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Expansion and characterization of cancer stem-like cells in squamous cell carcinoma of the head and neck

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SUMMARY

Evidence has accumulated indicating that only a minority of cancer cells with stem cell properties, cancer stem cells (CSCs), are responsible for maintenance and growth of the tumor. CD44 is currently used to identify CSCs as one of the cell surface markers for solid tumors. Here we report the identification, expansion, and characterization of CD44+ cancer stem-like cells from a permanent squamous cell carcinoma of the head and neck (SCCHN) cell line. Under serum-free medium culture conditions, a small population (less than 3%) of CD44+ cells in a permanent cancer cell line was dramatically increased up to around 40%. The CD44+ cell population also showed higher expression of CD133 and ABCG2 as compared with the CD44− cell population. Moreover, CD44+ cells possess not only a marked capacity for forming tumor spheres, proliferation, migration, and invasion *in vitro*, but also resistance to chemotherapeutic agents. Four genes related to chemoresistance, ABCB1, ABCG2, CYP2C8, and TERT, were up-regulated in a CD44+ cell population. Our findings indicate that a subpopulation of CSCs is maintained in the SCCHN cell line, and the presence of such CSCs has an important clinical implication for head and neck cancer treatment. Further characterization of CSCs may provide new insights for novel therapeutic targets and prognostic markers.

Introduction

Evidence has accumulated indicating that only a minority of cancer cells with stem cell properties, cancer stem cells (CSCs), are responsible for maintenance and growth of the tumor.[1–3] Recent advances in stem cell biology enable the identification of CSCs in solid tumors as well as putative stem cells in normal organs.[4–7] CD44 is currently used to identify CSCs as one of the cell surface markers for solid tumors.[4–6] With respect to squamous cell carcinoma of the head and neck (SCCHN), Prince et al. demonstrated that a small population of CD44+ cancer cells obtained from fresh tumor tissues, but not CD44− cancer cells, gave rise to new tumors in immunodeficient mice.[8] Interestingly, CSCs have been also identified in cultured SCCHN cell lines. Pries et al. demonstrated that permanent SCCHN cell lines constitutively expressed CD44.[9] Alternatively, Zhou et al. have shown that CD133+ cells in a laryngeal carcinoma cell line possess the capacity for self-renewal, extensive proliferation, and multilineal differentiation potency *in vitro*.[10] These findings suggest that a subpopulation of CSCs can persist even with long-term culture *in vitro*; however, CSCs are not only a small or rare subpopulation in tumors, but are also maintained in a hierarchy together with a spectrum of cells at different stages of differentiation; therefore, it is too difficult to maintain an enriched status of CSCs in long-term culture. Recent reports have demonstrated that CSCs from epithelial organs can be expanded as sphere-like cellular aggregates in serum-free medium (SFM) containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF).[11–13] In the current study, we first sought to detect CD44+ cells in the established SCCHN cell line, the Gun-1 cell line and, as expected, CD44+ cells represented a minority of the tumor cell population. Surprisingly, CD44+ cells were able to be propagated *in vitro* under SFM containing EGF and bFGF culture conditions. We then focused on whether such expanded CD44+ cells have stem cell properties by comparing with CD44− cells.

Materials and methods

Cell line and culture conditions

A SCCHN cell line, Gun-1, was established from a squamous cell carcinoma of the hypopharynx.[14] Gun-1 was cultured in either RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (all reagents were from Invitrogen, Grand Island, NY), or Serum Free

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Expansion Medium (StemCell Technologies, Inc., Vancouver, Canada) supplemented with EGF (Calbiochem, Darmstadt, Germany) and bFGF (Calbiochem) (20 ng/ml each). For tumor sphere culture, cells were seeded at a density of 1×10^3 cells/ml in uncoated plastic dishes and cultured for 7 days.

Flow cytometry

The trypsinized cells were resuspended, incubated with monoclonal antibodies for 30 min at 4 °C, washed twice with phosphate-buffered saline (PBS) containing 0.1% FBS and 0.1% NaN_3, and fixed with 1% paraformaldehyde in PBS. The antibodies used were anti-CD44− fluorescein isothiocyanate (FITC), -phycoerythrin (PE), or -allophycocyanin (APC), anti-CD24-PE, anti-HLA class I-FITC, anti-CD133-PE, anti-ABCG2 (all purchased from BD Pharmingen). FITC-conjugated goat anti-mouse monoclonal antibody was used as a secondary antibody in some experiments. Respective immunoglobulin G (IgG) isotype-matched controls (BD Pharmingen) were used as negative controls.

