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Mobility of the Arg-Gly-Asp ligand on the outermost surface of biomaterials suppresses integrin-mediated mechanotransduction and subsequent cell functions

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ABSTRACT

Mechanotransduction in the regulation of cellular responses has been previously studied using elastic hydrogels. Because cells interact only with the surface of biomaterials, we are focusing on the molecular mobility at the outermost surface of biomaterials. In this study, surfaces with the mobile Arg-Gly-Asp-Ser (RGDS) peptide have been constructed. Cell culture substrates were coated with ABA-type block copolymers composed of poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate) segments (A) and a polyrotaxane (PRX) unit with RGDS bound to α-cyclodextrin (B). Adhesion, morphological changes and actin filament formation of human umbilical vein endothelial cells were reduced on the surfaces containing mobile PRX-RGDS in comparison to the immobile RGDS surfaces constructed from random copolymers with RGDS side groups (Prop-andom-RGDS). In the neurite outgrowth assay using rat adrenal pheochromocytoma cells (PC12), only ~20% of adherent PC12 cells had neurites on PRX-RGDS surfaces, but more than 50% did on the Random-RGDS surface. The beating colony of dimethyl-sulfoxide-treated mouse embryonic carcinoma cells (P19CL6) were found 10 and 14 days after induction on PRX-RGDS and Random-RGDS surfaces, respectively. After 22 days, the beating colony disappeared on PRX-RGDS surfaces, but many colonies remained on Random-RGDS surfaces. These data suggest that the molecular mobility of the cell-binding ligand on the outermost surface of materials effectively suppresses the actin filament formation and differentiation of these functional cell lines, and may be used as a culture substrate for immature stem cells or progenitor cells.

1. Introduction

Highly functional scaffolds have been increasingly accelerating progress in the field of regenerative medicine [1,2]. These scaffolds are expected to function as a temporary extracellular matrix (ECM) for controlling cell adhesion and for defining the shape and size of regenerated tissues. The recent integrated understanding of cell–biomaterial interactions has given rise to the idea that well-designed scaffolds would lead to more effective tissue regeneration by modulating cell adhesion, migration, proliferation and stem cell differentiation. Cells sense and respond to a wide variety of chemical, physical and biological features of the scaffolds such as wettability, electric charge, topology and biological activity of various ligands; this phenomenon allows for regulation of cellular functions [3–5].

Recently, it was shown that matrix elasticity plays an important role in regulating numerous cell functions [6–9]. For instance, endothelial cells on the stiff hydrogel become stiffer, and their production of podosomes is enhanced, as is the maturation of the actin cytoskeleton [10,11]. Stem cell fate is also affected by the matrix elasticity [8,9]. Human mesenchymal stem cells (hMSCs) were reported to be directed specific lineages when cultured on hydrogels of varying elasticity [8,12]. The hMSCs differentiate into neurogenic, myogenic and osteogenic cells on the hydrogels with elasticity similar to that of brain, muscle and bone tissues. The proliferation and differentiation of rat neural stem/progenitor cells

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are stimulated better on the surfaces with moduli of 3.5 kPa than 1 kPa [13]. Furthermore, rat embryonic cardiomyocytes function longer on surfaces with moduli of 11–17 kPa, which is similar to the elastic modulus of normal myocardial tissue [14].

On the other hand, maintaining the undifferentiated state of various stem cells or progenitor cells is also necessary for some research and clinical applications. Bone-marrow-derived hMSCs maintain an ability for self-renewal and remain multipotent when cultured on collagen-coated polyacrylamide (PAAm) soft substrates (0.25 kPa) [15]. Gilbert and coworkers reported that skeletal muscle stem cells also keep the undifferentiated state with a self-renewal ability on the laminin-immobilized soft polyethylene glycol (PEG) hydrogel (12 kPa) [16]. In addition, Chowdhury et al. successfully cultured mouse embryonic stem cells as homogeneous undifferentiated colonies with high expression of Oct3/4 and alkaline phosphatase on the collagen-coated PAAm soft substrate (0.6 kPa) [17].

Cells adhere to the hydrogels coated with an ECM protein such as collagen, fibronectin or laminin through focal adhesion (FA). Previous researchers reported that cells also sense the surface stiffness via FA points, and then integrin-mediated mechanotransduction induces morphological changes, adhesion and differentiation [18–22]. The integrin-mediated mechanotransduction is closely related to the dynamic integrin clustering at FAs [23,24]. In fact, an intercellular traction force increases in proportion to the elasticity of microspot substrates via reorganization of an actin filament network through integrin clustering [25–27]. Trappmann et al. evaluated the human epidermal and mesenchymal stem cell behaviors on the collagen coated substrate with the elastic modulus of 0.1 kPa-2.3 MPa through the well-defined anchoring density [28]. Interestingly, they showed that the cell spreading and differentiation were affected by the collagen anchoring density, but not by the substrate elastic modulus. That is, stem cells decide their own fate by sensing the integrin-ligand mobility of collagen fibers. Tsai and Kam reported that the lateral mobility of E-cadherin enhances the Rac1 recruitment of Madin–Darby canine kidney epithelial cells [29]. Moreover, Andreasson-Ochsner et al. also demonstrated that the lateral mobility of E-cadherin influences cellular responses [30]. They evaluated the adhesion behavior of Chinese hamster ovary (CHO) cells on the supported lipid bilayer platform having laterally mobile or immobile E-cadherin ligands. The spreading and the actin filament formation of CHO cells were suppressed by enhancing E-cadherin mobility. Although the molecular mobility of the outermost surface of substrates is one of the candidates for their key factors, these hydrogels and microspot substrate features affecting cell adhesion continue to be studied.

