Biomaterials 161 (2018) 106–116

Contents lists available at ScienceDirect

Biomaterials

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

Lower fluidity of supported lipid bilayers promotes neuronal differentiation of neural stem cells by enhancing focal adhesion formation

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ARTICLE INFO

Article history:
Received 19 July 2017
Received in revised form
4 January 2018
Accepted 20 January 2018

Keywords:
Supported lipid bilayers
Fluidity
Neural stem cells
Differentiation

ABSTRACT

Extensive studies have been performed to understand how the mechanical properties of a stem cell's microenvironment influence its behaviors. Supported lipid bilayers (SLBs), a well-known biomimetic platform, have been used to mimic the dynamic characteristics of the extracellular matrix (ECM) because of their fluidity. However, the effect of the fluidity of SLBs on stem cell fate is unknown. We constructed SLBs with different fluidities to explore the influence of fluidity on the differentiation of neural stem cells (NSCs). The results showed that the behavior of NSCs was highly dependent on the fluidity of SLBs. Low fluidity resulted in enhanced focal adhesion formation, a dense network of stress fibers, stretched and elongated cellular morphology and increased neuronal differentiation, while high fluidity led to less focal adhesion formation, immature stress fibers, round cellular morphology and more astrocyte differentiation. Mechanistic studies revealed that low fluidity may have enhanced focal adhesion formation, which activated FAK-MEK/ERK signaling pathways and ultimately promoted neuronal differentiation of NSCs. This work provides a strategy for manipulating the dynamic matrix surface for the development of culture substrates and tissue-engineered scaffolds, which may aid the understanding of how the dynamic ECM influences stem cell behaviors as well as improve the efficacy of stem cell applications.

1. Introduction

Cells reside in a complex and dynamic extracellular matrix (ECM) environment that has characteristics such as dynamic display of ligands, cell-induced remodeling and no predefined spatial patterns [1–4]. Tremendous effort has been made to replicate the ECM, such as modifying materials with bioactive factors and modulating a material's physical characteristics such as stiffness or topography [5–7]. However, these works are always reported with statically displayed ligands, which cannot mimic the natural dynamic ECM very well. The mobility or fluidic properties of polymers [8–11] and supported lipid bilayers (SLBs) [12] have been used to mimic the dynamic characteristics of the ECM. The mobility properties of polymers can be controlled by defining chain length [11,13] or the sliding of cyclic compounds that are threaded onto linear polymeric chains [8–10]. In other words, the range of the mobility of these polymers is limited by the chain length. SLBs are a well-known biomimetic platform because of their similarity to the cell membrane. In addition to the advantages of their non-fouling nature and good biocompatibility, SLBs have a long range of fluidity, where the lipids can diffuse freely within the membrane, which cannot be achieved by polymers. Benefiting from this long

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https://doi.org/10.1016/j.biomaterials.2018.01.034
0142-9612/

range fluidity, SLBs allow surface-linked ligands to diffuse freely within membranes to complement cell surface cognate receptors and enable cell-mediated ligand clustering and rearrangement [14,15]. This has considerable influence on cell adhesion, cytoskeleton organization, cancer cell invasion potential and so on. For example, nontransformed fibroblasts develop podosome-like adhesions when spread onto fluid SLBs modified with Arg-Gly-Asp (RGD) peptides as opposed to habitually focal adhesions on RGD-modified static glass surfaces [16]. Chinese hamster ovary (CHO) cells cultured in SLB-coated polydimethylsiloxane (PDMS) microwells can adhere to SLBs through tethered E-cadherin, and the enhanced mobility of SLBs significantly decreases the formation of actin bundles and results in more diffuse actin organization [17]. EphA2-expressing human breast cancer cells cultured on SLBs modified by ephrin-A1 ligands show less cancer cell invasion potential when the fluidity of SLBs is restricted [18].

Stem cells are unspecialized and have the potential to differentiate into somatic cells, which is an attractive prospect for tissue engineering and regenerative medicine [19]. Various factors including biochemical and biophysical cues have been shown to influence stem cell fate [20]. Recent studies have been focused on the effect of biophysical cues such as topography, elasticity and stiffness on stem cell behaviors [21–24]. However, to the best of our knowledge, the influence of SLB fluidity on the differentiation of stem cells is not clear. NSCs can generate a multitude of neuronal and glial lineages [25], which has great potential in treating brain disorders and injuries. In this work, NSCs were used as a model to explore the influence of SLB fluidity on the differentiation of stem cells. To avoid the interference of other factors besides fluidity, different SLBs were constructed with the same lipid composition. We developed SLBs on piranha treated glass (P), chitosan-modified glass (Cs) and cholesteryl chloroformate tether-modified glass (CC) based on the consideration that hydrophilicity, electrostatic interaction and the presence of tethers can affect the fluidity of SLBs [26–29]. In addition, fibronectin, as one of the extracellular matrix protein, is commonly used to interface the living cells and material surfaces through its interaction with integrin in the cell membrane. In this work, we modified SLBs with fibronectin to examine the effect of fluidity on NSC adhesion, morphology and differentiation. The possible signal transduction pathways were also elucidated. Our results revealed that the fluidity of SLBs plays an important role in NSCs responses.

