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Quantitating distance-dependent, indirect cell–cell interactions with a multilayered phospholipid polymer hydrogel

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ABSTRACT

Multilayered polymer hydrogels containing living cells were assembled for assessing the distance-dependent effects of soluble factors secreted by stroma cells on tumor cell cycle progression *in vitro*. A layer of tumor cells and a layer of stroma cells were separated with finely controlled spacing in a multilayered sandwich composed of a 2-methacryloyloxyethyl phosphorylcholine polymer and poly(-vinyl alcohol) hydrogel. We demonstrated the utility of this tool for investigating intercellular communication between human cervical cancer HeLa cells and supportive stromal L929 fibroblast cells in co-culture. Time-lapse microscopic analyses of HeLa cells showed short distances (15 μm) between tumor cells and stroma cells induced a continuous increase in the percentage of HeLa cells in the S/G2/M phases of the cell cycle, while longer distances (70 μm) between the cells caused a slower increase followed by a sharp increase in the percentage of cells in S/G2/M phases. One possible explanation is gradient formation in the diffusion-dominant multilayer hydrogels by water-soluble factors such as those inducing growth, differentiation, and proliferation. This study provides insights into the potential effects of diffusion of soluble factors and related distance-dependent effects on cell behavior, which may contribute to the design of future co-culture systems.

1. Introduction

Advances in tissue engineering, particularly the use of well-defined synthetic hydrogels, have recently led to the design of tightly controlled models that can be used to study disease pathogenesis, for example, in tumors [1–3]. Tumor cells *in vivo* integrate and respond to cues in their microenvironment that vary in both time and space. These cues come from direct contact with contiguous cells, from soluble factors, such as growth, differentiation, and proliferation factors, secreted by neighboring or distant cells, and from the surrounding extracellular matrix (ECM). In particular, such interactions can regulate tumorigenesis, metastasis, and chemoresistance in cancer tissues [4–6]. Although conventional dish co-culture systems have played major roles in observing cell–cell interactions, these systems can only evaluate whether the presence or absence of such interactions contributes to a cell phenotype. There are therefore emerging approaches to develop 3-dimensional model systems that replicate the *in vivo* architecture and allow control of the degree of homotypic and heterotypic cell–cell interactions, enabling more accurate quantitative studies [7–10]. However, the application of these technologies to elucidate the underlying tumor cell biology is still a challenge.

The main reason for the slow progress in this area is the complexity of the tumor microenvironment. For example, when the effect on cell behavior of one defined extracellular stimulus (a soluble cytokine) is focused on, it is necessary to recognize that this input stimulus is not processed in isolation; the signal molecule network constantly receives additional inputs from changing environmental conditions, such as cell–cell contact and exposure to the ECM. Therefore, for more detailed information on indirect cell–cell interactions, they should be separated from other parameters and studied following the "cue–signal–response" rules in a simplified environment. Another probable reason is that microfabrication technologies are complex and require instrumentation not commonly available in biology laboratories; biologists tend to use cheap, simple, and reliable experimental systems they are familiar with, even if these systems are not at the cutting edge. Development of an experimentally convenient method using instrumentation commonly available in biology laboratories is emerging.

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We used a simple dropping/spinning-assisted layer-by-layer (LbL) procedure for preparing a cellular microenvironment *in vitro* that allows cell patterning by forming a sandwich of 2 cell-laden layers separated by a poly(MPC-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-vinylphenylboronic acid (VPBA)) (PMBV)/poly(vinyl alcohol) (PVA) multilayer hydrogel [11]. The PMBV/PVA hydrogel system is particularly suitable for the study of cell–cell indirect interactions. The MPC units are well known for their biocompatibility and high ability to suppress protein adsorption and subsequent biological reactions, so cells do survive well in PMBV/PVA hydrogel but they are passively attached to the interface of the hydrogel and unable to spread and flatten, and exhibit round morphology after encapsulation, which inhibits cell proliferation [12–14]. Another advantage is non-specific affinities between biomolecules and the hydrogels seldom happen due to antifouling property of the MPC units [15–17], which favor us who want to focus on the effect of only diffusion distance. Therefore, this hydrogel creates a blank milieu to decouple cell–cell indirect interactions from other parameters (cell–cell contact and cell–ECM interaction). Cell–soluble factors interactions may be quantitated by holding tumor and stroma cell layers at variable distances and measuring a cell response that corresponds to distances. As described in our previous report, the distance between two hydrogel layers containing living cells can be finely controlled [11]. Here, we used human cervical cancer HeLa cells stably expressing fluorescent ubiquitination-based cell cycle indicator (Fucci) [18] fusion proteins as cell cycle sensors to perform a quantitative analysis of the kinetics of cell activities as a function of time [19].

The aim of this study was to utilize the PMBV/PVA multilayer hydrogel system to gain a quantitative insight into an important variable "distance" regulating indirect cell–cell interactions in the tumor microenvironment, and point the way towards improving technologies to address fundamental biological questions about spatiotemporal regulation of tumor behavior.

2. Materials and methods

2.1. Materials

MPC, which was synthesized by a previously reported method [20], was obtained from NOF Corporation (Tokyo, Japan). BMA was purchased from Kanto Chemicals (Tokyo, Japan), VPBA was purchased from Tokyo Chemical Industry (Tokyo, Japan), and PVA (mean polymerization degree = 1000, saponification degree = 96.0 mol%) was purchased from Wako Pure Chemical (Osaka, Japan). Other organic reagents and solvents were commercially available reagents of extra-pure grade and were used without further purification.

