active cycling CBCs and the quiescent +4 position cells, have been suggested to exist in the intestinal epithelium; the presence of Lgr5 or Bmi1 distinguishes these 2 cell types [1, 24, 33]. However, recent findings have suggested that Bmi1 expression overlaps, at least partially, with Lgr5 expression [12, 20]. Additionally, Bmi1-positive cells are actively cycled in organoid cultures [33]. These finding suggest that, except for quiescent stem cells, actively cycling stem or progenitor cells may be marked by the presence of Bmi1. AR cells did not proliferate in Matrigel (data not shown), even in the presence of culture medium containing Wnt3A, EGF, R-spondin and Noogin. Similar media conditions allow the growth of organoids from single Lgr5-or Bmi1-positive cells [25, 33]. Therefore, actively cycling stem or progenitor cells, positive for Lgr5 or Bmi1, may be sensitive to anoikis. To clarify whether or not AR cells are quiescent stem cells, they need to be stained with other specific markers. However, Munoz et al. reported robust expression of proposed +4 position cell markers in Lgr5-positive cells [20]. Thus, at the present time, this point cannot be further elucidated.

In conclusion, the present study demonstrated that mouse colonic epithelium contains AR epithelial cells that express the stem or progenitor cell markers CD133 and DCLK1. Although further studies are needed to determine whether AR cells are quiescent stem cells or tuft cells, we have shown a possible approach for isolating stem cells from the intestinal epithelium.

Fig. 6. Localization of DCLK1- and CD133-positive cells in mouse colon. Mouse proximal colon tissues were double-immunostained for DCLK1 (Red) and CD133 (Green). DAPI was used to stain the nuclei (Blue). Arrows and arrowheads indicate DCLK1/CD133 double positive cells in apical and basal crypts, respectively. Bars: 50 µm.
ACKNOWLEDGMENTS. This work was supported by the Uehara Memorial Foundation, the Mishima Kaiun Memorial Foundation and a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES


