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Preferential adsorption of cell adhesive proteins from complex media on self-assembled monolayers and its effect on subsequent cell adhesion

Yusuke Arima, Hiroo Iwata *

Institute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT

We examined the effect of surface chemistry on adsorption of fibronectin (Fn) and vitronectin (Vn) and subsequent cell adhesion, employing self-assembled monolayers (SAMs) of alkanethiols carrying terminal methyl (CH_3), hydroxyl groups (OH), carboxylic acid (COOH), and amine (NH_2). More Fn and Vn adsorbed to COOH- and NH_2-SAMs than to CH_3- and OH-SAMs from a mixture with bovine serum albumin (BSA) and from 2% fetal bovine serum. Adhesion of human umbilical vein endothelial cells (HUVECs) on CH_3- and OH-SAMs preadsorbed with Fn and BSA decreased with decreasing adsorbed Fn; however, HUVECs adhered to COOH- and NH_2-SAMs even in the presence of BSA at 1000-fold more than Fn in a mixture because of the preferential adsorption of Fn and/or displacement of preadsorbed BSA with Fn and Vn in a serum-containing medium. SAMs coated with a mixture of Vn and BSA exhibited adhesion of HUVECs regardless of surface functional groups. A well-organized focal adhesion complex and actin stress fibers were observed only for COOH- and NH_2-SAMs when SAMs were preadsorbed with Vn and BSA. These results suggest that COOH- and NH_2-SAMs allow for both cell adhesion and cell spreading because of the high density of cell-binding domains derived from adsorbed Vn.

Statement of Significance

Adsorption of cell adhesive proteins including fibronectin (Fn) and vitronectin (Vn) plays an important role in cell adhesion to artificial materials. However, for the development of biomaterials that contact with biological fluids, it is important to understand adsorption of Fn and Vn in complex media containing many kinds of proteins. Here, we focused on adsorption of Fn and Vn from complex media including mixed solution with albumin and fetal bovine serum, and its role on cell adhesion using self-assembled monolayers (SAMs). Our result demonstrates that SAMs carrying carboxylic acid or amine allow for both cell adhesion and cell spreading because of preferentially adsorbed Vn. The result provides insights into surface design of cell culture substrates and tissue engineering scaffolds.

1. Introduction

Much effort has been devoted to understanding cell interactions with artificial materials to facilitate the development of medical devices and scaffolds for tissue engineering. However, in most cases, cell-material interactions are not clearly understood, and many studies are required to thoroughly characterize many factors relevant to the cell–material interactions for a particular system. These factors include material surface energy, surface electrostatic properties, macro- and micro-surface morphology, surface heterogeneity, functional groups, and the mobility of functional groups on surfaces. Systematic studies of biological responses to artificial materials require surfaces with well-controlled properties. Self-assembled monolayers (SAMs) of alkanethiols, $HS(CH_2)_nX$, where X denotes various functional groups [1,2], are suitable for studying correlations between biological responses and surface properties. It was reported that cell adhesion behavior is highly dependent on the outermost functional groups of SAMs [3–10].

The process of cell adhesion onto a material's surface includes the interaction between integrins in the cell membrane and adhesive proteins, such as fibronectin (Fn) and vitronectin (Vn), adsorbed on the surface. Role of Fn and Vn in cell adhesion on material surfaces has been extensively studied using polymer substrates [11–16] and SAMs [17–22]. Pretreatment of artificial materials with a solution containing either Fn only or Vn only improves

* Corresponding author.
E-mail address: iwata@frontier.kyoto-u.ac.jp (H. Iwata).

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cell adhesion to the surfaces. Realistically, however, cell adhesion onto surfaces occurs in tissue fluids, plasma, or culture media supplemented with serum, all of which contain various kinds of proteins. The adsorption behaviors of Fn-only and Vn-only solutions differ from that of complex media, because concentrations of cell adhesive proteins in serum (Fn: 30 μg/mL [23]; Vn: 200 μg/mL [24]) are several orders of magnitude lower than some other proteins, such as bovine serum albumin (BSA, 35–55 mg/mL) and immunoglobulin G (IgG, 0.8–1.8 mg/mL), which lack the capacity to interact with cells. This would imply that a protein-adsorbed layer mainly consists of serum proteins that do not mediate cell adhesion.

We aimed to understand how minute amounts of Fn and Vn in complex media can mediate cell adhesion to SAMs presenting different surface properties. In this study, we examined adsorption of Fn and Vn to SAMs in the case of mixed solutions with BSA or fetal bovine serum (FBS), as well as displacement of initially adsorbed proteins by Fn and Vn. We also tested adhesion of human umbilical vein endothelial cells (HUVECs) and formation of focal adhesion complexes on these SAMs to elucidate the relationship between adsorption of Fn and Vn and cellular behavior.

2. Materials and methods

2.1. Materials

1-Dodecanethiol (CH_3: Wako Pure Chemical Industries, Ltd., Osaka, Japan), 11-mercapto-1-undecanol (OH: Sigma–Aldrich, St. Louis, MO, USA), 11-mercaptoundecanoic acid (COOH: Sigma–Aldrich), and 11-amino-1-undecanethiol hydrochloride (NH_2: Dojindo Laboratories, Kumamoto, Japan) were used as received. Dulbecco's (D) phosphate-buffered saline (PBS) (DPBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was purchased from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). Human plasma Fn (F2006), bovine plasma Vn (V9881), and BSA (A4503) were purchased from Sigma–Aldrich and used as received. Antibodies against integrin α_5 (AB1928, Millipore, Billerica, MA, USA) and integrin α_v (ab16821, Abcam, Cambridge, UK) were used as received. Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 488 anti-mouse IgG, and Alexa Fluor 594 phalloidin were purchased from Molecular Probes, Inc. (Eugene, OR, USA).