PMBV was synthesized by radical random copolymerization of corresponding monomers, as previously described [12,21,22]. The mole fractions of MPC, BMA, and VPBA units in the obtained PMBV were determined to be 0.60, 0.25, and 0.15, respectively, using ^{1}H NMR (JNM-NR30; JEOL, Tokyo, Japan). The weight-averaged molecular weight of PMBV was 1.7×10^4, as determined by gel permeation chromatography (Jasco, Tokyo, Japan), with poly(ethylene oxide) standards. The chemical structure of PMBV and the gelation mechanism between PMBV and PVA are shown in Fig. 1(a) and (b), respectively.

2.2. Cell culture, cell staining, and collection of conditioned medium

HeLa-Fucci cells (HeLa-Fucci) and L929 fibroblast cells (a murine fibroblast cell line established from connective tissue) were purchased from Riken Cell Bank (Ibaraki, Japan). Both were cultured with DMEM (Sigma–Aldrich, St. Louis, MO) supplemented with 10% FBS. All cells were cultured at 37 °C in a 5.0 vol% CO_2 humidified atmosphere.

For labeling cells, L929 cells were stained using the red fluorescent dye PKH26 (Sigma–Aldrich, St. Louis, USA) and green fluorescent dye PKH67 (Sigma–Aldrich), respectively, as per the manufacturer's instructions.

For the collection of L929 fibroblast cell conditioned medium (LCM), L929 cells were first grown to 80% confluence in DMEM supplemented with 10% FBS. Since the LCM was to be concentrated for study, it was desirable not to have serum in the LCM. Therefore, the confluent cells were washed with PBS and then fed serum-free DMEM. After 24 h of incubation, the LCM was collected.

2.3. Preparation of multilayer cell culture hydrogels with HeLa-Fucci cells associated with stromal fibroblast cell conditioned medium

The PMBV/PVA multilayered hydrogel platform utilized here was prepared by a layer-by-layer (LbL) procedure similar to that previously reported [11]. The objective was to hold HeLa-Fucci cells at finely controlled separations from LCM. This platform was designed to determine whether the soluble factors secreted by L929 cells can affect cell cycle progression in HeLa-Fucci cells, and how the soluble factors influence cycle progression as a function of diffusion distance.

Briefly, the platform was prepared by successive assembly of hydrogel layers onto a layer of HeLa-Fucci cells immobilized in hydrogel, making use of the spontaneous gelation between PMBV and PVA [11–14]. The PMBV (5.0 wt%) and PVA (5.0 wt%) solutions were prepared in DMEM without Phenol Red (Gibco, Grand Island, USA). All hydrogel layers were built in a 35-mm diameter cell culture dish (Iwaki Co., Tokyo, Japan).

In a modification of a previously reported method [11], 10 μL HeLa-Fucci cell suspension (2.5×10^6 cells/mL) containing PMBV was dropped onto the PMBV/PVA precursor hydrogel layer and allowed to flow by gravity with the dish tilted to a 45° angle, thus forming a thin membrane with a larger cell distribution area and most of the cells embedded in the hydrogel membrane. The HeLa-Fucci cell-laden layer was

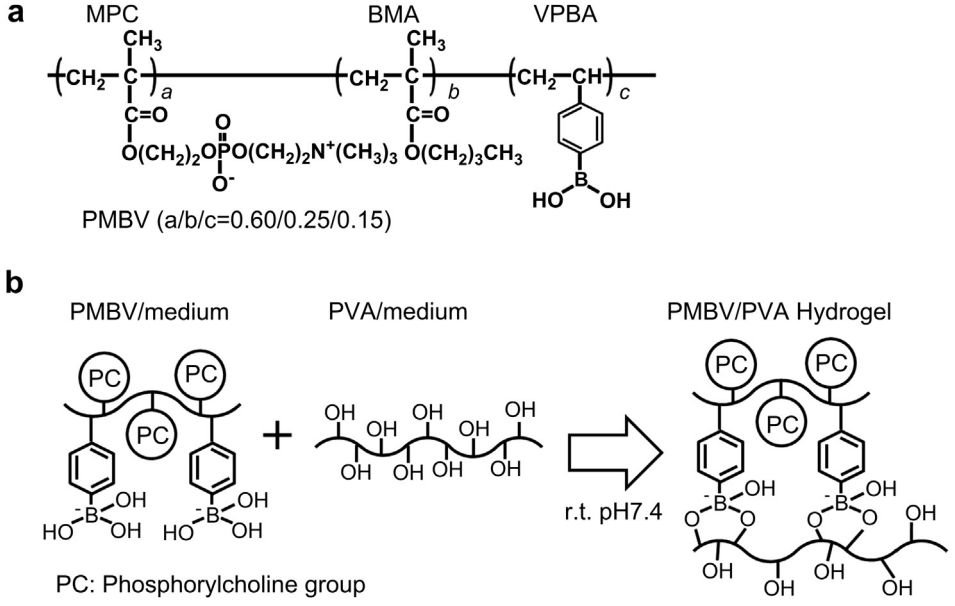

Fig. 1. (a) The chemical structure of PMBV, and (b) gelation mechanism between PMBV and PVA.