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

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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.

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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.

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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

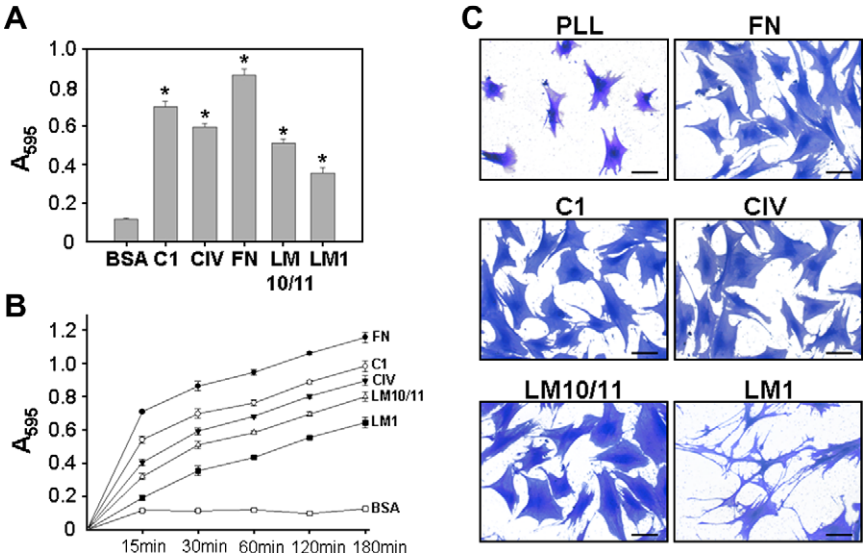

Fig. 1. Cell adhesion assays. (A) Static 30 min cell adhesion assay was performed with MSCs in culture plates pre-coated with ECM proteins collagen-1 (C1), collagen-IV (CIV), fibronectin (FN), laminin-1 (LM1), and laminin-10/11 (LM10/11). Heat-inactivated BSA coated wells served as controls. (B) Time course study of cell adhesion was carried out from 15 min to 180 min. The data is represented as mean ± SEM from three independent experiments done in triplicate. ∗ indicates $p < 0.05$ compared with BSA. (C) MSCs were cultured for 24 h on ECM protein-coated plates in serum-free medium devoid of growth factors/differentiating agents and monitored for neurite outgrowth. Cells were fixed and stained with 0.3% Coomassie brilliant blue. Plates coated with poly-L-lysine were used as controls. Scale bar: 100 μm.

outgrowth. As shown in Fig. 1C, after 24 h of incubation on laminin-1, MSCs showed prominent neurite outgrowth with 32% of the cells showing long cytoplasmic extensions. Cellular processes longer than cell body diameters were scored as neurites and the average length of neurite was found to be 186 μm. Many of the neurite bearing cells showed typical neuronal morphology. In contrast, neurite outgrowth was not observed in cells seeded on poly-L-lysine, collagen-1, collagen-IV, fibronectin, and laminin-10/11. The cells plated on these ECM proteins exhibited flattened, fibroblastic morphology resembling MSCs cultured in complete culture medium. Neurite formation on laminin-1 was blocked by function-blocking antibodies against integrin subunits α6 and β1 (20 μg/ml) which confirmed the involvement of integrin α6β1 in neurite outgrowth (*Supplementary* Fig. S3B). These findings led us to conclude that while all ECM proteins were able to support MSCs adhesion, interaction with laminin-1 only was effective in promoting neurite outgrowth in MSCs in growth factors/differentiating agents-free conditions.

Activation of FAK, MEK, and ERK is essential for laminin-1- induced neurite outgrowth

ECM proteins activate integrin signaling pathways resulting in tyrosine phosphorylation of FAK and its associated molecules such as paxillin, tensin, and Src [15]. FAK is a non-receptor cytoplasmic protein tyrosine kinase which becomes autophosphorylated at Tyr 397 site upon attachment of cells to ECM proteins [16]. To examine the possible role of FAK in the regulation of neurite outgrowth, MSCs plated on laminin-1 were lysed and probed for FAK phosphorylation at Tyr 397 site by Western blotting. FAK Tyr 397 phosphorylation was prominently detected in cells plated on laminin-1 but not in cells plated on poly-L-lysine or in cells held in suspension (Fig. 2A). FAK signaling pathway can activate mitogen-activated protein kinase (MAPK) cascades for eliciting specific biological responses [7]. The role of MAPK signaling pathways in ECM-stimulated neurite outgrowth has been well documented [17,18]. We therefore examined the activation of three major MAPK pathways: the MEK/ERK, JNK/SAPK, and the p38 kinase pathways in cells plated on laminin-1. We found that cells seeded on laminin-1 had elevated levels of phosphorylated-MEK (p-MEK) and phosphorylated-ERK (p-ERK) compared to cells plated on poly-L-lysine or cells held in suspension (Fig. 3B). Interestingly, there was no significant difference in the levels of phosphorylated-JNK (p-JNK) in cells plated on laminin-1 and poly-L-lysine. Phosphorylated p38 was not detected in cells plated on laminin-1 or on poly-L-lysine (data not shown).

