Biochemical and Biophysical Research Communications 391 (2010) 43–48

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Laminin-1 induces neurite outgrowth in human mesenchymal stem cells in serum/differentiation factors-free conditions through activation of FAK–MEK/ERK signaling pathways

S. Mruthyunjaya [a], Rumma Manchanda [b], Ravibhushan Godbole [b], Radha Pujari [a], Anjali Shiras [a], Padma Shastry [a,*]

[a] National Centre for Cell Science, Ganeshkhind, Pune 411007, India
[b] Department of Pathology, KEM Hospital, Rasta Peth, Pune 411011, India

ARTICLE INFO

Article history:
Received 9 October 2009
Available online 4 November 2009

Keywords:
Human mesenchymal stem cells
Laminin-1
Neurite outgrowth
MEK/ERK
FAK
Extracellular matrix

ABSTRACT

Mesenchymal stem cells (MSCs) can be differentiated into cell types derived from all three germ layers by manipulating culture conditions *in vitro*. A multitude of growth and differentiation factors have been employed for driving MSCs towards a neuronal phenotype. In the present study, we investigated the potential of extracellular matrix (ECM) proteins—fibronectin, collagen-1, collagen-IV, laminin-1, and laminin-10/11, to induce a neuronal phenotype in bone marrow derived human MSCs in the absence of growth factors/differentiating agents. All of the ECM proteins tested were found to support adhesion of MSCs to different extents. However, direct interaction only with laminin-1 triggered sprouting of neurite-like processes. Cells plated on laminin-1 exhibited neurite out growth as early as 3 h, and by 24 h, the cells developed elaborate neurites with contracted cell bodies and neuronal-like morphology. Function-blocking antibodies directed against α6 and β1 integrin subunits inhibited neurite formation on laminin-1 which confirmed the involvement of integrin α6β1 in neurite outgrowth. Mechanistic studies revealed that cell adhesion to laminin-1 activated focal adhesion kinase (FAK), and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) signaling pathways. Abrogation of FAK phosphorylation by herbimycin-A inhibited neurite formation and also decreased activities of MEK and ERK. Pharmacological inhibitors of MEK (U0126) and ERK (PD98059) also blocked neurite outgrowth in cells plated on laminin-1. Our study demonstrates the involvement of integrin α6β1 and FAK–MEK/ERK signaling pathways in laminin-1-induced neurite outgrowth in MSCs in the absence of serum and differentiation factors.

Introduction

Human mesenchymal stem cells (MSCs) are a heterogeneous population of cells capable of differentiating into cell lineages derived from all three germ layers [1]. MSCs represent a promising autologous source of stem cells for regenerative medicine. MSCs derived from human, mouse, and rat bone marrow have been reported to differentiate into neuron-like cells [2,3]. Clinical improvement with transplantation of MSCs has been demonstrated in animal models of brain injury and neurological disorders [4,5]. Such studies have suggested that MSCs are a potential tool for transplantation in trauma and neurodegeneration [6].

Extracellular matrix (ECM) is an important component of cellular environment that regulates crucial functions such as cell motility, proliferation, and survival by interaction with integrins. ECM–integrin interactions activate a number of signaling pathways similar to those stimulated by growth factors and cytokines [7]. Accumulating evidence in the recent years demonstrates that ECM is involved in development of neurons by regulating neuronal migration and outgrowth [8]. To date, various neurotrophic factors and chemical stimulators in combination with ECM proteins have been used to induce neurite-like extensions and neuronal morphology in human MSCs [9,10]. However, heterogeneity within the MSCs population results in diverse cell responses to multiple differentiation factors and hence does not result in yielding consistent and reproducible results. Recent findings demonstrated that direct contact of MSCs with laminin-5 induced osteogenic gene expression [11], and interaction of MSCs with vitronectin and collagen-1-induced osteogenic differentiation [12]. The current study aimed to

Abbreviations: C1, collagen-1; CIV, collagen-IV; FN, fibronectin; LM1, laminin-1; LM10/11, laminin-10/11; Herb.A, herbimycin-A; Cell susp, cell suspension.

* Corresponding author. Fax: +91 20 25692259.
E-mail address: padma@nccs.res.in (P. Shastry).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.10.158

investigate the potential of purified ECM proteins—fibronectin, collagen-1, collagen-IV, laminin-1, and laminin-10/11, to induce neurite outgrowth in human MSCs in growth factors/differentiating agents-free conditions. We report here that laminin-1-induced neurite outgrowth and neuronal morphology in MSCs under serum-free conditions devoid of differentiation factors. We demonstrate for the first time that laminin-1-induced neurite outgrowth in human MSCs is mediated through α6β1 integrin and involves enhanced activation of FAK–MEK/ERK pathway.

Materials and methods

Materials. ECM proteins, poly-L-lysine and histopaque were purchased from Sigma–Aldrich (St. Louis, MO). Mouse monoclonal antibodies to CD markers were obtained from BD Biosciences (Bedford, MA). Antibodies to integrin subunits, nucleostemin, bmi1, and Cy3-conjugated secondary antibodies were procured from Millipore (Billerica, MA). Antibodies to MAP kinases and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA) and Bio-Rad (Hercules, CA), respectively. Oregon Green conjugated secondary antibodies, DMEM and neurobasal/B27 media were from Invitrogen (Carlsbad, CA). Inhibitors herbimycin-A, U0126, and PD98059 were purchased from Calbiochem (San Diego, CA).

