Acta Biomaterialia 42 (2016) 157–167

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Chitosan-hyaluronan based 3D co-culture platform for studying the crosstalk of lung cancer cells and mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form 2 June 2016
Accepted 9 June 2016
Available online 11 June 2016

Keywords:
Lung cancer
Mesenchymal stem cells
Chitosan-hyaluronan
3D culture
Zebrafish transplantation

ABSTRACT

The controversial roles of mesenchymal stem cells (MSCs) in lung cancer development are not yet resolved because of the lack of an extracellular environment that mimics the tumor microenvironment. Three-dimensional (3D) culture system is an emerging research tool for biomedical applications such as drug screening. In this study, MSCs and human non-small cell lung carcinoma cells (A549) were co-cultured on a thin biomaterial-based substratum (hyaluronan-grafted chitosan, CS-HA; ~2 μm), and they were self-organized into the 3D tumor co-spheroids with core-shell structure. The gene expression levels of tumorigenicity markers in cancer cells associated with cancer stemness, epithelial-mesenchymal transition (EMT) property, and cell mobility were up-regulated for more than twofold in the MSC-tumor co-spheroids, through the promoted expression of certain tumor enhancers and the direct cell-cell interaction. To verify the different extents of tumorigenicity, A549 cells or those co-cultured with MSCs were transplanted into zebrafish embryos for evaluation *in vivo*. The tumorigenicity obtained from the zebrafish xenotransplantation model was consistent with that observed *in vitro*. These evidences suggest that the CS-HA substrate-based 3D co-culture platform for cancer cells and MSCs may be a convenient tool for studying the cell-cell interaction in a tumor-like microenvironment and potentially for cancer drug testing.

Statement of Significance

Mesenchymal stem cells (MSCs) have been found in several types of tumor tissues. However, the controversial roles of MSCs in cancer development are still unsolved. Chitosan and hyaluronan are commonly used materials in the biomedical field. In the current study, we co-cultured lung cancer cells and MSCs on the planar hyaluronan-grafted chitosan (CS-HA) hybrid substrates, and discovered that lung cancer cells and MSCs were rapidly self-assembled into 3D tumor spheroids with core-shell structure on the substrates after only two days in culture. Therefore, CS-HA based 3D co-culture platform can be applied to exploration of the relationship between cancer cells and MSCs and other cancer-related medical applications such as drug screening.

1. Introduction

Lung carcinoma is a major cause of cancer-related death in the world though some target-drugs have been developed. According to the different pathological features, lung cancer is commonly classified into two types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC accounts for approximate 80% of all lung cancer cases, and the patients have only 15% five-year survival and 35–50% recurrence rates [1,2]. Extensive research has been focused on the mechanism and therapeutic treatment for NSCLC.

Recent findings have demonstrated that solid tumor development is not only regulated by the behavior of tumor cells, but also determined by the microenvironment that tumor cells reside in. The microenvironment includes inflammatory cytokines and growth factors [3–6]. In addition, cancer stem cells (CSCs) and mesenchymal stem cells (MSCs) also play crucial roles during cancer development. CSCs are a small subpupolation of cells that can self-renew and present aggressive tumorigenicity within solid tumor [7]. The existence of CSCs may cause the local recurrence

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http://dx.doi.org/10.1016/j.actbio.2016.06.014
1742-7061/

and distal metastasis of solid tumors [8]. CSCs have been recently discovered in lung and other types of cancer, and the possible mechanism and signaling pathway that CSC involved for cancer progression were also elucidated in many reports [9–12]. Unlike CSCs, the roles of MSCs are still disputed in the regulation of cancer development. It has been reported that the population of CD133-positive cancer stem cells increased when prostate cancer cells were co-cultured with MSCs *in vitro* [13]. For the pancreatic cancer cells, MSCs promote the expression of certain epithelial-mesenchymal transition (EMT) and mobility markers, such as Snail1 and MMP9, resulting in the enhanced sphere-forming ability of these cancer cells [14]. Furthermore, MSCs may be recruited and transformed to promote cancer progression [15,16] or they may inhibit certain cancers by decreasing proliferation of cancer cells or releasing antiangiogenic factors [17,18]. In NSCLC, MSCs displayed the alterations of cell properties and gene expression patterns to promote cancer development [19]. Meanwhile, treatment with the conditioned medium from MSCs reduced the stemness of lung cancer cells [20]. Therefore, it is still difficult to unravel the complicated relations between cancer cells and MSCs.

Three-dimensional (3D) cell culture system draws significant attention in the field of cell and tumor biology. The development of organ or solid tumor *in vivo* is proceeded in a 3D environment. A 3D culture system is thus a powerful tool for studying tumorigenesis. Several strategies to create a 3D culture environment for cancer-related studies have been reported [21], such as self-assembled tumor spheroids from cells seeded on the hyaluronan-grafted chitosan (CS-HA) substrates [22] or embedded within gelatin methacrylamide (GelMA)-based hydrogels [23].

