The expression of cancer stem cell markers in human colorectal carcinoma cells in a microenvironment dependent manner

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ABSTRACT

Numerous lines of evidence support the hierarchical model of cancer development and tumor initiation. According to the theory, cancer stem cells play a crucial role in the formation of the tumor and should be targeted for more effective anticancer treatment. However, cancer stem cells quickly loose their characteristics when propagated as 2D cell culture, indicating that the 2D cell culture does not provide the appropriate settings to maintain an in vivo environment. In this study we have investigated the expression of self-renewal, cancer stem cell and epithelial to mesenchymal transition markers after the transfer of human colorectal carcinoma cell DLD1 and HT29 lines from 2D cell cultures to scaffold-attached laminin rich extracellular matrix and scaffold-free multicellular spheroid 3D culture models. Based on the up-regulated expression of multipotency, CSC and EMT markers, our data suggests that human colorectal carcinoma cells grown in 3D exhibit enhanced cancer stem cell characteristics. Therefore, in order to design more efficient targeted therapies, we suggest that 3D cell culture models should be employed in cancer stem cell research.

1. Introduction

According to the hierarchical model of tumor development, tumors are initiated and maintained by a small population of slow cycling cells that possess stem cell-like characteristics. These cells are considered as cancer stem cells (CSCs) and are defined by an undifferentiated cell status, plasticity and self-renewing, like normal somatic stem cells [1,2]. CSCs display a highly heterogeneous population, which sustains dynamic clonal modifications during tumor progression. Thus, CSCs are involved in the resistance to conventional therapeutic agents leading to the repopulation of tumor cells following initial reduction [3]. The resilience of CSCs is typically maintained by an elevated expression of drug efflux proteins or increased DNA repair activity. In addition, the plasticity of CSCs might reduce the therapeutic efficiency as non-stem like tumor cells are capable of a spontaneous conversion to CSC and vice versa [4]. Furthermore, recent studies have linked CSCs with the epithelial-mesenchymal transition (EMT) [5] and tumor metastasis [6] indicating that metastasizing cells could possess or gain CSC features during tumor invasion, eventually leading to the initiation of secondary tumors. However, the CSC-driven molecular mechanisms, which orchestrate tumor progression and resistance to anti-cancer therapies, still remain unclear. Therefore, a better

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understanding of CSC biology could lead to a better optimization of new cancer treatment strategies.

In order to study the biological functions and the response to various types of therapeutic treatment, an appropriate cell model for CSCs is required. In most in vitro studies, two-dimensional (2D) cancer cell culture models are applied. However, such cell culture models poorly represent the complexity of the tumor, as cancer cells grown in 2D predominantly interact with the plastic surface in a growth medium enriched with concentrated nutrients, lacking the appropriate cell-ECM and cell-cell interactions [7]. As tumor initiation is one of the main characteristics defining CSCs, the orthotopic transplantation of tumor cells remains a central model for the CSC research [8]. However, the tumor microenvironment and growth factor background in the xenograft model may not be adequate to the conditions found in patients' tumor. In addition, this technique requires harsh experimental procedures testing for the presence of the most robust growing tumor cells. All these limitations could alter tumor cell growth in the xenograft assay.

By contrast, three-dimensional (3D) cell models provide conditions more akin to those that exist in vivo. These models exhibit regions of hypoxia, zones of quiescent and proliferating cells, chemical gradients and 3D cell-cell and cell-ECM interactions, all of which are maintained in a solid tumor microenvironment [9]. In addition, the response of cancer cells grown under 3D conditions to therapeutic agents is thought to be more representative of actual tumor cells [10]. Furthermore, it was demonstrated that hypoxic niches formed under 3D cell culture conditions could promote CSC conversion or at least self-renewal of already existent CSCs, increase the enrichment of cancer cells positive for CSC markers and enhance cellular tumorigenicity in mice compared to cells grown in a monolayer [11—13]. These observations indicate that increased drug resistance observed in cancer cells grown in a 3D microenvironment could be related to the expansion of CSCs. Therefore, the characterization of cell culture models that better maintain the CSC niche could promote the development of new anti-cancer therapy strategies based on targeted treatment against CSCs.