2.2. Preparation of SAMs

Cover glasses (diameter; 15 mm, Matsunami Glass Ind., Ltd., Osaka, Japan) were cleaned by oxygen plasma treatment using a plasma reactor (PA300AT, O-kuma Engineering Co. Ltd., Fukuoka, Japan) for 1 min and rinsed with highly purified water (18.2 MΩ) and with ethanol three times. Cleaned cover glasses were coated with a chromium underlayer of 1 nm and then a gold layer of 19 nm in thickness by a thermal evaporation apparatus (V-KS200, Osaka Vacuum Instruments, Osaka, Japan). The gold-coated cover glasses were immediately immersed in a 1 mM solution of alkanethiols overnight to form SAMs. The cover glasses were then rinsed with ethanol and water twice and finally with ethanol, and then dried under a stream of nitrogen gas.

2.3. Surface characterization

Elemental compositions of the SAM surfaces were determined by X-ray photoelectron spectroscopy (XPS) using an ESCA 850V (Shimadzu Co., Kyoto, Japan) equipped with a Mg Kα source. The take-off angle was 90°, and the operating pressure was lower than 1×10^{-5} Pa. All spectra were shown referring to Au ($4f_{7/2}$) at 83.8 eV.

Static water contact angles were determined by the sessile drop method using a contact angle meter (CA-X; Kyowa Interface Science Co. Ltd., Saitama, Japan) at room temperature. A droplet (10 μL) of water was placed on a SAM surface, and 10 s later, the contact angle was determined three times. This procedure was repeated five times at different sites on the same surface, and the contact angle of a sample was expressed as the mean value of five contact angle measurements.

2.4. Adsorption of Fn and Vn

Fn and Vn were radiolabeled using the chloramine-T method [25]. Five microliters of $Na^{125}I$ (Iodine-125, PerkinElmer Inc., Boston, MA, USA) and 100 μL of chloramine-T (Nacalai Tesque, Kyoto, Japan; 0.2 mg/mL in 0.5 M phosphate buffer containing 0.15 M NaCl, pH 7.4) was added to 200 μL of Fn or Vn (500 μg/mL in 0.5 M phosphate buffer containing 0.15 M NaCl). For Vn, 50 μg/mL of stock solution was concentrated by ultrafiltration (Amicon Ultra-0.5, nominal molecular weight limit: 10,000; Millipore) before radiolabeling. The reaction mixture was incubated for 2 min at room temperature. Then 100 μL of sodium metabisulfite (Nacalai Tesque; 4 mg/mL in water) was added to stop the reaction. Labeled proteins were separated by size-exclusion chromatography using a Sephadex G-25 column (PD-10; GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Fractions were collected and examined for radioactivity using a gamma counter (ARC-380CL, Aloka Co., Ltd., Tokyo, Japan). Protein concentration was also determined by the Micro BCA™ protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). Labeled proteins were mixed with unlabeled proteins, and specific activity (cpm/ng-protein) was determined. The sample was stored at −20 °C until use. A control protein adsorption experiment was performed using ^{125}I-labeled proteins with a different ratio to non-labeled proteins. The adsorbed amount of proteins was constant regardless of the ratio (data not shown), suggesting that the iodination of Fn and Vn does not change their adsorption behavior.

A 100 μL droplet of protein solution, dissolved in DPBS containing 10 mM NaI (PBSI) [26], was placed on a 35-mm non-treated polystyrene dish (Asahi Techno Glass Corp., Tokyo, Japan), and substrate-carrying SAM was immediately floated on the droplet so that the SAM surface faced the protein solution. The substrate was placed on the droplet immediately in order to avoid formation of protein layer at air–water interface of the droplet. The concentration of Fn or Vn was kept constant at 0.01 mg/mL while BSA concentration was varied at 0.01–10 mg/mL in PBSI. After 30 min incubation at 37 °C, the substrates were rinsed with PBSI and placed in polystyrene tubes. The radioactivities (cpm) were measured by the gamma counter and converted to the amount of adsorbed Fn or Vn (ng/cm^2). In some experiments, to observe displacement of adsorbed proteins, SAMs were first incubated with 10 mg/mL BSA for 30 min and then rinsed with PBSI followed by incubation with either ^{125}I-labeled Fn or Vn for 30 min.

Protein adsorption to SAMs from FBS was also tested. Fn contained in native FBS was removed using gelatin Sepharose [27]. FBS was mixed with gelatin Sepharose 4B (GE Healthcare) and incubated for 2 h at room temperature. Fn-depleted FBS was collected by centrifugation at 1000 × g for 2 min using a filter unit (ULTRAFREE-MC; Millipore). Removal of Fn was confirmed by ELISA. ^{125}I-labeled Fn was then added to a concentration of 30 μg/mL. For Vn, ^{125}I-labeled Vn was added to native FBS at a concentration of 20 μg/mL, which is one tenth of the Vn concentration in native FBS [24]. A protein adsorption experiment was performed as described above. The amount of adsorbed Fn was calculated from radioactivity while that of adsorbed Vn was calculated from radioactivity and then multiplied by 11 because the FBS included ^{125}I-labeled Vn and native Vn with a presumed ratio of 1:10.