2. Materials and methods

2.1. Materials

Glass coverslips were purchased from Haimen Kangtai Experimental Equipment Factory. Low molecular weight chitosan (MW = 10,000, degree of deacetylation: 85.31%) was a gift from Jinan Haidebei Marine Bioengineering Co., China. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(succinyl) (Succinyl-PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), Cholesterol and polycarbonate filters with a pore size of 50 nm were purchased from Avanti Polar Lipids. Cholesteryl chloroformate, (3-Aminopropyl)triethoxysilane, glutaraldehyde, Fibronectin (Fn), Retinoic acid (RA), paraformaldehyde, anti-Tuj-1 and DAPI were purchased from Sigma-Aldrich. Tissue culture plastic ware was purchased from Corning. Cell culture media was purchased from HyClone. EGF and bFGF were purchased from PeproTech Asia. B27 and FBS were purchased from Gibco. Anti-Glial Fibrillary Acidic Protein (Anti-GFAP) was purchased from Merck Millipore. Rhodamine-conjugated phalloidin was purchased from Invitrogen. U0126, anti-MAPK (ERK1/2) and Phospho-p44/42 MAPK (pERK1/2) were purchased from Cell Signaling. Anti-vinculin was purchased from R&D Systems. Anti-FAK, Anti-FAK (phospho Y397) and anti-GAPDH were purchased from Abcam. RIPA Lysis Buffer, Phenylmethanesulfonyl fluoride (PMSF), nitrocellulose membrane and the ECL Western blot substrate kit were purchased from Beyotime. TRIeasy™ Total RNA Extraction Reagent, Hieff™ First Strand cDNA Synthesis Super Mix and Hieff™ qPCR SYBR Green Master Mix were purchased from YEASEN, China. Sulfo-Cyanine5 NHS ester (Cy5) was purchased from LiTTLE-PA Sciences, China.

2.2. Surface modification

Glass coverslips were first treated with piranha solution (a mixture of sulfuric acid and hydrogen peroxide and the volume ratio was 3:1) for 30 min to create the piranha-treated coverslips (P). These coverslips were further modified by (3-Aminopropyl) triethoxysilane (1 ml (3-Aminopropyl)triethoxysilane mixed with 15 ml absolute ethyl alcohol) for 2 h, glutaraldehyde (1 ml glutaraldehyde mixed with 10 ml phosphate buffer) for 1 h and chitosan (10 mg/ml) [30] for 1 h respectively at room tempreature to generate chitosan-modified coverslips (Cs). Finally, the chitosan-modified coverslips were modified by cholesteryl chloroformate (30 mM cholesteryl chloroformate in N,N-Dimethylformamide/dichloromethane (1:1) in the presence of 0.5 mM pyridine) for 5 h at room temperature to produce cholesteryl chloroformate-modified coverslips (CC).

2.3. Construction of SLBs

The small unilamellar vesicles (SUVs) containing DMPC, succinyl-PE and cholesterol (20:1:3 in moles) were prepared by extrusion through a polycarbonate filter with a pore size of 50 nm. For fluorescence recovery after photobleaching (FRAP) measurements, SUVs containing NBD-PE (1% mol) were prepared. SLBs were formed by incubating SUVs on different modified surfaces for 1 h at 37 °C. For NSC culture, SLBs were further incubated with 10 μg/ml fibronectin (Fn) in 37 °C for 3 h.

2.4. Fluorescence recovery after photobleaching measurements

A Leica SP5 was used for FRAP measurements. Pictures were taken using a 60× objective lens with a scanning speed of 500 Hz and a resolution of 256*256, and the laser intensity was below 20%. Before bleaching, 3 pictures were taken with a speed of 1.318 s per frame. Then, a region with a diameter of 15 μm was bleached by adjusting the laser intensity to 100% for 10 frames. Ten pictures were taken immediately after bleaching. Then 15 pictures were taken with 30 s intervals. After that, 15 to 50 pictures were further taken with 60 s intervals.

2.5. NSCs culture

Neural stem cells were isolated from the hippocampus of neonatal ICR mice, and the cells between passages 3 and 10 were used. NSC suspension was cultured in proliferation medium (serum-free DMEM/F12 medium containing EGF (20 ng/ml), bFGF (20 ng/ml) and B27 supplement (2% (v/v)). After 4–5 days of culture, the neural spheres were dissociated into single cells and seeded onto SLBs in a density of 64,000 cells/cm^2 in proliferation medium. After 12 h, the proliferation medium was replaced by differentiation medium (serum-free DMEM/F12 medium supplemented with RA (1 μM), FBS (1% (v/v)) and B27 (2% (v/v)). The differentiation medium was replaced every 3 days. After 6 days, cells were processed for immunofluorescent staining.