Colloids and Surfaces B: Biointerfaces 126 (2015) 188–197

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Effect of surface chemistry on the integrin induced pathway in regulating vascular endothelial cells migration

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ARTICLE INFO

Article history:
Received 31 August 2014
Received in revised form 5 December 2014
Accepted 9 December 2014
Available online 30 December 2014

Keywords:
Surface chemistry
Cell adhesion/migration
Focal adhesion
FAK
Rho GTPases

ABSTRACT

The migration of vascular endothelial cells (ECs) is essential for reendothelialization after implantation of cardiovascular biomaterials. Reendothelialization is largely determined by surface properties of implants. In this study, surfaces modified with various chemical functional groups (CH_3, NH_2, COOH, OH) prepared by self-assembled monolayers (SAMs) were used as model system. Expressions and distributions of critical proteins in the integrin-induced signaling pathway were examined to explore the mechanisms of surface chemistry regulating EC migration. The results showed that SAMs modulated cell migration were in the order $CH_3 > NH_2 > OH > COOH$, determined by differences in the expressions of focal adhesion components and Rho GTPases. Multiple integrin subunits showed difference in a surface chemistry-dependent manner, which induced a stepwise activation of signaling cascades associated with EC migration. This work provides a broad overview of surface chemistry regulated endothelial cell migration and establishes association among the surface chemistry, cell migration behavior and associated integrin signaling events. Understanding the relationship between these factors will help us to understand the surface/interface behavior between biomaterials and cells, reveal molecular mechanism of cells sensing surface characterization, and guide surface modification of cardiovascular implanted materials.

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1. Introduction

The importance of reendothelialization and vascular repair after arterial injury followed by cardiovascular stent implantation is widely recognized [1]. It is conceivable that reendothelialization is essential for inhibition of smooth muscle cell (SMC) proliferation and intimal hyperplasia induced in-stent restenosis (ISR) [2]. The migration of vascular endothelial cells (ECs) from adjacent healthy endothelium is essential for reendothelialization after implantation of cardiovascular biomaterials, which is largely determined by surface properties of implants. Therefore, it is important to properly design implanted surface to favor EC migration.

It has been previously demonstrated that cell adhesion/migration behavior is affected by chemical composition and surface micropatterning of the substrates [3,4]. Self-assembled monolayer (SAM) is molecular assemblies formed spontaneously on surfaces by adsorption and is organized into well-controlled chemical functional groups. Meanwhile, the substrates are quite smooth with equivalent rigidity, which are suitable for cells adhesion and migration studies as model substrates. Using SAM substrates, adhesion of a variety of cell types have been described [5], and the long-term stability and biocompatibility for stent modification was focused and discussed [6,7]. Although many works solely described that different surface properties regulated cell adhesion or migration behaviors for endothelialization, the potential mechanisms for how surface chemistry influenced cell migration induces specific molecular signals are unclear.

Several signaling events contributed to cell migration have been characterized [8]. Especially, integrins transmit extracellular

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http://dx.doi.org/10.1016/j.colsurfb.2014.12.019
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chemical signals into the cell (outside-in signals), providing information on its location, local environment, adhesive state and surrounding matrix [9]. Surface properties of cardiovascular implants trigger a cascade of biochemical and biophysical events at the cell–matrix interface. The cascade subsequently regulates cell behavior and function and determines the integrity of endothelium. It is well documented that absorption of extracellular matrix (ECM) proteins is the first event occurring at the biomaterial–cell interface, that typically provides structural and biochemical support to the surrounding cells [10]. Integrins are transmembrane receptors that bind to ECM through extracellular domains of α subunits on cell membranes. This induces conformational change and transduction of intracellular signals. Around activated integrin clusters, cytoskeletal proteins such as Talin, Paxillin and Vinculin, as the ligands of β integrin cytoplasmic tails assemble together. At the regions of cell–substratum contacts, they form focal adhesion (FA) plaques and provide enough adhesive sites to support stable cell attachment [11]. FA plaque disassembly drives the migration cycle through activated Rho-family GTPases including RhoA, Rac1 and Cdc42. This results in direct local actin assembly by regulating stress fibers, lamellipodia or filopodia. In addition, the integrin-binding Talin and Paxillin recruit focal adhesion kinase (FAK) via C-terminal focal-adhesion targeting domain and phosphorylated FAK at initial Y397 tyrosine site, which leads to a cascade of activation of other downstream signals [12]. Consequently, intercellular FA formation, activation of Rho-family GTPases, and associated cellular FAK signaling events likely participate in determining cell adhesion/migration behavior on the surface of implanted materials.

