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Substrate-dependent modulation of 3D spheroid morphology self-assembled in mesenchymal stem cell-endothelial progenitor cell coculture

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The structural evolution of three-dimensional spheroids self-assembled from two different types of cells on selective biomaterials is demonstrated in this study. The two types of cells involved in the self-assembly are human mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). When seeded in different population ratios, they can create a variety of cellular patterns on different biomaterial substrates. When the two populations are matched in initial numbers, they are self-assembled in co-spheroids with different morphologies (i.e. randomly mixed, bumped, or concentric spheroids). The morphologies are influenced by the specific cell-substrate interaction possibly through integrin signaling, as well as a substrate-dependent regulation of heterophilic cell–cell interaction possibly through Notch signaling. In particular, the self-assembled core–shell concentric spheroids from adipose-derived MSCs and EPCs show a greater angiogenic effect in vitro. This study reveals the possibility to modulate the self-assembled morphology as well as the effect of cocultured cells by changing the cell culture substratum.

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1. Introduction

Cell sorting and organization is a complicated process. Cell patterns may be generated by gene control, cellular modulation, or induced by a specific niche. Among all pattern forming processes in nature, blood vessel formation (angiogenesis) is important because it enables nutrient supplies to the site of ischemia, as well as maintains the vitality of the implanted tissue [1]. The process requires the formation of an integrated endothelial cell (EC) layer supported by the other types of cells. Endothelial progenitor cells (EPCs) participate in vascular remodeling and angiogenesis by migration to the site in need of blood vessels [2]. Mesenchymal stem cells (MSCs) contribute to EC proliferation, survival, mobility, and help recruit EPCs for angiogenesis [3–5]. Besides, MSCs have the potential to differentiate into a variety of cell types for blood vessels including smooth muscle cells [6,7].

Coculture of different types of cells can produce beneficial effects under many circumstances because of the synergistic effect [8–10]. Most works regarding cell culture or coculture involve two-dimensional (2D) monolayer culture, while three-dimensional (3D) culture better simulates the natural environment in living organisms [11]. 3D multicellular spheroids of human ECs generated by hanging drop may increase the cell survival, release paracrine factors, and enhance the angiogenesis of ischemic animals [12,13]. Previously, we have observed that MSCs grown on certain materials including chitosan (CS) and hyaluronan-modified chitosan (CS-HA) were self-assembled into 3D spheroids [14]. The process of spheroid formation was associated with increased cell–cell interaction on these materials [14,15]. We therefore hypothesize that cell–cell interaction between two different types of cells may also be modulated or facilitated when they are cocultured on proper biomaterial substrates. The different heterophilic cell–cell interaction and cell-material interaction may lead to formation of different cellular patterns. Moreover, a favorable pattern may promote the synergy of cocultured cells and offer beneficial effects. In

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the case involving EPC coculture, these beneficial effects may be on the angiogenic properties.

In this study, human MSCs and EPCs were cocultured in different ratios on substrates made of selective polymeric biomaterials. We first demonstrated that a variety of patterns may be generated from the two types of cells grown on the substrates. When the numbers of the two cell populations were closely matched, the self-assembled morphologies were in the form of various co-spheroids (mixed, bumped, or concentric), depending on the substrate employed. The cell-material interaction, heterophilic cell–cell interaction, and the coculture effect were then investigated in terms of integrin activation and Notch signals. Finally, we explored if the substrate-induced self-assembled MSC-EPC co-spheroids had different in vitro properties, e.g. angiogenesis capacities.

