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Cancer stem cells

Expression of CD133 in ovarian cancer

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Introduction

Recently, much attention has been focused on the role of cancer stem cells (CSCs) in the formation and progression of malignancies¹. The existence of antigenic profiles distinctive of tumor cells with stem cell properties would be of the utmost importance to facilitate CSC selection. CSCs have been isolated from several solid tumors. Stem cells isolated from nervous system, prostate, colon and hepatocellular carcinomas express the CD133 antigen²⁻⁵. The CD133 antigen, a 120 kDa membrane glycoprotein, was first detected in CD34⁺ hematopoietic stem cells⁶ and since then has been widely used to identify⁶ and to facilitate the isolation of hematopoietic stem and progenitor cells⁷. The aims of this study were to investigate the expression of the CD133 antigen in primary ovarian tumors and to assess phenotypic characteristics, self-renewal capability, and capacity to maintain CD133 expression in serum-free culture.

Material and methods

Tissue collection, isolation and culture of CD133-expressing cells

Tissue specimens from primary ovarian tumors and normal ovaries were obtained at time of primary surgery from 41 patients. Tumors were mechanically and enzymatically dissociated. Depletion of CD45⁺ cells from single-cell suspensions using CD45 MicroBeads (Miltenyi Biotec) was performed in order to remove hematopoietic cell contaminants. CD133⁺ cells were obtained using the CD133 MicroBead Kit, human (Miltenyi Biotec) in combination with the autoMACS[®] Separator. Phycoerythrin (PE)-conjugated

CD133/1 or CD133/2 antibodies (Miltenyi Biotec) were used to evaluate the efficiency of magnetic separation by flow cytometry. CD133⁺ and CD133⁻ cell populations were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and used for limiting dilution and cell proliferation assays.

Limiting dilution assay

The limiting dilution assay was conducted as previously described⁸, with minor modifications. CD133⁺ and CD133⁻ cells were plated at a final density of one cell/well in 96-well plates⁹. Colonies (each one comprising more than 30 cells) were counted at day 14 and expressed as the percentage of the number of wells plated.

Cell proliferation assay

The cell proliferation assay was performed by plating CD133⁺ and CD133⁻ cells in T25 flasks with RPMI 1640 supplemented with 10% fetal bovine serum at a density of 90,000 cells/mL. Cells were counted on days 6 and 12. Cell viability was determined by the Trypan Blue exclusion method.

Flow cytometric analysis of CD133⁺ cells

Aliquots of tumor tissue cells were incubated for 10 min in the dark at 4 °C with FcR Blocking Reagent (Miltenyi Biotec), the monoclonal antibody CD133/1 (AC133)-PE or CD133/2 (293C3)-PE (Miltenyi Biotec), and a fluorescein isothiocyanate (FITC)-conjugated CD45 antibody (Becton Dickinson). Appropriate fluorochrome-conjugated, isotype-matched antibodies were used as control to establish background staining. To identify dead cells, samples were incubated with 20 µg/mL 7-aminoactinomycin-D (7-AAD; Molecular Probes) for 10 min at room temperature; 7-AAD⁺ dead cells were systematically excluded from the analysis. In order to confirm the epithelial nature of purified CD133⁺ cells, cells were fixed and the expression of cytokeratin 7 (CK7) was measured by incubation for 1 h at 4 °C with a monoclonal antibody to human CK7 (Dako). An anti-mouse FITC-conjugated antibody (Dako) was used as secondary antibody. Cells were analyzed flow cytometrically as described⁹.