In this study we examined the changes in gene expression related to the maintenance of CSCs including genes implicated in hypoxia, CSC marker, multipotency and EMT in human colorectal carcinoma (CRC) cell lines DLD1 and HT29 grown in 3D versus 2D cell culture conditions. In order to elucidate the changes in gene expression we employed two 3D cell-culturing methods: 3D laminin enriched extracellular matrix (ECM) and multicellular spheroid (MCS) culture formed by liquid overlay technique. Our present data indicate that CRC cells grown in 3D culture models exhibit enhanced expression of genes regulating CSC properties.

2. Materials and methods

2.1. Cell lines

Human colorectal carcinoma DLD1 and HT29 cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were maintained in an RPMI-1640 (DLD1) or DMEM (HT29) cell culture medium (Gibco, Germany), respectively. Both media were supplemented with 10% fetal bovine serum (Gibco, Germany), 2 mM glutamine (Gibco, Germany), 1 mM sodium pyruvate (Gibco, Germany), 100 UI/mL penicillin (Sigma) and 0.1 mg/mL streptomycin (Sigma). Cell culturing was carried out in 25 cm^2 plastic cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

2.2. 3D cell culture experimental design

For the evaluation of changes in gene expression between 2D and 3D, cells, initially grown as monolayer, were transferred to 3D ECM and MCS cultures. All experiments were performed following 2 days or 6 days of cell growth and repeated at least 3 times. For the 2D cell culture, DLD1 and HT29 cells were plated in 9.5 cm^2 plastic 6 well cell culture plates and cell density was as follows: 2.52×10^5 and 1.26×10^4 cells for 2 and 6 days of cell growth, respectively. For 3D ECM cell culture, 0.5×10^5 of DLD1 and HT29 cells were embedded in 1 mL of 0.5 mg/mL lr-ECM protein mixture Geltrex (ThermoFisher Scientific, USA) in culture medium in 24 well plates as described previously [14]. To avoid cell attachment to the well bottom, each well was pre-coated with 1% agarose in sterile water. Multicellular spheroids (MCS) were formed using liquid-overlay technique as described previously [15]. Briefly, 0.7×10^4 DLD1 cells and 3.5×10^4 HT29 cells were suspended in 200 μL cell culture medium and plated in each well of 96 round-bottom well plates pre-coated with 1% agarose in water gel and centrifuged at $1000 \times g$ for 10 min. Cells were photographed every second day with inverted optical microscope Eclipse TS100 and digital camera DS-Fi2 (Nikon, Japan). MCS size was evaluated using SpheroidSizer 1.0 [16].

2.3. RNA extraction

After 2 days and 6 days of cell growth, cells grown in 2D, lr-ECM 3D and MCS were harvested. Total RNA was isolated from approximately 2×10^6 cells using Quick RNA MiniPrep (Zymo Research, USA) according to manufacturer's instructions. The quantity and quality of RNA were measured with Nanodrop (ThermoFisher Scientific, USA).

2.4. RT-qPCR

cDNA were synthesized using Revert Aid RT Kit (ThermoFisher Scientific, USA) according to manufacturer's instructions. A total of 1 μg RNA was used for cDNA synthesis. Quantitative real-time PCR was performed using Realplex4 Mastercycler thermocycler (Eppendorf, USA) and Kapa SYBR Fast qPCR Master Mix (2×) (Kapa Biosystems, USA) according to manufacturer's instructions. Briefly, for each reaction in 96 well plate 1 μL of 5 times diluted cDNA, 5 μL Kapa SYBR FAST qPCR Master Mix, 3.8 μL nuclease-free water and 2 μM forward and reverse primer was used. PCR cycling conditions included polymerase activation at 95 °C for 3 min, followed by 40 2-step amplification cycles consisting of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. The relative changes in gene expression were calculated by ΔΔCt method using HPRT1 as the housekeeping gene for sample normalization [17]. Primer sequences are shown in supplementary file 1.

2.5. Statistical analysis

Each data point is displayed as the mean ± standard deviation of three independent biological experiments. Student's *t*-test was applied to determine the differences between groups and statistical significance was considered at p < 0.05.

3. Results

3.1. Three-dimensional cell culture growth conditions induce hypoxia related gene expression

In order to compare cells grown under 2D and 3D cell culture conditions, CRC, DLD1 and HT29 cells were grown as a plastic-attached monolayer (2D), scaffold-attached cells in laminin-rich extracellular matrix (3D ECM) and scaffold-free multicellular spheroids (3D MCS).