Treatment of cells plated on laminin-1 with1 μM and 5 μM herbimycin-A, resulted in reduction of levels of p-FAK (Y397) by 61% and 78%, respectively. Interestingly, abrogation of FAK phosphorylation by herbimycin-A decreased levels of p-ERK (Fig. 2A) and p-MEK (Fig. 2B) in cells plated on laminin-1. In cells cultured on poly-L-lysine, both phosphorylated as well as total forms of FAK, MEK, and ERK were reduced significantly. This is quite understandable as internalization and recycling of receptors, required for the maintenance of signaling proteins, does not occur on poly-L-lysine in the absence of serum and differentiation factors [19].

Further studies were directed to evaluate the functional roles of FAK, MEK, and ERK in neurite outgrowth. For this purpose, inhibitors to these kinases were added to cells plated on laminin-1 and monitored for 24 h. Herbimycin-A (1 μM) (Fig. 2C), U0126, and PD98059 (40 μM) (Fig. 3A) inhibited neurite outgrowth on laminin-1 and the cells retained a fibroblastic morphology. Collectively, these findings suggest that neurite outgrowth and neuronal morphology induced in MSCs by laminin-1 involves enhanced activation of FAK and MEK/ERK signaling pathways. These data also demonstrate that activation of MEK/ERK signaling pathways is dependent on tyrosine phosphorylation of FAK and its associated molecules.

Levels of phosphorylated-FAK, -MEK, and -ERK were higher on laminin-1 compared to other ECM proteins

Since FAK, MEK, and ERK were found to be involved in neurite outgrowth on laminin-1, it was of interest to examine the effect of other ECM proteins on the activities of these kinases. As depicted in Fig. 4, levels of p-FAK (Y397), p-MEK, and p-ERK were

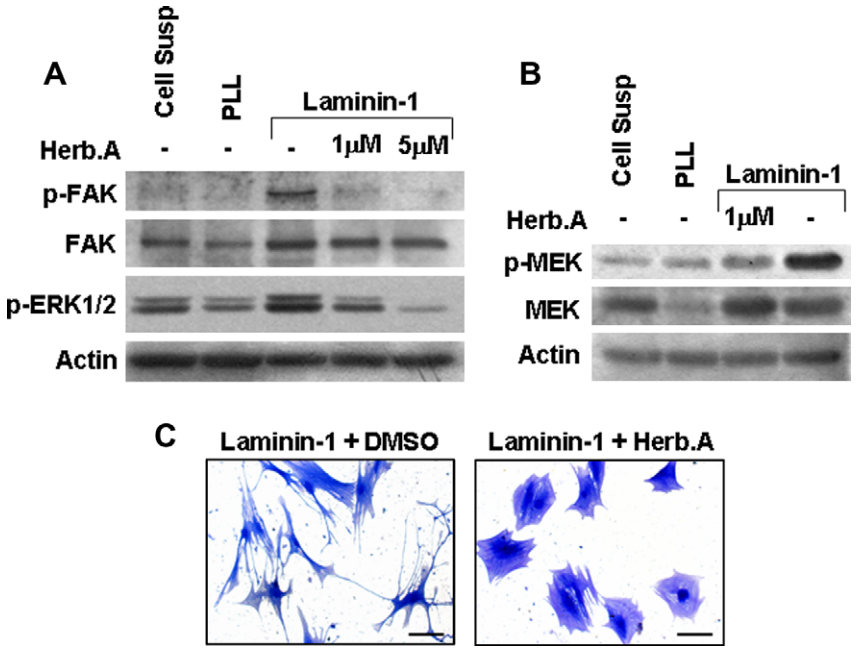

Fig. 2. Adhesion to laminin-1 activated FAK and MEK/ERK signaling pathways. (A) Cell lysates were prepared from MSCs held in suspension or plated on laminin-1 or poly-L-lysine (PLL) for 1 h. Samples were analyzed by SDS–PAGE and immunoblotted with anti-phosphorylated-FAK Tyr 397 and total FAK antibodies. Herbimycin-A inhibited activation of FAK significantly and also reduced levels of p-ERK. (B) MEK activity was determined in cells plated on laminin-1 for 1 h in the presence or absence of herbimycin-A. β-Actin served as loading control. (C) Neurite outgrowth and morphology of cells plated on laminin-1 was assessed in the presence of herbimycin-A (1 μM). Cells treated with DMSO served as vehicle control. 0.3% Coomassie brilliant blue was used to stain the cells. Scale bar: 100 μm.