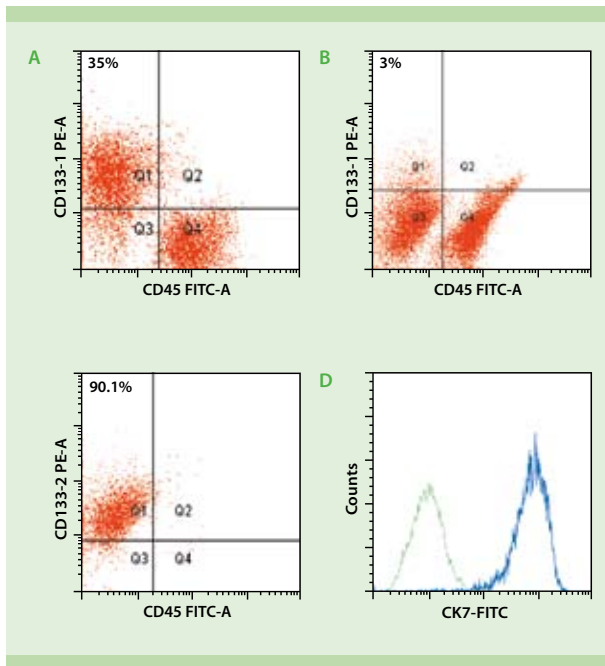


Figure 1 Two representative experiments depicting CD133 expression in primary ovarian carcinoma. A, B) CD133-1-expressing cells in the whole tissue preparation and C) after cell purification by MACS[®] Technology. D) Flow cytometric analysis of CK7 expression of isolated CD133⁺ cells from ovarian carcinoma (frequency of CK7-positive cells = 92.4%). The first peak represents the negative control lacking the primary antibody, while the second peak refers to the incubation of cells with the CK7 antibody.

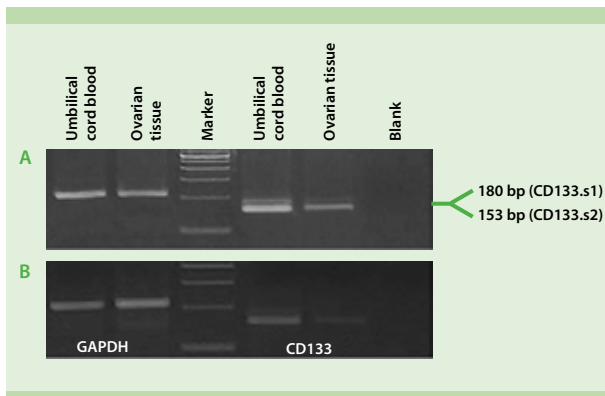


Figure 2 Representative experiment showing semiquantitative RT-PCR analysis of Prom-1 splice variant expression in ovarian cancer (A) and in normal ovarian tissues (B). The lower band (153 bp) and the upper band (180 bp) correspond to the CD133.s2 and CD133.s1 variants, respectively.

Reverse transcription-polymerase chain reaction studies

Total RNA was extracted using the RNeasy[®] Mini Kit (Qiagen) from homogenized tissues or cells, and 1 µg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (PE Applied Biosystem). Two microliters of the product were used for the amplification with 1 U of AmpliTaq Gold[®] (PE Applied Biosystems) in the presence of primers specific for the two CD133 splice variants using the PTC-0200 DNA Engine[®] (MJ Research)¹⁰. For the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers 5'-TGACATCAAGAAGGTGGTGA-3' and 5'-TCCACCACCTGTTGCTGTA-3' (synthesized by m-Medical) were used. PCR products were analyzed on 3% agarose gels.

Immunohistochemistry

Immunostaining was performed on 3-µm, formalin-fixed, paraffin-embedded cancer tissue sections, mounted on SuperFrost[®] Plus microscope slides. The slides were deparaffinized in xylene and rehydrated conventionally; the endogenous peroxidase was blocked with 3% H₂O₂ for 5 min. To reduce non-specific binding, sections were incubated with 20% normal goat serum for 30 min at room temperature. Cells expressing CD133-1 were identified after overnight incubation at 4 °C using the mouse monoclonal CD133/1 (AC133) antibody (Miltenyi Biotec) at a 1:50 dilution. CD133-1 detection was performed using anti-mouse EnVision[™] System-HRP (Dako) for 30 min at room temperature. Diaminobenzidine was used as a chromogen (DAB Substrate System from Dako). Sections were counterstained with hematoxylin.

Results and discussion

Single-cell suspensions from dissociated tumor tissue and normal ovaries were flow cytometrically analyzed for expression of the CD133 antigen. The frequencies of CD133-expressing cells in normal ovaries ranged from 0.06 to 0.5% (median value = 0.16%) and were not significantly different from the values obtained in benign ovarian tumors (range: 0.1–5.40%; median value = 0.30%) but differed from those measured in primary ovarian cancer (frequencies of CD133⁺ cells ranging from 0.01 to 42.1%; median value = 1.2%; $p < 0.01$). The data were asymmetrically distributed and ranged from very low to high percentages. For this reason we present the median values.