Immunocytochemistry

Dishes containing tumor spheres were washed with PBS (Invitrogen), and incubated with antibodies against CD44-FITC (BD Pharmingen, San Diego, CA), CD133-PE (Miltenyi Biotec, Gladbach, Germany), and ABCG2 (BD Pharmingen) for 30 min. FITC-conjugated goat anti-mouse monoclonal antibody was used as the secondary antibody for ABCG2 staining. After two additional washes, fluorescence microscopy was performed.

Magnetic cell sorting

The cells were incubated with anti-CD44-biotin (BD Pharmingen) for 5 min at 4 °C. After washing once, 20 μl of anti-biotin microbeads (Miltenyi Biotec)/1×10^7 cells was added for 15 min at 4 °C. Subsequently, cells were washed once, resuspended, and applied into MACS separation columns (Miltenyi Biotec). Positive (CD44+) and negative (CD44−) fractions were resuspended in SFM with EGF and bFGF for further experiments, respectively.

Cell proliferation assays

Cell proliferation was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Cells were plated at a density of 500 cells/well in 96 flat-bottomed plates, and cell proliferation assays were performed on days 1, 3, 5, and 7 using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS. Twenty microliters of MTS solution was added to each well and the plate was incubated for 2 h at 37 °C, and viable cells were quantified by measuring absorbance at 490 nm.

Migration and Invasion assays

The sorted cells were plated at a density of 2.5×10^4 cells/well onto 8-μm Transwell filters in a 24-well plate. Medium containing 10% FBS was added to the bottom wells as a chemoattractant. Twenty-four hours later, the filters were removed and, then stained with a Diff-Quik kit (Sysmex Corp., Kobe, Japan) according to the manufacturer's instructions. The migratory cells were counted in four random fields per insert under a microscope at 20× magnification. The invasion assay was performed in a similar fashion using BD BioCaot Matrigel Invasion Chamber (BD Bioscience), and the results are expressed as the total number of cells that had invaded each filter.

Drug sensitivity assay

The number of viable cells following drug treatment was assessed using a MTS proliferation assay as described above. Cells were plated at a density of 5×10^3 cells/well in 96 flat-bottomed plates, allowed to attach overnight, and finally chemotherapeutic agents at various concentrations were added. The cultures were incubated at 37 °C for 48 h, 20 μl of MTS solution was added for the last 2 h, and absorbance at 490 nm was measured. The chemotherapeutic agents tested were carboplatin (kindly supplied by Bristol-Myers Squibb), paclitaxel, docetaxel (both kindly supplied by Sanofi-Aventis), 5-fluorouracil (kindly supplied by Kyowa Hakko), and cisplatin (kindly supplied by NIPPON KAYAKU).

Real-time quantitative RT-PCR

To assess the expression of genes related to stem cell and cancer drug resistance in CD44+ cells, real-time quantitative RT-PCR was performed. We used the commercially available RT^2 *Profiler*™ PCR Array (Super Array Bioscience Corp). A list of genes on this PCR array is available from http://www.superarray.com/rt_pcr_product/HTML/PAHS-405A.html and http://www.superarray.com/rt_pcr_product/HTML/PAHS-004A.html. The relative expression level of the target gene in CD44+ cells to that in CD44-cells was determined by $2^{-\text{deltadelta Ct}}$ method.

Statistical analysis

Two-tailed Student's *t* test was used for statistical analysis of data. *p*-values <0.05 were considered significant.

Results

Identification and expansion of CD44+ cell in Gun-1 cell line

We first examined CD44 expression in a SCCHN cell line, Gun-1, using flow cytometry, and it was detected in 2.1%, as shown in Figure 1A. Gun-1 was then cultured in SFM containing EGF and bFGF. After 5 weeks of culture, the CD44+ population was increased up to around 40%, as shown in Figure 1B.

Expression of CSC-related markers on CD44+ and CD44− cells

To examine the difference between CD44+ and CD44− subpopulations, the expression of CSC-related markers was analyzed by flow cytometry (Fig. 2). The expression of CD133 and ABCG2 on CD44+ cells was higher than on CD44− cells. In contrast, the expression of CD24 was lower in CD44+ cells. There was no difference in the expression of HLA class I molecules between these two subpopulations.

Tumor sphere and expression of CSC-related markers

After plating at low densities in uncoated dishes, tumor cells readily proliferated and formed spheres in within 5 days (Fig. 3A and B), whereas the original parental cells cultured with RPMI-1640 containing 10% FBS neither proliferated nor formed spheres (data not shown). Moreover, as expected, tumor spheres showed immunoreactivity for CD44, CD133, and ABCG-2 (Fig. 3C–E).

CD44+ cell population exhibits higher potential for proliferation, migration, and invasion

To investigate the biological significance of the CD44+ population, we sorted CD44+ and CD44− cell populations using magnetic bead cell sorting. After sorting, CD44+ cells were only 0.8% in CD44− populations (data not shown). CD44+ and CD44− popula-