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Acquisition of epithelial–mesenchymal transition and cancer stem-like phenotypes within chitosan-hyaluronan membrane-derived 3D tumor spheroids

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ABSTRACT

Cancer drug development has to go through rigorous testing and evaluation processes during pre-clinical in vitro studies. However, the conventional two-dimensional (2D) in vitro culture is often discounted by the insufficiency to present a more typical tumor microenvironment. The multicellular tumor spheroids have been a valuable model to provide more comprehensive assessment of tumor in response to therapeutic strategies. Here, we applied chitosan-hyaluronan (HA) membranes as a platform to promote three-dimensional (3D) tumor spheroid formation. The biological features of tumor spheroids of human non-small cell lung cancer (NSCLC) cells on chitosan-HA membranes were compared to those of 2D cultured cells in vitro. The cells in tumor spheroids cultured on chitosan-HA membranes showed higher levels of stem-like properties and epithelial–mesenchymal transition (EMT) markers, such as NANOG, SOX2, CD44, CD133, N-cadherin, and vimentin, than 2D cultured cells. Moreover, they exhibited enhanced invasive activities and multidrug resistance by the upregulation of MMP2, MMP9, BCRC5, BCL2, MDR1, and ABCG2 as compared with 2D cultured cells. The grafting densities of HA affected the tumor sphere size and mRNA levels of genes on the substrates. These evidences suggest that chitosan-HA membranes may offer a simple and valuable biomaterial platform for rapid generation of tumor spheroids in vitro as well as for further applications in cancer stem cell research and cancer drug screening.

1. Introduction

Lung cancer is the most aggressive malignant tumor by high incidence and mortality worldwide. There are two major types of lung cancer according to histological features, i.e. small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC; also known as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma) [1]. NSCLC accounts for 85% of lung cancer cases and of which about 40% cannot be resected [2]. Therefore, development of therapeutic agents is important for treating NSCNC.

Development of new drug has to go through rigorous testing and evaluation during pre-clinical in vitro studies. However, the traditional two-dimensional (2D) in vitro culture lacks the proper cell–cell and cell–extracellular matrix (ECM) interactions as seen in the microenvironment in vivo, which have significant effects on tumor progression and treatment efficacy [3,4]. Consequently, the efficiency of drug screening and testing by 2D in vitro culture is often discounted by the insufficiency to present a more typical tumor microenvironment. The multicellular tumor spheroids have been a valuable model to provide more comprehensive assessment of tumor in response to therapeutic strategies. It mimics the tumor in vivo in many aspects, such as the protein expression, pH and oxygen gradients, the permeation rate of growth factors within the spheroids, and interactions with ECM [5,6]. In addition, the accessibility of therapeutic agents into tumor spheroids is also limited by hypoxia and poor vascularization, as occurs in solid tumors in vivo [7,8]. These characteristics demonstrate that tumor spheroids are more suitable models for drug penetration studies in tumors in comparison to 2D in vitro culture. However, the applications of tumor spheroids are limited due to long incubation time, inconvenient culturing technique, unequal-size spheroids, and failure to

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form rigid aggregates [9]. These factors greatly affect the precise evaluation of biological or biochemical endpoints in drug screening [10]. Hence, a simple and well-characterized platform for rapid generation of tumor spheroids is necessary to improve the limitations of tumor spheroid culture system in drug screening processes.

