Adhesion of Human Mesenchymal Stem Cells and Differentiation of SH-SY5Y Cells on Amyloid Fibrils

Reeba S. Jacob,* Shamik Sen, Samir K. Maji*

Summary: Amyloids are protein/peptide aggregates traditionally associated with many human disorders. However recent evidences suggest that amyloids are also capable of performing various native biological functions in host organisms. Many lower organisms including bacteria and algae use the highly ordered and adhesive property of amyloids for adhesion to host/substrates and/or for colonization, thus suggesting amyloids could serve as extracellular matrices (ECMs). Recently, we had reported that mammalian cells can adhere on amyloids using membrane-fibril interactions and integrin-based focal adhesion. In the current study, we demonstrate the nanotopography of amyloid fibrils for adhesion of human mesenchymal stem cells (hMSCs). Further, we also investigate the amyloid surface for differentiation of neuroblastoma cells SH-SY5Y. Our present study delineates the role of amyloid self-assembly in cell adhesion and differentiation.

Keywords: amyloid; cell adhesion; differentiation; extracellular matrix; stem cells

Introduction

Amyloids are protein aggregates and are considered as an ancient protein fold associated with disease as well as native functions in host organisms. Amyloids are fibrillar in nature and their ultra-structure is composed of cross-β-sheet motifs giving them unique surface properties with combination of sequence-dependant hydrophilic and hydrophobic surfaces. Though initially associated with diseases, recent evidences suggest that the existence of amyloid structure in many organisms with functional roles. Examples of such functional amyloids include curl amyloids in E.coli, which help in mediating surface adhesion and Orb2 protein in Drosophila, which helps in memory and learning. Recently, protein/peptide hormones have also been reported to be stored in amyloid like state in secretory granules of mammals. Due to their superior material properties, many recent studies have probed the utility of non-toxic amyloids as biomaterials for nanotechnology as well as tissue engineering applications.

Previous studies have shown that amyloid fibrils alone and also tagged with functional moieties from ECM proteins such as fibronectin can be used as substrates for cell adhesion and proliferation. In this regard, Yan et al also have shown that fibroblasts can adhere and proliferate on amyloid hydrogels without the use of any cell recognition peptide or ECM protein coating. Recently nanocomposites containing amyloid fibrils have also been developed for cell adhesion, where amyloid fibrils along with hydroxyapatite composite were used as a replacements for collagen in scaffolds synthesized for bone tissue regeneration. Moreover, studies to understand cell adhesion on amyloids revealed that the nanotopography of amyloids could support cell adhesion with amyloids showing density-dependent cell adhesion responses similar to ECM proteins. In a recent study,
we tested the mechanism of cell adhesion on amyloid fibrils from various protein/peptides. The study demonstrated that mammalian cells were capable of adhering and spreading on a wide range of amyloids fibrils including amyloids fibrils of α-synuclein (α-Syn) and Aβ42 associated with Parkinson’s and Alzheimer’s disease, respectively.\[22\] Interestingly, we found enhanced integrin signaling in cells adhered on amyloids compared to that on collagen (native ECM protein).\[22\] The study thus suggests that cell adhesivity might be a generic property of amyloid fibrils and adhesion could be mediated by a combination of cell membrane-fibril interactions as well as integrin mediated focal adhesion complex formation.\[22\] In the current study, we demonstrate that amyloid fibrils can also support cell adhesion of hMSCs similar to other cell lines and the fibrillar nature of amyloid fibrils can support differentiation of SH-SY5Y cells to neurons.

**Results and Discussion**

**Amyloid Formation by Kassinin and Substance P**
The amyloid fibrils of kassinin and Sub P were prepared by incubating 2 mg/ml solution of kassinin and Substance P (Sub P) at 37°C, and the amyloid formation was monitored using circular dichroism (CD) spectroscopy and Thioflavin T (ThT) binding assay. CD spectra of both kassinin and Sub P alone showed unstructured states immediately after dissolution (Figure 1A and B). Previous studies reported that while kassinin alone incubated for 15 days forms amyloid, Sub P forms amyloid in the presence of heparin.\[23\] To prepare Sub P amyloid, 1500 μM

![Figure 1](image)

**Figure 1.**
Amyloid formation by kassinin and Sub P peptides. A) CD spectra of kassinin alone at day 0 and day 15. B) CD spectra of Sub P in absence and presence of heparin at day 0 and 15. C) ThT fluorescence of kassinin at day 0 and 15 showing weak fluorescence signal. D) and E) ThT fluorescence of Sub P in absence and presence of heparin. In presence of heparin, Sub P shows increased ThT binding indicating amyloid formation. F) Results of MTT assay showing cytocompatibility of kassinin and Sub P amyloid fibrils.