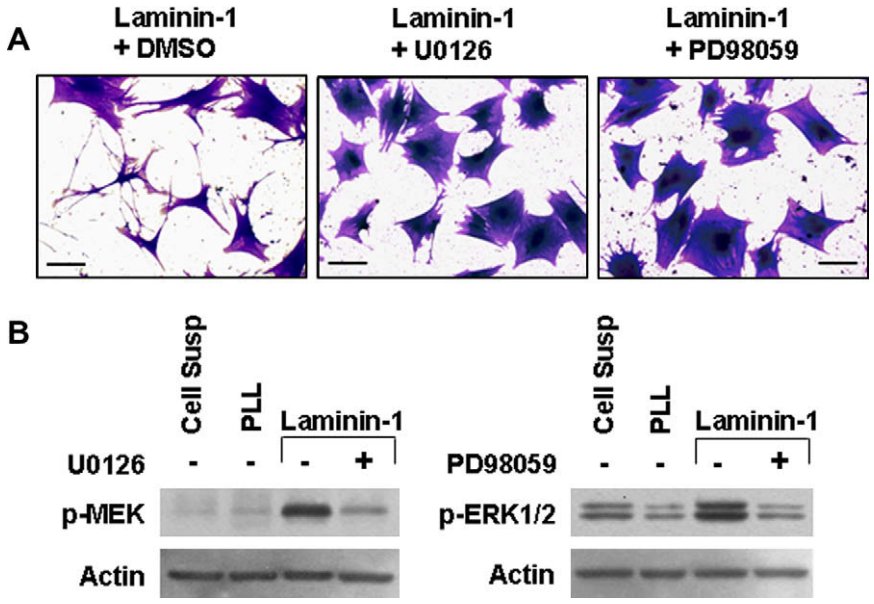

Fig. 3. Inhibitors of MEK and ERK inhibited laminin-1-induced neurite outgrowth. (A) MSCs were cultured for 24 h on laminin-1 in the presence or absence of 40 μM of U0126 or PD98059. DMSO was used as vehicle control. Scale bar: 100 μm. (B) Cells were seeded on laminin-1 for 1 h in the presence or absence of U0126 or PD98059 (40 μM) and levels of phosphorylated-MEK and -ERK were determined by immunoblotting. β-Actin served as loading control.

significantly higher in cells plated on laminin-1 compared to cells plated on collagen-1, collagen-IV, fibronectin, and laminin-10/11.

Discussion

The molecular mechanisms underlying ECM-induced-neurite outgrowth in differentiation factors-free conditions have not been reported in human MSCs. Our results suggest that integrin α6β1–laminin-1 interactions and enhanced activation of FAK–MEK/ERK signaling pathways were essential for the induction of neurite outgrowth in human MSCs. Preliminary findings showed that fibronectin and collagen-1 promoted adhesion of cells better than other ECM proteins. Consistent with an earlier report [20], our findings revealed that MSCs expressed α5, α1, and β1 integrin subunits abundantly and low levels of α2, α3, α4, α6, and β4 subunits. Higher expression of α5β1 and α1β1 integrins may explain higher adhesion efficiency of MSCs to fibronectin and collagen-1.

Neurite outgrowth is regarded as a consequence of differentiation of precursor cells to a mature neuronal phenotype [21]. Laminin-1 but not other ECM proteins induced formation of neurites in

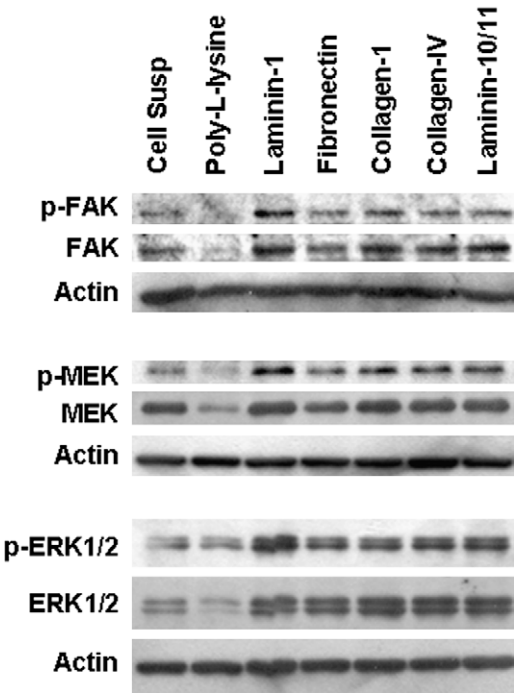

Fig. 4. Comparison of phosphorylated-FAK, -MEK, and -ERK levels on ECM proteins. MSCs were plated on different ECM proteins for 1 h and the levels of phosphorylated-FAK, -MEK, and -ERK were measured by immunoblotting. β-Actin served as loading control.