Several recent studies have shown that cancer cells cultured in three-dimensional (3D) lyophilized scaffolds exhibit higher malignancy and drug resistance than the same cells cultured in 2D due to the increased cell communication and ECM signaling [11,12]. Although 3D scaffolds can to some extent mimic the ECM, the fabrication process is more complicated. Hyaluronan (HA) is a natural anionic polymer and widely utilized in biomedical applications because of its biocompatibility and water adsorption [13,14]. HA is the major ligand of CD44 and an abundant ECM component [15]. The high HA level in the tumor cells and stroma of NSCLC patients is associated with poor tumor differentiation and a high recurrence rate [16]. HA has been shown to facilitate tumor progression, epithelial to mesenchymal-like phenotype transition (EMT), and drug resistance as it interacts with CD44, and to activate a wide range of intracellular signaling pathways including cell migration and invasion [15,17]. Chitosan is a widely used natural cationic polymer derived from crustacean shells and has broad applications in tissue engineering because of its biocompatibility, biodegradability, and hydrophilicity. Chitosan is also inexpensive and readily available [18]. Moreover, the amine group of chitosan allows it to graft with the carboxyl group of HA to form a stable covalent bond. A previous study has revealed the ability of chitosan substrates grafted with HA to promote the formation of 3D multicellular spheroids from 2D mesenchymal stem cells [19]. The 3D spheroids have greater expression of stemness genes as well as higher differentiation capacities [19]. Therefore, we considered that chitosan substrates grafted with HA may have further applications in cancer research and the substrates may present different effects on different types of NSCLC cells.

In this study, the HA grafted chitosan (CH) membranes were employed to mimic the microenvironment of human NSCLC tumor. We expected that human NSCLC cells cultured on CH membranes would show enhanced malignancy and invasiveness as compared to the traditional 2D culture and would simulate the clinical and histologic features of human NSCLC in vivo. The 3D model may be a platform for providing more accurate prediction in drug screening and treatment efficiency.

2. Materials and methods

2.1. Preparation of chitosan-hyaluronan (CH) membranes

Chitosan powder from crab shells was purchased from Sigma–Aldrich (USA). The molecular weight of the chitosan was 510 kDa, and the degree of deacetylation was 77%. The molecular weight of hyaluronan (sodium salt) was about 2500 kDa, and was acquired from SciVision Biotech Inc. (Kaohsiung, Taiwan). 1% acetic acid was used to dissolve chitosan for acquiring a 1 wt% chitosan solution. The solution (1.5 ml) was coated on each well of 6-well plate (Greiner Bio-One). The formation of chitosan membranes was by evaporating the solvent in a laminar flow cabinet for 24 h. Chitosan membranes were immersed in 0.5 N sodium hydroxide for 30 min, and then washed three times with phosphate buffered saline (PBS). To prepare CH membranes, 1.5 ml of HA solution containing different amounts of HA was added on each chitosan-coated well so the amount of HA was 0.1, 0.5 or 2.5 mg per cm^2 of chitosan membranes. The HA coated chitosan membranes were then cross-linked by 2 ml of ethyl (dimethylaminopropyl) carbodiimide/Nhydroxysuccinimide (EDC/NHS) solution with a weight ratio of HA/EDC/NHS adjusted to 1:1.84:0.23 at pH 5.5 which was added and shaken for 48 h at 4 °C. After that, unbound HA was removed by washing three time with PBS, and the membranes were later lyophilized. The CH membranes grafted with three different doses of HA were abbreviated as CH–L, CH–M, CH–H respectively [19].

2.2. Cell culture and tumor spheroid formation

Human non-small cell lung carcinoma cell lines A549 and H1299 were grown in continuous culture in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 10 mg/l penicillin-streptomycin. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2, and subcultured twice per week. Cells ($1.25 \cdot 10^5$) were seeded on each CH membrane in 6-well culture plates for three days or in the blank culture well (tissue culture polystyrene, TCPS) as the control. The morphology was observed by an inverted microscope (Leica, DMIRB). The size of tumor spheroids was computed based on image analysis (by the software ImageJ).

2.3. Cell proliferation assay

Cell proliferation was measured by the water soluble tetrazolium salt WST-8 assay (Dojindo Labs, Tokyo, Japan). A549 and H1299 cells were seeded at 5000 cells/well in 96-well plates in the complete medium at 37 °C on day 0. After adding 10 μl of the WST-8 solution to triplicate wells every 24 h for 3 days, the absorbance was measured at 450 nm using a microplate reader (SpectraMax® M5, USA).