2. Materials and methods

2.1. Preparation of biomaterial substrates

Polyvinyl alcohol (PVA, Sigma) with a molecular weight 70–100 kDa was dissolved in 90 °C double-distilled water at 1%. A volume of 500 μL was added to each well of the 24-well tissue plate and air-dried for cell studies. The chitosan-based substrates were prepared as described previously [14,15]. Chitosan powder (Fluka) with a molecular weight ~510 kDa and degree of deacetylation (defined by NMR) 77.7% was dissolved in 1% acetic acid to obtain 1% chitosan solution. Chitosan membranes (CS) were prepared by coating 300 μL solution on 15 mm-diameter coverslip glass placed in petri dish. Samples were evaporated in a laminar cabinet for 24 h, immersed in sodium hydroxide (0.5 N) for 15 min, washed extensively with double-distilled water, and air-dried. Hyaluronan powder (HA sodium salt, SciVision Biotech, Taiwan) with a molecular weight ~2500 kDa was dissolved in double-distilled water. To prepare chitosan-hyaluronan membranes (CS-HA), CS substrates were added with 300 μL of HA solution and further crosslinked with 150 μL of ethyl(dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) solution with a weight ratio of HA/EDC/NHS adjusted to 1:1.84:0.23 at pH 5.5 for 48 h at 4 °C. They were washed extensively and air-dried. The actual density of HA on CS-HA substrates was 0.5 mg/cm^2. CS and CS-HA substrates were placed in 24-well tissue plates for cell studies.

The substrates were characterized for physico-chemical properties. The elastic modulus was measured by a dynamic mechanical analyzer (DMA; Q800, TA Instruments) at 1 Hz and 0.5% strain in wet state. The surface hydrophilicity was evaluated by a contact angle meter (FTA-1000B, First Ten Angstrom, USA). The surface zeta potential was determined by electrophoretic light scattering (Delsa™ Nano C, Beckman Coulter). The surface phase and topography diagrams were obtained using atomic force microscopy (AFM; CP-II, Bruker). The surface functional group was analyzed by attenuated total reflectance-infrared spectroscopy (ATR-IR; Spectrum 100, Perkin Elmer).

2.2. Cell culture

All human tissues were obtained with approval from the Institutional Review Board. Two types of human MSCs were used in this research. The umbilical cord derived MSCs (UCMSCs) were supplied by BIONET Corp. (Taiwan). Cells were cultured in α-minimum essential medium (α-MEM, Invitrogen) with 10% fetal bovine serum (Gibco). The adipose-derived adult stem cells (ADSCs) were isolated from the subcutaneous adipose tissue discarded during surgery. All procedures were approved by the institutional review board. The adipose tissue was minced into several pieces and treated with 2000 U/mL type I collagenase (Sigma) in phosphate buffered saline (PBS) at 37 °C for 60 min with gentle agitation [14]. Cells were cultured in Dulbecco's modified Eagle medium-low glucose (DMEM-LG, Gibco)/F12 (Gibco)/MCDB-201 (Sigma) (1:1:2) with 10% FBS. Cells were labeled with green fluorescence by PKH67 (green fluorescent cell linker kit, Sigma) following the manufacturer's instruction prior to coculture with EPCs. Human MSCs (UCMSCs as well as ADSCs) of passages 2–6 were used.

Human EPCs was isolated following the method described [16]. Progenitor cells were isolated from human umbilical cord blood by magnetic microbead selection using direct CD34 progenitor isolation beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and MACS LS-columns (Miltenyi Biotech), and were subsequently cultured. Cells were cultured in the endothelial cell growth medium-2 (EGM-2, Lonza, Switzerland) with 20% fetal bovine serum (FBS, Caisson) in a T-flask coated with 2 μg/cm^2 fibronectin (BD, USA). Cells were labeled with red fluorescence by PKH26 (red fluorescent cell linker kit, Sigma) before coculture with MSCs. Human EPCs of passages 10–15 were used.

Surface markers for each type of cells were quantified by flow cytometry (FACS Caliber, BD Biosciences), using CD31, CD34, CD45, CD73, CD90, and CD105 antibodies. Proliferation of each type of cells (in monoculture) on different substrates for 48 h was analyzed by DNA assay with the initial seeding density of 5×10^6 cells/well in 24-well tissue culture plates.

