Acta Biomaterialia 31 (2016) 231–240

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Generation of a patterned co-culture system composed of adherent cells and immobilized nonadherent cells

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ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form 25 November 2015
Accepted 10 December 2015
Available online 11 December 2015

Keywords:
Patterned co-culture
Serum albumin
Cancer microenvironment
Cell membrane anchoring reagent
Cell immobilization
Protein adsorption

ABSTRACT

Patterned co-culture is a promising technique used for fundamental investigation of cell–cell communication and tissue engineering approaches. However, conventional methods are inapplicable to nonadherent cells. In this study, we aimed to establish a patterned co-culture system composed of adherent and nonadherent cells. Nonadherent cells were immobilized on a substrate using a cell membrane anchoring reagent conjugated to a protein, in order to incorporate them into the co-culture system. Cross-linked albumin film, which has unique surface properties capable of regulating protein adsorption, was used to control their spatial localization. The utility of our approach was demonstrated through the fabrication of a patterned co-culture consisting of micropatterned neuroblastoma cells surrounded by immobilized myeloid cells. Furthermore, we also created a co-culture system composed of cancer cells and immobilized monocytes. We observed that monocytes enhanced the drug sensitivity of cancer cells and its influence was limited to cancer cells located near the monocytes. Therefore, the incorporation of nonadherent cells into a patterned co-culture system is useful for creating culture systems containing immune cells, as well as investigating the influence of these immune cells on cancer drug sensitivity.

Statement of significance

Various methods have been proposed for creating patterned co-culture systems, in which multiple cell types are attached to a substrate with a desired pattern. However, conventional methods, including our previous report published in Acta Biomaterialia (2010, 6, 526–533), are unsuitable for nonadherent cells. Here, we developed a novel method that incorporates nonadherent cells into the co-culture system, which allows us to precisely manipulate and study microenvironments containing nonadherent and adherent cells. Using this technique, we demonstrated that monocytes (nonadherent cells) could enhance the drug sensitivity of cancer cells and that their influence had a limited effective range. Thus, our technique is useful for recreating complex tissues in order to investigate cellular interactions involving nonadherent cells.

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1. Introduction

Tissues and organs consist of different types of cells acting in concert to perform specialized functions. Cellular functions and fates in such intricate organization are highly affected by surrounding cells through soluble bioactive molecules and direct cell-to-cell contact. The arrangement of multiple cell types according to a desired pattern on a substrate, called patterned co-culture, is a promising technique used for understanding cell–cell communication. It is also used in tissue engineering applications for the creation of organized structures. A large number of studies have demonstrated the utility of patterned co-culture techniques [1,2]. One study using patterned co-culture composed of micropatterned hepatocytes surrounded by fibroblasts showed that the liver-specific functions of hepatocytes were maintained by the presence of fibroblasts. Interestingly, that study also showed that the level of increased liver-specific function was several-folds higher in patterned co-culture than the randomly distributed control co-culture sample [3]. Moreover, unlike randomly distributed co-culture, patterned co-culture enables us to regulate the degree of interaction between hepatocytes and fibroblasts by changing

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http://dx.doi.org/10.1016/j.actbio.2015.12.016
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the pattern configuration [4]. Patterned co-culture composed of various different types of cells, such as neurons and astrocytes or cancer cells and endothelial cells has also been generated to mimic multi-cellular organisms [1,5,6]. Diverse methods of generating patterned co-culture systems have been demonstrated. These include utilizing photolithography and soft lithography techniques, or unique materials that have switchable surface properties for cell-adhesion in response to the deposition of polyelectrolytes in a layer-by-layer manner or external stimuli such as light, voltage, heat, and microelectrodes [1,2]. So far, adherent cells, such as hepatocytes, fibroblasts, neurons, astrocytes, and endothelial cells, have been used as components of co-culture systems; therefore, these conventional methods are inapplicable to nonadherent cell types, such as blood cells. However, interactions between adherent and nonadherent cells are important in some physiological events. For example, cancer cells release chemotactic factors and actively recruit immune cells, such as monocytes and lymphocytes, to their surrounding environments. Mekata et al. demonstrated that blood mononuclear cells influence the sensitivity of cancer cells to anticancer drugs [7]. Thus, incorporation of nonadherent cells into co-culture systems might be useful for recreating the cancer microenvironment *in vitro* and investigating the influence of immune cells in the tumor stroma on cancer drug sensitivity.

