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The usefulness of three-dimensional cell culture in induction of cancer stem cells from esophageal squamous cell carcinoma cell lines

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ABSTRACT

In recent years, research on resistance to chemotherapy and radiotherapy in cancer treatment has come under the spotlight, and researchers have also begun investigating the relationship between resistance and cancer stem cells. Cancer stem cells are assumed to be present in esophageal cancer, but experimental methods for identification and culture of these cells have not yet been established. To solve this problem, we created spheroids using a NanoCulture® Plate (NCP) for 3-dimensional (3-D) cell culture, which was designed as a means for experimentally reproducing the 3-D structures found in the body. We investigated the potential for induction of cancer stem cells from esophageal cancer cells.

Using flow cytometry we analyzed the expression of surface antigen markers CD44, CD133, CD338 (ABCG2), CD318 (CDCP1), and CD326 (EpCAM), which are known cancer stem cell markers. None of these surface antigen markers showed enhanced expression in 3-D cultured cells. We then analyzed aldehyde dehydrogenase (ALDH) enzymatic activity using the ALDEFLUOR reagent, which can identify immature cells such as stem cells and precursor cells. 3-D-cultured cells were strongly positive for ALDH enzyme activity. We also analyzed the expression of the stem cell-related genes Sox-2, Nanog, Oct3/4, and Lin28 using RT-PCR. Expression of Sox-2, Nanog, and Lin28 was enhanced. Analysis of expression of the hypoxic surface antigen marker carbonic anhydrase-9 (CA-9), which is an indicator of cancer stem cell induction and maintenance, revealed that CA-9 expression was enhanced, suggesting that hypoxia had been induced. Comparison of cancer drug resistance using cisplatin and doxorubicin in 3-D-cultured esophageal cancer cells showed that cancer drug resistance had increased. These results indicate that 3-D culture of esophageal squamous cell carcinoma lines is a useful method for inducing cancer stem cells.

1. Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth leading cause of cancer death, but is one of the least studied of all cancers [1,2]. Prognosis for this cancer remains poor, even with multimodal treatment, due to high resistance to chemotherapy and radiotherapy.

Cancer stem cells have recently been recognized as a contributing factor to the resistance to multimodal treatment for cancer therapy [3–5]. According to the definition proposed by Reya et al. at the 2006 meeting of the American Association for Cancer Research, cancer stem cells are considered to have four characteristics: tumorigenicity, multipotency, self-renewal, and drug resistance [6,7]. These cells have been identified using intracellular metabolic activity and cell surface CD antigens, and have been studied using cells isolated from cultured cell lines and clinical samples. The following findings have been garnered from a range of cancers (hematological malignancy, colon cancer, brain tumors, breast cancer, etc.). (1) CD44, CD133, CD338 (ABCG2), CD318 (CDCP1), and CD326 (EpCAM) are candidates for surface CD antigens in cancer stem cells [8–12]. (2) The enzymatic activity of aldehyde dehydrogenase (ALDH) is elevated in cancer stem cells and precursor cells, and by measuring this enzymatic activity, it is possible to identify cells with the multipotency characteristic of cancer stem cells [13–18]. (3) Sox-2, Nanog, Oct3/4, and Lin28 have been identified as stem cell-related genes, and cells that strongly express these genes have acquired resistance to chemotherapy [19–22]. (4) Cancer stem cells are induced and maintained in hypoxic conditions [11,23]. However, with research on esophageal cancer having failed so far to identify cancer stem cells, there is a

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need to develop new methods for inducing esophageal cancer stem cells.

Tumors in the body have a 3-dimensional (3-D) structure, and tumor cells form a microenvironment through interactions with their surroundings in three dimensions. Cancer drug resistance studies in other cancers have reported the benefits of using spheroids formed in 3-D cell culture, which experimentally reproduces the 3-D structure of cancerous tissue in the body, instead of using conventional 2-dimensional (2-D) cell culture on a flat plate [24–26].

In this study, we investigated the usefulness of 3-D cell culture in inducing esophageal cancer stem cells by culturing esophageal cancer cell lines in 2-D and 3-D culture and comparing cell surface antigen markers, expression of strongly analyzed ALDH-positive cells, gene expression, and sensitivity to anti-cancer drugs.

2. Materials and methods

2.1. Tumors and cell culture methods

The human esophageal squamous cell carcinoma lines TE2 and TTn (supplied by Professor Hisahiro Matsubara (Fig 1A), Department of Frontier Surgery, Graduate School of Medicine, Chiba University) [27–29] were cultured in a 75-cm^2 flask (Greiner Bio-One, Frickenhausen, Germany) at 37 °C in 5% CO_2 in RPMI-1640 cell culture medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Gibco; Grand Island, NY) and the antibiotics penicillin (5000 units/mL) and streptomycin (5000 μg/mL) (Gibco).