Chitosan and hyaluronan (HA) are natural products with high biocompatibility [24,25]. HA is a main component of extracellular matrix (ECM), and the cell receptor for HA is CD44 [26]. The HA-CD44 interaction has been proposed to promote the tumor progression through altering the physiological properties of cancer cells such as EMT, cell migration, and invasion [27,28]. In the previous study, we have demonstrated that CS-HA substrates can be easily fabricated, and the tumorigenicity of lung cancer cells was elevated when they formed cell spheroids on the CS-HA substrates. In addition, the formation of tumor spheroids on CS-HA substrates was quite rapid, indicating the CS-HA substrates may be an efficient 3D culture platform [22]. Furthermore, different types of cells can be assembled into cell spheroids on CS-HA substrates to elucidate the cell-cell interaction in a 3D space [29]. So far 3D co-culture systems used for evaluating the effects of non-tumor cells on cancer development are still rare. In this work, we intended to investigate whether a tumor spheroid comprising not only cancer cells but also MSCs could be generated to mimic the properties of solid tumor *in vivo* using the CS-HA substrates.

In this study, we intended to establish a 3D co-culture system of lung cancer cells and MSCs on CS-HA substrates. We co-cultured MSCs and A549 lung cancer cells on the CS-HA substrates, and examined if cell spheroids formed. To evaluate the tumorigenicity of A549 cells co-cultured with MSCs on CS-HA substrates, we analyzed the expression levels of several representative markers for stemness, EMT, and mobility in A549 cells *in vitro* as well as verified the cell mobility of A549 cells in the zebrafish xenotransplantation model. The crosstalk of lung cancer cells and MSCs could be further elucidated using the CS-HA co-culture platform.

2. Materials and methods

2.1. Preparation of CS-HA substrates on culture plates

Chitosan powder (molecular weight of 510 kDa) originated from crab shell was purchased from Sigma-Aldrich (USA), 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). The preparation procedure for CS-HA substrates was performed as previously described [22]. Briefly, 1 wt% chitosan was prepared in 1% acetic acid solution, and then chitosan solution (1.5 ml) was coated onto each well of the 6-well tissue culture polystyrene (TCPS) plates. To form chitosan membrane, the solvent was evaporated in a laminar flow cabinet for 24 h. To prepare CS-HA membranes, the HA solution (1.5 ml, 0.65 mg/ml) was then added onto chitosan-coated wells and air-dried in a laminar flow cabinet for 24 h. The HA-coated chitosan membranes were crosslinked by 4.5 ml of ethyl (dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS, weight ratio 1.84:0.23) solution with the concentration of 6 mg/ml for 48 h. After crosslinking, the final membranes were washed with phosphate buffered saline (PBS) and stored at 4 °C before use. The grafting density of HA on the membranes was 0.1 mg/cm^2 determined by the glucuronic acid assay [30]. The thickness of CS-HA membranes was ~2 μm determined by the Microfigure Measuring Instrument (Surfcorder ET3000, Kosaka Laboratory, Kosaka, Japan).

2.2. Cell culture

The non-small cell lung carcinoma cell line A549 was cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Caisson Labs, USA). Mesenchymal stem cells (MSCs) were isolated from the adipose tissue of three 12-week-old male Sprague-Dawley rats (body weight from 350 g to 500 g). The protocols used for MSCs isolation and identification were performed as previously described [30]. In this study, MSCs of the third to sixth passages were used. The MSCs were cultured in the mixture of low-glucose Dulbecco's modified Eagle medium (DMEM) and F12 medium (Gibco, USA) in 1:1 vol ratio, and supplemented with 10% FBS (Gibco, USA), 20 mM HEPES (Gibco, USA), 50 mg/ml bovine serum albumin fraction V (Gibco, USA), 10 mg/l L-glutamine (Gibco, USA), and 1% penicillin-streptomycin (Caisson Labs, USA). The medium used for co-culture of A549 cells and MSCs was composed of respective medium combined with each other in 1:1 vol ratio. Both cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

2.3. Cell seeding and tumor spheroids observation

To observe the formation of tumor spheroids, cells were seeded with the density of 3×10^5 cells/well in the 6-well TCPS plates that were coated with or without CS-HA on the well surfaces. The morphologies of cells cultured on each substrate were observed by an inverted microscope (Leica, DMIRB). In the co-culture groups, A549 cells and MSCs were seeded with different ratios as indicated. To characterize the tumor spheroids formed by A549 cells and MSCs, A549 cells and MSCs were respectively labeled by PKH26 (red color) and PKH67 (green color) Fluorescence Cell Linker Kits (Sigma-Aldrich, USA). In these kits, the fluorescent dyes are synthesized with long aliphatic tails for incorporating into the cell membrane, and the fluorescence is stable and not affected by pH within physiological ranges. It has been reported that the half-life of PKH was over one month *in vitro* [31], and the dye has been used for long-term cell tracking in our previous study [32]. The labeling procedure was performed according to manufacturer's instruction. Briefly, cells were suspended in the Diluent C iso-osmotic solution (provided in the kit), and then stained with PKH fluorescent dye with the final concentration of 2 μM for 3 min. After staining, the cells were washed with 10 ml complete culture medium twice before seeding. The organization of tumor spheroids was directly