Recently, we have succeeded at controlling the molecular mobility of biomaterial surfaces by means of polyrotaxane (PRX)-based supramolecules and evaluated their function *in vitro* [31–37]. PRX has highly mobile structure, in which ring-shaped α-cyclodextrins (α-CDs) are threaded by a PEG chain, and the α-CDs are able to slide and rotate on the PEG chain. We found that the mobility of the PRX structure facilitates specific ligand–receptor interactions [38]. ABA-type block copolymers consisting of amphiphilic anchoring segments (A) that bind on various hydrophobic substrates via hydrophobic interactions and a central PRX segment (B) have been successfully designed and used for coating the surface of materials [33–37]. These triblock copolymers were useful for controlling the molecular mobility of the outermost surface irrespective of the substrate elastic modulus. We figured out that the mobility factor (Mf) measured using quartz crystal microbalance (QCM-D) analysis is useful for describing the mobility of a surface and strongly correlates with behavior the adherent cells [31]. The surface mobility is an influential feature because it causes conformational changes of adsorbed proteins and the morphology of adherent platelets and other types of cells. In a previous study, we prepared the surfaces with mobile Arg-Gly-Asp-Ser (RGDS) ligands by means of RGDS bearing PRX (PRX-RGDS) [37]. The integrin–RGDS interaction was accelerated on PRX-RGDS surfaces at the very early stage (within the first ~20 min).

In the present study, the effects of the mobility of RGDS ligands on several features of cell behavior were evaluated for a much longer period of time in order to assess the downstream mechanoresponse. Three types of cell functions – adhesion of human umbilical vessel endothelial cells (HUVECs), neuronal differentiation of rat adrenal pheochromocytoma cells (PC12) and differentiation of mouse embryonic carcinoma cells (P19CL6) into beating colonies – were evaluated. The HUVEC is known to dynamically alter its morphology in response to the mechanical environments such as the shear stress of blood flow and the tensile stress of the vascular wall [39]. PC12 cells which discontinue proliferating and begin the neurite outgrowth by the treatment of nerve growth factor (NGF) are widely used as a model of neural stem cells [40]. The neurite outgrowth of PC12 cells is greatly affected by the cell adhesion and morphology in regulating integrin activation [41]. Furthermore, P19Cl6 cells are differentiated into beating cardiomyocytes with dimethyl sulfoxide (DMSO) treatment. The cardiac differentiation and maturation of P19CL6 is also affected by cell–material interaction [42]. ABA-type block copolymers consisting of a PRX block with RGDS-bearing α-CDs (A segment) and amphiphilic anchoring terminal blocks of poly(2-methacryloyloxyethyl phosphorylcholine-*co*-n-butyl methacrylate) (PMB; B block) were synthesized and used for constructing mobile surfaces (Fig. 1A). To prepare immobile RGDS surfaces, random copolymers composed of 2-methacryloyloxyethyl phosphorylcholine (MPC) and *n*-butyl methacrylate (BMA) containing RGDS (Random-RGDS) were synthesized (Fig. 1B). The morphology of adherent HUVECs was examined after staining for F-actin and FA points. Neurite outgrowth of NGF-stimulated PC12 cells and differentiation of P19CL6 cells into beating colonies after DMSO induction were also analyzed.

2. Materials and methods

2.1. Materials

Monotosylated α-CD, BMA, 2,2′-azobis(isobutyronitrile) (AIBN), methacryloyl chloride, 4-azidobutanol and organic solvents were purchased from Tokyo Kasei Co. (Tokyo, Japan). The organic solvents were of high purity chemical grade and were used without further purification. MPC was obtained from NOF Co. (Tokyo, Japan), which was prepared according to a previously published method [43].

For peptide synthesis, Fmoc-Gly-OH (where Fmoc is 9-fluorenylmethyloxycarbonyl group), Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Gly(propargyl)-OH, Barlos resin (1.57 mmol g^{-1}, 100–200 mesh), N,N-diisopropyl ethylamine (DIEA), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-1H-benzotriazole hydrate (HOBt), triisopropylsilane (TIS), piperidine, trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Acetic anhydride was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

HUVECs were obtained from Cell Application, Inc. (San Diego, CA, USA). Rat adrenal pheochromocytoma cells (PC12) and mouse embryonic carcinoma cells (P19CL6) were purchased from the Riken BioResource Center (Ibaraki, Japan). For the culture of HUVECs, the EBM-2 basal medium and EGM-2 SingleQuots kit including growth factors (GFs) and fetal bovine serum (FBS) were obtained from Lonza (Walkersville, MD, USA). For the culture of