2.2. 3-D cell culture using NanoCulture® Plates (NCP)

3-D culture was done for 2 weeks in RPMI-1640 medium supplemented with 6.7% FCS using a 24-well NCP inoculated with 5×10^4 cells per well. The spheroids formed in 3-D culture were harvested by pipetting, re-inoculated onto a flat culture plate, and cultured for 2 days.

2.3. Analysis of cancer stem cell surface CD antigens

A FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was used to analyze cell surface antigen markers. Fluorescence-labeled antibodies CD44 [5], CD133 [6], CD326 [8] (eBioscience, San Diego, CA), CD318 [7], and CD338 [9] (Biolegend, San Diego, CA) were used for surface markers.

2.4. ALDEFLUOR assay

The ALDEFLUOR® kit (StemCell Technologies, Inc., Vancouver, BC, Canada) was used for ALDEFLUOR analysis. Cells were incubated for 60 min at 37 °C in a buffer containing 100 U/mL BODIPY-aminoacetaldehyde (BAAA), washed twice, and analyzed with flow cytometry. When analyzing the differences in ALDH enzymatic activity, we confirmed that the activity of ALDH-positive cells was reduced by diethylaminobenzaldehyde, which inhibits ALDH activity.

2.5. Analysis of gene expression

The expression of Sox-2, Nanog, Oct3/4, and Lin28 was analyzed with real-time PCR after extraction of mRNA. Primers with the following sequences were used.

Sox2-forward: 5′-ATGGACAGTTACGCGCACAT-3′, Sox2-reverse: 5′-GACTTGACCA

CCGAACCCAT-3′, Nanog-forward: 5′-GACTTGACCACCGAACCCAT-3′, Nanog-reverse: 5′-CTGGATGTTCTGGGTCTGGT-3′, Oct3/4-forward: 5′-GACAACAATGA GAACCTTCA-3′, Oct3/4-reverse: 5′-GA CAACAATGAGAACCTTCA-3′, LIN28-forward: 5′-AAAGGAGACAGGT GCTAC-3′, LIN28-reverse: 5′-ATATGGCTGATGCTCTGG-3′, GAPDH forward: 5′-AGCCACATCGCTCAGACACC-3′, GAPDH reverse: 5′-GT ACTCAG CGGCCAGCATCG-3′. mRNA extraction, PCR, and electrophoresis were done using a Total RNA Minikit® (ATP Biotech Inc.

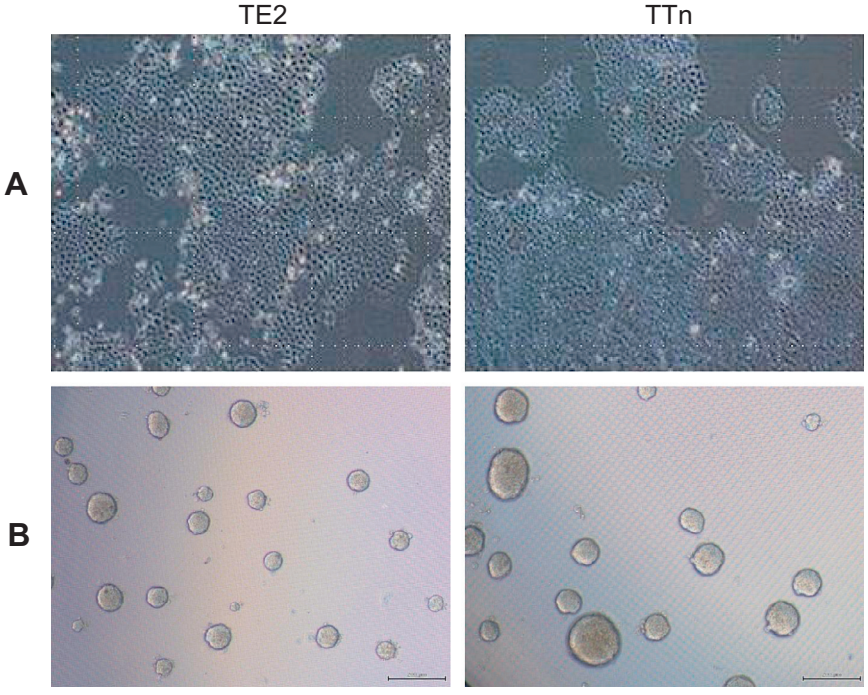

Fig. 1. Morphological changes in 2-D and 3-D cell culture of the esophageal squamous cell carcinoma lines TE2 and TTn. (A) 2-D culture. (B) 3-D culture. Spheroids formed in 3-D culture.