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim  www.ms-journal.de
of Sub P peptide solution was incubated in the presence of 400 μM heparin. The CD spectra of the resultant solution showed two minima, one at ~206 nm and another at ~226 nm at day 0 (Figure 1B). After 15 days of incubation, the CD spectra of kassinin alone solution showed a structural transition with a single minima (Figure 1A). As for Sub P, while the CD spectra of Sub P in absence of heparin remained in random coil structure, in the presence of heparin after 15 days of incubation, Sub P exhibited increased helicity with two minima at ~206 nm and ~226 nm similar to that of day 0 (Figure 1B). To check for amyloid formation, ThT dye binding study was performed. Amyloid fibrils after binding to ThT show a characteristics ThT fluorescence emission peak at 480 nm, when excited at 450 nm.[24] Intriguingly, ThT fluorescence signals remained weak in the kassinin alone samples both at day 0 and at day 15 (Figure 1C). However, Sub P incubated in the presence of heparin showed significant ThT binding at day 0, which further decreased after 15 days of incubation (Figure 1E). The decrease in ThT fluorescence upon incubation of Sub P in presence of heparin could be because of inaccessibility of binding sites due to the lateral association of amyloid fibrils. The precipitation of peptide aggregates could be another reason for lowering the ThT fluorescence. Sub P incubated alone did not show any β-sheet signal in CD or ThT binding (Figure 1B and D). To further check the morphology of incubated samples, the morphology of 2-week-old kassinin sample and Sub P in presence of heparin was visualized using electron microscopy (EM). The EM analysis showed the formation of thin elongated fibrils after 2 weeks of incubation in both kassinin alone sample as well as in Sub P incubated with heparin (Figure 2A). Finally, the toxicity of kassinin and Sub P fibrils was evaluated using MTT assay, which showed that these fibrils are mostly non toxic (Figure 1F) and hence suitable for cell adhesion studies.

Adhesion of Stem Cells on Amyloid Fibrils

Given the attachment and spreading response of neuronal and other cell lines on amyloid fibrils,[22] we hypothesized that amyloid fibrils might also be conducive for culturing mesenchymal stem cells (MSCs). To test this, we took amyloid fibrils formed by two tachykinin peptides, kassinin and its human counterpart Sub P (Figure 2A). Equal number of hMSCs was cultured on coverslips coated with amyloid fibrils at identical coating density. Collagen, a native ECM protein was used as control. The spreading response of hMSCs was studied on collagen, kassinin and Sub P fibrils after 24 hours in culture (Figure 2B). When quantified the cell spreading area using Image J software, the data showed that hMSCs exhibited enhanced spreading on kassinin fibrils compared to collagen (Figure 2C). However, hMSCs on Sub P fibrils showed lesser spreading area than on collagen. Although, we are not certain at this point about the reason(s) behind the difference in cell spreading area on individual amyloid fibrils. However, we hypothesize that it could be due to the differences in fibril stiffness and/or in nanotopography of individual amyloid fibrils dictated by its amino acid sequence. Additionally, the morphology of the adhered cells was quantified by measuring circularity of cells in each condition. The study showed that compared to amyloid fibrils, cell spreading was more anisotropic (i.e., cells were more elongated) on collagen (Figure 2D).

Further, characterization of cytoskeletal organization and focal adhesions of hMSCs on different substrates was performed to compare the cell adhesion on collagen coated substrates and amyloid fibrils (Figure 3). The data showed well developed stress fibers across all the conditions (Figure 3A). When focal adhesion complex formation was studied using immunofluorescence using focal adhesion kinase (FAK) antibody, the data showed that the size distribution of focal adhesions was markedly different on collagen and
Figure 2.
Adhesion and spreading of hMSCs on amyloid surfaces. A) Representative TEM image of collagen and amyloid fibrils, scale bars are 200 nm. B) Phase contrast image of hMSCs adhered on collagen and amyloid fibrils; scale bar is 100 μm. C) and D) Quantitative analysis of cell spreading area and circularity of hMSCs on different substrates. Statistical significance: **p < 0.05.

Figure 3.
Spreading and cytoarchitecture of stem cells on collagen-coated and kassinin fibril-coated substrates. A) Actin organization (green) and focal adhesion formation (red) in hMSCs cultured on different substrates. Scale bar = 20 μm. B) Quantitative analysis of size and number of FAK-positive adhesions. Cells on amyloid fibrils displayed stronger adhesions compared to those on collagen.
amyloid fibrils. Specifically, greater number and larger focal adhesions were observed on both amyloid substrates compared to that on collagen (Figure 3B). Interestingly though, the spreading area of hMSCs was less on the Sub P fibrils, more number of larger sized focal adhesion complexes were found in these cells, which indicates strong cell adhesion on Sub P fibrils compared to collagen. This result is similar to our previous study where we found enhanced formation of focal adhesion complexes in SH-SY5Y cells cultured on amyloid fibrils.\[22\] Previous reports suggest that the hydrophobic nature of amyloids facilities deposition of serum proteins on the fibril surface, which can contribute to enhanced cell adhesion on amyloid fibrils.\[20,21\] Together these results suggest that though the spread area and morphology of hMSCs adhering on amyloid fibrils may vary depending on surface topography of amyloids, the outside-in signaling received by cells on amyloid surface might be similar. This is indicated by the increased FAK expression of cells adhered on amyloids and further suggests the possibility of similar cell-specific recognition of amyloid features by different cell types.