2.3. Cell seeding and pattern observations

MSCs or/and EPCs were seeded (5×10^4 cells per well) in 24-well tissue culture polystyrene (TCPS) plates, or those containing PVA, CS, and CS-HA substrates. TCPS served as the control material. In the coculture group, MSCs and EPCs were seeded with a total of 5×10^4 cells in various ratios (0:4, 1:3, 2:2, 3:1, 4:0, i.e. 2:2 means 2.5×10^4 cells each per well). The culture medium for coculture was a mixture of the respective culture media based on the same ratio [17]. The culture time was 48 h. The dynamics of spheroid formation was recorded by the real-time Cultured Cell Monitoring System (CCM-Multi, Astec, Japan).

The morphologies at 48 h for MSC-EPC coculture in various ratios and on different substrates were observed by an inverted fluorescence microscope (DMIRB, Leica). The change of relative cell population ratios after 48 h of incubation was measured by flow cytometry. The co-spheroids formed at 1:1 ratio were further examined in detail by a confocal microscope (TCS SP2, Leica).

The number of cells in co-spheroid groups after coculture was determined by reseeding the spheroids onto TCPS and counted by an automated cell counter (Invitrogen) after trypsinization. Based on the above data and the population ratios obtained above, the individual population at 48 h could be estimated. The spreading of co-spheroids after reseeding was recorded at 24 h. Among all groups, the cell migration rates on CS-HA substrates were computed based on real-time images.

The gene expression levels of cadherins, stromal cell-derived factor 1 (SDF1)/C-X-C chemokine receptor type 4 (CXCR4), vascular endothelial growth factor (VEGF), and Notch ligands/receptors were analyzed at 48 h and those of integrins were analyzed at 12 h by the real-time RT-PCR with details described in Supplementary Table S1.

2.4. In vitro Matrigel assay for cocultured cells

The formation of vascular networks was evaluated in vitro using the Matrigel assay. Spheroids generated from MSCs and EPCs (1:1, seeded at 5×10^4 cells in total) cocultured on substrates for 48 h were retrieved and tested by the assay. The spheroids suspended in 100 μL were laid on the gelled 1:1 medium-premixed GFR Matrigel (100 μL, #354263 BD Biosciences) in each well of a 96-well tissue plate. After 8 days, the formation of vascular networks was examined by the inverted fluorescence microscope. The number of branches and the spreading diameter were computed based on image analysis.

2.5. Statistical analysis

Data from multiple samples were collected in each experiment. Numerical values were expressed as the mean ± standard deviation. Three experiments were repeated independently for each type of experiment. Reproducibility was confirmed for cells form at least three different donors. Statistical differences among the experimental groups were evaluated by analysis of variance followed by one-way analysis of variance (ANOVA). p-values <0.05 were considered statistically significant.

3. Results

3.1. Characterization of the substrates

The properties of the three biomaterial substrates prepared in this study (PVA, CS, and CS-HA) are shown in Table 1 and Fig. 1. The modulus of the substrates is listed in Table 1. The bulk modulus of CS-HA was the highest (probably due to crosslinking), while the surface hardness (HA) estimated by the phase shift of the AFM phase diagrams (Fig. 1a) showed that CS-HA surface was the least rigid. The surface of PVA and CS-HA was similarly hydrophilic, while that of CS was more hydrophobic with a larger contact angle. The surface zeta potential of PVA and CS was almost neutral while that of CS-HA was negative. The surface topography obtained by AFM is shown in Fig. 1a. The average roughness value calculated from the topography images was close (1–2 nm), indicating that

Table 1
The surface properties of substrates.

	Contact angle (°)	Surface zeta potential (mV)	Average roughness (nm)	Elastic modulus (MPa)
PVA	27.9 ± 4.9	−1.3 ± 1.4	2.1 ± 1.4	109.4 ± 4.3
CS	54.3 ± 5.0	−2.5 ± 2.4	2.1 ± 1.3	94.0 ± 5.4
CS-HA	29.5 ± 5.8	−47.9 ± 1.9	1.4 ± 1.0	231.3 ± 6.3

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