In this study, we describe an adaptable method for creating a patterned co-culture system composed of adherent and nonadherent cells. In the most common methods for co-culturing adherent cells with nonadherent cells, the nonadherent cells are placed on top of the adherent cells either directly or on a porous filter insert [8,9]. However, it is difficult to control the position of nonadherent cells precisely using such approaches. Our strategy for controlling the position (i.e., preparation of the pattern) of nonadherent cells is to immobilize nonadherent cells on a substrate and control its spatial localization. Recently, novel cell immobilization techniques have been developed, enabling nonadherent cells to be immobilized on the substrate just like adherent cells attach to the substrate [10,11]. In the method developed by Nagamune et al., the cell membrane anchoring reagent (CMAR) was bonded to the substrate surface, followed by the immobilization of nonadherent cells on the substrate via the interaction between hydrophobic units in the CMAR and the cell membrane. Here, we used a CMAR and protein conjugate for immobilizing the nonadherent cells on the substrate. To control deposition of CMAR–protein conjugates and subsequent immobilization of nonadherent cells in spatially defined patterns, we employed cross-linked albumin (cl-albumin) film, which has unique surface properties for protein adsorption behavior. A water-insoluble, cl-albumin film maintains the properties of native albumin, such as resistance to protein adsorption, and this bioinert surface property was easily converted to allow protein adsorption by exposing the film to cationic polymer solutions or UV light. Making use of this convertible surface property, micropatterns of the proteins, including the fibronectin and antibody, were successfully created in previous studies [12,13]. In addition, the micropattern of adherent cells could also be fabricated on the albumin film through the selective attachment of cells to the cell adhesion protein-adsorbed region but not to the surrounding bioinert film surface [12,14,15]. To our knowledge, this is the first report of a method for generating a patterned co-culture composed of adherent cells and immobilized nonadherent cells. Here, we demonstrate the utility of our approach through the generation of a patterned co-culture consisting of micropatterned neuroblastoma cells (adherent cells) surrounded by immobilized myeloid cells (nonadherent cells). A co-culture system composed of colon cancer cells and immobilized monocytes was also created to examine the influence of monocytes on the drug sensitivity of cancer cells.

2. Materials and methods

2.1. Preparation of CMAR–albumin conjugate

The CMAR and serum albumin conjugate was prepared for the immobilization of nonadherent cells on the substrate. CMAR is composed of three different units: (i) an oleyl group that interacts with the cell membrane via hydrophobic interactions; (ii) a polyethylene glycol (PEG) chain of hydrophilic spacers; and (iii) *N*-hydroxysuccinimide (NHS) as a reactive group that allows CMAR to bind with other molecules (Fig. 1A). Bovine serum albumin (Sigma, St Louis, MO) was dissolved in phosphate-buffered saline (PBS) to make a 15.2 μM solution. Then, 20 μl CMAR (SUNBRIGHT OE-040CS; $M_n = 4000$, NOF CORPORATION, Tokyo, Japan) in dimethylsulfoxide was added to 10 ml of albumin solution at a final concentration of 0.1 mM. After reacting for 1 h at room temperature, the reaction mixture was poured into centrifugal filter devices (Amicon® Ultra-15, molecular weight cut off: 10 kDa, Merck Millipore, Billerica, MA) and centrifuged at 5000×*g* for 30 min to remove the unreacted CMAR. The prepared CMAR–albumin conjugate was adjusted to a final concentration of 14 μM by the addition of PBS and filtered through a 0.22 μm filter for sterilization. Protein concentration was determined by absorbance at 280 nm using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan).

2.2. Quantification of amino groups

The CMAR–albumin conjugate or native albumin solution in PBS (3 μM, 100 μl) was mixed with 100 μl of 4% sodium bicarbonate (pH = 8.5) and 100 μl of 0.1% 2,4,6-trinitrobenzenesulfonic acid (Wako, Osaka, Japan) in water [16]. The mixture was reacted at 40 °C in the dark. After 30 min, 100 μl of 3% sodium dodecyl sulfate was added, followed by 50 μl of 1 M HCl. The absorbance was then measured at 340 nm using a Multiskan Ascent plate reader (Thermo Labsystems, Helsinki, Finland). The reaction efficiency of CMAR and albumin was calculated using the following equation: Reaction efficiency (%) = (Na − Nc/Na) × 100, where Na or Nc is the absorbance of the native albumin sample or CMAR–albumin conjugate sample, respectively. The average and standard deviation of reaction efficiency were calculated from three independent experiments.

2.3. Circular dichroism spectroscopy

The circular dichroism (CD) spectra of native albumin and the CMAR–albumin conjugate were measured using a CD spectrophotometer (J-820, JASCO Co. Ltd., Tokyo, Japan) and a 1 cm path length quartz cuvette at 22 °C with an accumulation of 4 scans. Albumin samples were dissolved in PBS at a concentration of 0.15 μM. Each spectrum was corrected for baseline by subtracting the spectral contribution of the buffer solution. The results are expressed as mean residue ellipticity, defined as $[\theta] = 100 \times \theta/(l \times C \times A)$, where θ is the observed ellipticity (degree), l is optical path length (cm), C is the protein concentration (M), and A is the number of amino acid residues of bovine serum albumin (=582) [17].

2.4. Cells and cell culture

Mouse myeloid 32D cells (RCB1145, Riken Cell Bank, Tsukuba, Japan) were maintained in RPMI 1640 medium (Nacalai, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), 10 U/ml recombinant murine interleukin-3 (BioVision, Inc., Milpitas, CA), and an antibiotic–antimycotic mixed solution which contained

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