Accordingly, in the present study various chemical functional groups (hydrophobic CH_3, positively charged NH_2, neutral hydrophilic OH and negatively charged COOH) prepared by SAMs were used as model substrates to examine surface chemistry regulating cell migration. Especially, expressions and distributions of the intracellular crucial signal molecules in integrin-FAs-FAK-Rho GTPase signaling pathway to surface chemistry were explored to determine the whole pathway participating in surface-induced endothelial cell migration. This work provides a broad overview of surface chemistry regulating EC migration and establishes associations among surface chemistry, cell adhesion/migration behavior and associated signaling events.

2. Materials and methods

2.1. Preparation of SAMs

Surfaces with various chemical functional groups using SAMs technique were used in this study as model materials. The alkanethiols 1-dodecanethiol (HS-$(CH_2)_{11}$-CH_3), 11-amino-1-undecanethiol hydrochloride (HS-$(CH_2)_{11}$-NH_2), 11-mercapto-1-undecanol (HS-$(CH_2)_{11}$-OH), and 11-mercaptoundecanoic acid (HS-$(CH_2)_{10}$-COOH) were purchased from Sigma–Aldrich (St. Louis, USA). Glass slides with 50 nm Au coating were prepared by Dongwei Biotechnology Co. Ltd. (Hangzhou, China). The techniques for preparation and characterization of SAMs were described in a previous study [13,14]. Briefly, prepared Au-coated slides were dipped in 1 mM alkanethiol solutions for SAMs self-assembly overnight. The slides with SAMs were dried in nitrogen for 10 min. The prepared surfaces were assessed by using a contact angle measurement system with a digital camera and image analysis software (VCA 2500XE, USA). The chemical structures were characterized using a Nicolet FTIR 6700 spectrometer from Thermo Electron Corporation (Waltham, MA, USA). Additionally, clean glass slides without any coating and with Au coating were used as parallel controls representing inorganic (glass) and metallic (Au) substrates, respectively.

2.2. Cell culture

Human vascular EC lines, EA.hy 926 cells instead of primary vascular ECs were used in this study. Cells were cultured in RPMI1640 medium (Invitrogen Company, USA) supplemented with 10% fetal bovine serum in a 5% CO_2 incubator at 37 °C. The normalized cells were resuspended and seeded at a concentration of 10^4 cells/mm^2 on slides with different surface chemistries and controls.

2.3. F-actin staining

After culturing cells for 48 h, the F-actin distribution (stained by Phallotoxins, Invitrogen™, USA) was examined. Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min and were incubated with Phallotoxins (Invitrogen™, USA) for 30 min, then DAPI (4′,6′-diamidino-2-phenylindole) with 1:800 dilution was added and co-incubated for 30 min at 37 °C. Samples were observed by laser scanning confocal microscopy (Leica TCS SP5, Germany).

2.4. Cell migration onto SAMs – scratch wound migration assay

Cell migration was measured using a monolayer scratch injury assay as previously described [15]. Briefly, the cells were cultured on the various slides or SAMs until monolayer confluence. Then the cells were starved and synchronized at serum-free conditions overnight. A uniform scratch (about 500 μm width) was done in the cell monolayer using a plastic cell scrapper. The slides were gently washed with PBS to remove suspended cells. The cells were cultured in an incubator containing 5% CO_2 at 37 °C. Three images of the wounds were randomly chosen at 0 h and photographed consecutively at 0, 2, 4, 8 and 12 h under static culture using an inverted microscope (CK2, Olympus, Japan). The cell migration distance at the end of each recording period was calculated as the difference between the end length and the original length of the wounded edge. Migrating cell numbers were derived from the average of the fields in triplicates. The samples were observed in half-hour periods for up to 12 h until cells reached confluence. The wound repair duration of each sample was determined.

2.5. Western blot analysis

Cells cultured for 48 h with 60–80% confluence were washed three times with PBS and disintegrated by 50 μL cell lysis solution. The total protein was collected and centrifuged with 10,000 rpm at 4 °C for 5–10 min, quantified by enhanced bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology Co. Ltd., Beijing, China). Equal amounts of protein (30 μg) were loaded onto each lane of an 8–12% SDS-PAGE gel. After gel electrophoresis and membrane transferring, polyvinylidene difluoride membranes (PVDF, GE Healthcare) were blocked for 2 h in 3% BSA in TBST buffer (20 mmol/L Tris–HCl [pH 8.0], 150 mmol/L NaCl, 0.05% Tween 20) at 37 °C. Membranes were incubated with primary antibodies overnight at 4 °C. HRP binding secondary antibodies were incubated for 2 h at 37 °C. Bands were visualized by enhanced chemiluminescence and Molecular Image® ChemiDoc™ XRS+ system with Image Lab™ Software. The tests were done three times and quantification was done and analyzed by Image J 1.44p software (National Institutes of Health, USA). The intrinsic controls (β-actin) were used to guarantee the uniformity of equal loaded protein among all groups. Detailed information of primary antibodies is shown in Table 1.

2.6. Immunofluorescence assay

After culturing for 48 h, cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 for 10 min. Following fixation and permeabilization, samples were blocked by

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