2.4. Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

The mRNA expression for markers associated with stemness, EMT, invasion, drug resistance, and anti-apoptosis was analyzed by qRT-PCR. Trizol® reagent (Invitrogen) was used to extract total RNA from cells according to manufacturer's instruction. Total RNA (1 μg) was reverse-transcribed into cDNA by the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). QRT-PCR was conducted in a Chromo 4 PTC200 Thermal Cycler (MJ Research, USA) using the DyNAmo Flash SYBR Green qPCR Kit (Finnzymes Oy, Espoo, Finland). QRT-PCRs were performed using primers for POU domain class 5 transcription factor 1 (POU5F1), SRY (sex determining region Y)-box 2 (SOX2), NANOG, CD44, CD133, aldehyde dehydrogenase 1 family, member A3 (ALDH1A3), matrix metallopeptidase 2 (MMP2), matrix metallopeptidase 9 (MMP9), vimentin, fibronectin, ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2), B-cell CLL/lymphoma 2 (BCL2), baculoviral inhibitor of apoptosis repeat-containing 5 (BCRC5), multidrug resistance protein 1 (MDR1), N-cadherin, twist family bHLH transcription factor 1 (TWIST1), snail family zinc finger 1 (SNAIL1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The expression levels were normalized to those of GAPDH. The normalized value was then expressed as the relative ratio to that in the TCPS group [19]. The primer sequences are listed in Table 1.

Table 1
The primer sequences used for qRT-PCR analyzes.

Gene	Primer sequences	Primer annealing temperature (°C)
POU5F1	F: ACATCAAAGCTCTGCAGAAA R: CTGAATACCTTCCCAAATAGAAC	62
SOX2	F: TGCGAGCGCTGCACAT R: GCAGCGTGTACTTATCCTTCTTCA	62
NANOG	F: AATACCTCAGCCTCCAGCAGAT R: TGCGTCACACCATTGCTATTCTT	62
CD44	F: CCTGTTCTCTCCTGTGAAAG R: TTTGCCAATCTCTTTCATTT	55
CD133	F: CACCATTGACTTCTTGGTGCTG R: TGCATGCCATTTCCAAGTGG	62
ALDH1A3	F: ACAGACAACATGCGGATTGC R: TTTTGTGAACACGGCTGCTG	62
MMP2	F: TGATGGTGTCTGCTGGAAAG R: AGCAAACCTCGAACAGATGC	62
MMP9	F: TCTTCCCCTTCACTTTCCTG R: TGTCGCTGTCAAAGTTCGAG	62
Vimentin	F: GAAATTGCAGGAGGAGATGC R: CGCATTGTCAACATCCTGTC	62
Fibronectin	F: ATGAGCTGCACATGTCTTGG R: TGGCACCGAGA ATTCCTTC	62
ABCG2	F: GCTGCAAGGAAAGATCCAAG R: GTGCCCATCACAACATCATC	62
BCL2	F: TCTGCGAAGAACCTTGTGTG R: CACTTTGAG CCATGCTGATG	62
BCRC5	F: TTTCTGCCACATCTGAGTCG R: AGGCTGGAGTGCATTTTCTG	62
MDR1	F: CCGCTGTTCGTTTCCTTTAG R: TCAAGATCCATTCCGACCTC	62
N-cadherin	F: TTTGAGGGCACATGCAGTAG R: ACTGTCCCATTCCAAACCTG	62
TWIST1	F: ACTGGCCTGCAAAACCATAG R: GCATTTTACCATGGGTCCTC	62
SNAIL1	F: CTGCGTGGGTTTTTGTATCC R: TCTGTCAGCCTTTGTCCTGTAG	62
GADPH	F: AACCTGCCAAATATGATGAC R: ATACCAGGAAATGAGCTTGA	62