MSCs in the absence of growth factors/differentiating agents. Laminin-1 has a neurite outgrowth-promoting site in its E8 fragment. E8 fragment of laminin-1 comprises of LG1–3 module of the α1 chain and the adjacent rod domain. Integrin α6β1, the sole laminin-1 receptor in many cell types, has been implicated in neurite outgrowth on laminin-1 [22]. Function-blocking antibodies directed against α6 and β1 integrin subunits blocked neurite outgrowth in MSCs on laminin-1. This was not unexpected as the E8 fragment of laminin-1 has been shown to interact with integrin α6β1 for functioning in cell attachment and induction of neurite outgrowth [23]. Laminin-1, in combination with various growth factors and differentiating agents, has been extensively used for induction of neural differentiation and promotion of neuritic regeneration [24,25]. Recently it has been reported that laminin-1 promoted neurite outgrowth in adult mouse DRG neurons in the absence of neurotrophins [26]. However, there are no reports on induction of neurite outgrowth by laminin-1 in MSCs in the absence of growth factors/differentiating agents. Our findings demonstrated efficient induction of neurite outgrowth by laminin-1 in human MSCs in differentiating agents-free conditions.

Laminin-10/11, the most abundant laminin isoform in the adult stage [27], has been shown to promote neurite outgrowth in ciliary ganglion neurons. Specific activity of laminin-10/11 in promoting neurite outgrowth was one-half of that of laminin-1 [28]. However, in our study, laminin-10/11 failed to induce neurite outgrowth in human MSCs. Also, other ECM proteins, specifically fibronectin, collagen-1, and collagen-IV, did not promote neurite outgrowth in MSCs. These findings are in accordance with earlier studies reporting that laminin-1 is a more potent inducer of neurite outgrowth compared to fibronectin and collagens [24,26].

The role of integrin signaling in ECM-stimulated neurite outgrowth is well documented. A key event during integrin signaling is FAK activation. FAK signaling pathways may activate MAPK cascades for eliciting specific cellular responses [7]. The three MAP kinase pathways—MEK/ERK, JNK, and p38 kinase pathways, have been implicated in regulation of neurite outgrowth on laminin-1 in different systems [17,29,30]. In our experimental system, cells plated on laminin-1 showed enhanced activation of FAK, MEK, and ERK. Phosphorylated p38 was not detected, and JNK activity was not significantly different in cells seeded on laminin-1 and poly-L-lysine suggesting that activity of these kinases was not obligatory for neurite formation on laminin-1.

Further studies revealed that 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 on laminin-1. Collectively, our data provide evidence for the role of FAK–MEK/ERK signaling pathway in laminin-1-induced neurite outgrowth in MSC in the absence of exogenous growth factors/differentiating agents. These results prompted us to investigate the effect of other ECM proteins on activities of these kinases. Levels of p-FAK (Y397), p-MEK, and p-ERK, stimulated by collagen-1, collagen-IV, fibronectin, and laminin-10/11, were found to be higher than levels stimulated by poly-L-lysine, but were lower compared to levels induced by laminin-1. Magnitude of ERK activation has been reported to govern the nature of the cellular responses such as neurite outgrowth [31]. Thus high intensity and maximum amplitude of MEK/ERK signaling, observed in cells plated on laminin-1, is indispensable for the induction of neurite outgrowth in MSCs. In addition, our studies with inhibitors showed that the concentrations that blocked neurite outgrowth effectively, did not inhibit MAP kinases activation completely. Thus the residual levels of MAP kinases do not induce neurite formation. Therefore it may be speculated that activation of MAP kinases beyond certain thresholds elicits cellular responses such as neurite outgrowth in human MSCs.

Conclusion

To our knowledge, this is the first study to provide evidence that laminin-1 induces neurite outgrowth in human MSCs through activation of FAK–MEK/ERK signaling pathways in conditions devoid of serum and differentiation factors. Hence, direct interaction with laminin-1 can be explored further for induction of neuronal differentiation in growth factors/differentiating agents-free conditions.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.10.158.

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