Isolation and characterization of human mesenchymal stem cells (MSC) derived from human bone marrow. This study was evaluated and approved by Institutional Ethical Committees of NCCS and KEM hospital. Human bone marrow aspirates were obtained from KEM hospital and mesenchymal stem cells were isolated using Rosettesep mesenchymal stem cell enrichment cocktail (Stem Cell Technologies, Canada). MSCs were expanded in a complete culture medium (DMEM-low glucose, 10% fetal bovine serum) and characterized on the basis of CD markers. All experiments were done with primary cultures (8–17 passages) established from bone marrow aspirates of four donors within the age group of 13–35 years.

Adhesion assays. 96-well culture plates were coated with collagen-1, collagen-IV, fibronectin, laminin-1, laminin-10/11 at 20 μg/ml for 2 h at 37 °C and blocking was done with 5% bovine serum albumin (BSA) for 30 min. Twenty-four hour cultures of MSCs were dislodged using trypsin–EDTA, washed and resuspended in serum-free DMEM with 0.1% BSA. Five thousand cells were seeded per well in plates pre-coated with ECM proteins and incubated for 30 min at 37 °C. Bound cells were fixed with 3.7% paraformaldehyde and stained with 0.1% crystal violet. The dye was eluted with 1% SDS solution and absorbance measured at 595 nm. Wells coated with heat-inactivated 5% BSA were used as negative controls.

Preparation of substrates and neurite outgrowth assay. Culture plates were coated with ECM proteins at 2.5 μg/cm^2 and poly-L-lysine at 1 μg/cm^2 as per manufacturers' instructions. Cells were plated at 50% confluency in neurobasal/B27 medium on dishes pre-coated with ECM proteins or poly-L-lysine. After 24 h, cells were fixed with 3.7% paraformaldehyde, washed, and stained with 0.3% Coomassie brilliant blue prepared in 10% acetic acid and 25% isopropanol. Culture dishes were washed thoroughly to remove excess dye, and cells were visualized for morphological changes under inverted phase contrast microscope (Nikon, Japan) and photographed.

Western blotting. MSCs were harvested by scraping and whole cell extracts were prepared with ice-cold radioimmunoprecipitation assay (RIPA) buffer. Protein samples were resolved by 8–10% SDS–polyacrylamide gels, transblotted to PVDF membranes, blocked with 5% non-fat dried milk in Tris-buffered saline with 0.1% Tween-20 and probed with primary antibodies. The blots were incubated with HRP-conjugated secondary IgG and immunoreactive bands were detected using super signal femto chemiluminescent detection reagent (Pierce, Rockford, IL).

Immunofluorescence staining. Cells were grown on glass coverslips (Sigma) at 50% confluency for 24 h, fixed with 3.7% paraformaldehyde, and made permeable with 0.02% Triton X-100. Blocking was done with 10% goat serum. Cells were probed with primary antibodies, washed thoroughly, and incubated with secondary antibodies conjugated to Cy3. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) and images were captured using Nikon fluorescent microscope.

Flow cytometry for expression of integrins. Cells were disaggregated by trypsin–EDTA and resuspended to 10^6 cells/ml. Blocking was done with 5% BSA and cells were probed with primary antibodies against integrin subunits for 1 h at 4 °C. Cells were incubated with secondary antibodies conjugated to Oregon Green for 45 min, washed and fixed with 2% paraformaldehyde. Data was acquired for 10,000 cells using FACS Calibur and analyzed using Cell Quest-Pro software (BD Biosciences).

Statistical analysis. Student's *t*-test was done using SigmaStat software (version 3.5), values of p less than 0.05 were considered statistically significant.

Results

Characterization of MSCs

See Supplementary material online data (*Supplementary* Fig. S1).

Adhesion of MSCs to ECM proteins

In order to study cellular attachment to ECM proteins, a static 30 min cell adhesion assay was performed with fibronectin, collagen-1, collagen-IV, laminin-1, and laminin-10/11. As depicted in Fig. 1A, adhesion of MSCs to ECM proteins was in the order fibronectin > collagen-1 > collagen-IV > laminin-10/11 > laminin-1. Time course study of cell adhesion showed that most of the cells adhered during the first 60 min of seeding (Fig. 1B). Further increase in absorbance was seen up to 180 min, which might be attributed to the increased spreading and flattening of cell bodies.

Expression of integrins on MSCs

Cells adhere to ECM proteins via integrin receptors. Expression pattern of integrins has profound impact on physical, biochemical, and morphological properties of cells in different microenvironments [13,14]. As MSCs showed higher adhesion efficiency to ECM proteins such as fibronectin and collagen-1, they were analyzed for the expression of integrin subunits α1, α2, α3, α4, α5, α6, β1, and β4. Analysis by flow cytometry revealed that MSCs had higher expression of integrin α5β1 (fibronectin receptor) and integrin α1β1 (collagen-1 receptor) (*Supplementary* Fig. S2). Cells showed lower expression of integrin α3β1 (laminin-10/11 receptor) and integrin α6β1 (laminin-1 receptor). These observations revealed that the expression pattern of integrins in MSCs is directly correlated with the extent of cell adhesion to ECM proteins.

Laminin-1 induces neurite outgrowth in MSCs in serum/differentiation factors-free condition

In the time course studies of cell adhesion it was observed that MSCs seeded in serum-free medium showed sprouting of neurite-like extensions after 3 h of incubation on laminin-1. This was not observed in cells plated on other ECM proteins (*Supplementary* Fig. S3A). Further experiments were done to evaluate the potential of ECM proteins for induction and maintenance of neurite