Figures 1A and 1B show two representative examples of flow cytometric analysis of CD133⁺ cells in the whole preparation of single cells from primary ovarian carcinoma, with high and low frequencies of CD133⁺ cells, respectively. Figure 1C depicts the analysis of CD133⁺ cells after MACS[®] Cell Separation of the preparation shown in figure 1A using the CD133 MicroBead Kit, yielding a high purity of CD133⁺ cells. The epithelial origin of isolated CD133⁺ cells was confirmed by flow cytometric analysis with the monoclonal anti-CK7 antibody, as shown in figure 1D. Limiting dilution experiments were performed in order to document the ability of isolated CD133⁺ cells to form colonies. CD133⁺ cells gave rise to a 4.7±0.9-fold higher number of colonies than the CD133⁻ population ($P < 0.05$). CD133⁺ cells were also capable of extensive proliferation in liquid culture: CD133⁺ cells had a 2.2-fold higher proliferative potential compared to CD133⁻ cells. Quantitation of the percentage of CD133⁺ cells in the cultures revealed that the frequency of CD133-expressing cells diminished with time of culture. However, their absolute number increased, thus sustaining their self-renewing capacity (data not shown).

Figure 2 shows a representative experiment of RT-PCR analysis of CD133 splice variant expression in ovarian cancer (fig. 2A) and in normal ovarian tissues (fig. 2B). The lower band (153 bp) and the upper band (180 bp) correspond to the CD133.s2 and CD133.s1 variants,

respectively. The data suggest that both splice variants are expressed in ovarian cancer, whereas the bands were barely detectable in samples from normal tissue. Immunohistochemical analysis was performed with the aim to investigate the localization of CD133-expressing cells in ovarian tissues. Specific immunostaining for CD133 was detected in scattered foci of tumor cells (fig. 3A), while no immunoreactivity was found in normal ovarian tissue (fig. 3B).

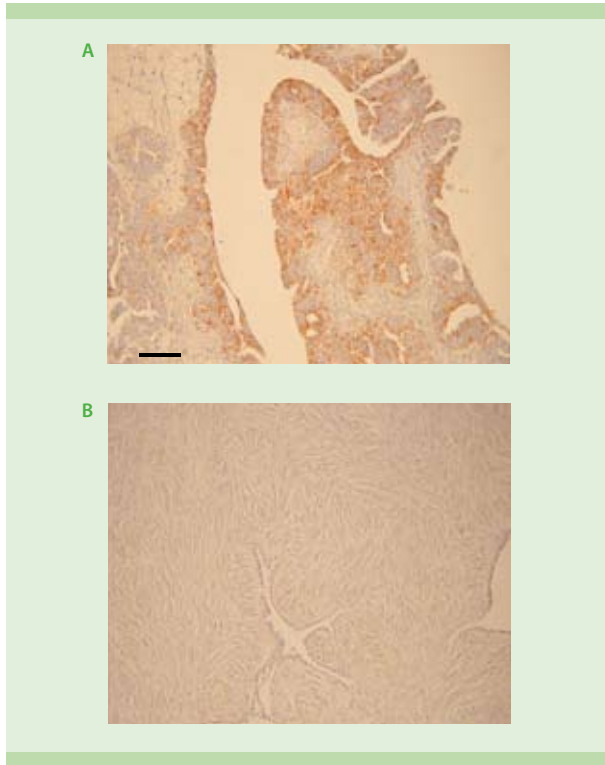


Figure 3: CD133-1 immunostaining in tissue sections of primary ovarian cancer (A) and normal ovarian tissue (B). The figure shows representative data. Magnification: 10x. The bar represents 200 μ m.

Conclusion

CD133-1 and CD133-2 are useful markers for the isolation of CD133⁺ ovarian tumor cells, which are characterized by a higher clonogenic efficiency and proliferative potential than CD133⁻ cells.

Isolation of CD133⁺ cells by MACS Technology using the CD133 MicroBead Kit yields a cell population of high purity. Isolated cells can be used for flow cytometric analysis. Moreover, cells can be cultured for analysis of their *in vitro* clonogenic and proliferative potential. The CD133 antigen is expressed in ovarian tissue as shown by immunohistochemistry using monoclonal CD133 antibodies from Miltenyi Biotec.

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