**Amyloids as Scaffolds for Promoting SH-SY5Y Differentiation**

Previous studies indicate that the nano- and microscale architecture of ECM fibrils manipulate cell polarity and promote directional migration by providing contact guidance cues.\[25–27\] For example, neurons in the peripheral nervous system were found to polarize along nanogrooves fabricated specifically to mimic the nanotopographic features of neurite bundles.\[28\] Nanotopography could be also a useful tool for guiding differentiation, as the features are more durable and can be modified to suit the desired application.\[29\] In line with these findings, we hypothesized that nanotopography of amyloid fibrils may support neurogenesis in neuroblastoma cells. To test this, we used human neuroblastoma SH-SY5Y cell line, which is used as a model cell line for understanding neurogenesis.\[30,31\] SH-SY5Y cells can be maintained in undifferentiated state and can be differentiated to mature neurons when treated with all trans-retinoic acid (RA).\[32,33\] To study the efficiency of amyloid fibrils in promoting neuronal differentiation, SH-SY5Y cells were cultured on collagen and kassinin amyloid fibrils. These cells were then treated with 10 µM RA for 48 hours, then fixed and stained with βIII tubulin, a neuron specific marker, which can detect the newly formed neurites.\[34\] Neurite outgrowth is considered as a marker of neuronal differentiation.\[35,36\] To measure this, we quantified the neurite length of SH-SY5Y cells differentiated on both collagen and kassinin amyloid fibrils. Our results showed that while on both collagen and kassinin amyloid fibril surfaces, SH-SY5Y cells were able to differentiate (Figure 4A), the cells differentiated on kassinin amyloid fibrils were more elongated in nature and possessed longer neurites (Figure 4B and C). The difference in increased neurite length of differentiated SH-SY5Y cells on kassinin fibrils could be due to the following factors: 1) the surface topography of amyloid fibrils supports neuronal differentiation of SH-SY5Y cells; 2) RA, immediately upon addition could get deposited on to the surface of amyloid fibrils, which may increase the local concentration of RA, thus making this differentiating factor more available to the cells. 3) Another possibility is that a combination of both nanotopography of amyloid fibril as well as the presence of the chemical cue (RA) could have favorably enhanced the differentiation process of SH-SY5Y cells. These results are in line with our recent work where we demonstrated that amyloid based hydrogels could drive neuronal differentiation of hMSCs even without the addition of any external chemical cue.\[37\] However addition of the chemical cue (RA) entrapped in amyloid based hydrogels drives the differentiation of hMSC to a different neuronal lineage.\[38\] Together, these studies suggest that while amyloids fibrils support neuronal
differentiation (probably by providing contact guidance) addition of external chemical cue could promote differentiation to a specific cell type.

**Conclusion**

In conclusion, our study suggests that hMSCs can attach and spread on amyloid fibril surfaces through integrin mediated activation of cell adhesion machinery. Moreover, the surface topography of amyloids can promote differentiation of SH-SY5Y cells to neurons. Based on these results, we propose that amyloids, in the course of evolution, may have served as templates for cell/tissue organization and also may have had significant functional roles in cells. Thus our studies demonstrate that amyloids have not only evolved for creating various diseases, but can also perform biological functions in the host organism. In line with these findings, proteins/peptide could be used as building blocks to construct amyloid based substrates that are cell adhesive without the use of any cell adhesive moiety and could signify a novel class of cell scaffolds that could have an important role in the biomaterial and tissue engineering sector.

**Experimental Section**

**Chemical and Reagents**

All chemicals and reagents for the experiments were purchased from Sigma. Deionized water was obtained from a Milli-Q system (Millipore Corp., Bedford, MA). The peptide hormones kassinin and Sub P were purchased from BACHEM (Switzerland).
Amyloid Formation by Kassinin and Sub P

The detailed description for preparation of kassinin and Sub P amyloid fibrils is already published elsewhere. Briefly, lyophilized powders of each peptide was dissolved in 0.5 ml of 5% D-Mannitol, 0.01% sodium azide, pH 5.5 at a concentration of 2 mg/ml in 1.5 ml eppendorf tubes and were placed into an EchoTherm model RT11 rotating mixture (Torrey Pines Scientific, USA) at 50 rpm and incubated inside a 37°C incubator for 15 days. To induce Sub P amyloid formation, 2 mg/ml peptide solution of Sub P was incubated in presence of 400 mM LMW heparin (5 kDa heparin, Thermo Fisher Scientific) at 37°C. CD, ThT binding assay were performed at regular intervals to monitor the aggregation and after 15 days, EM was performed using incubated peptide solutions to confirm the fibril formation. The biocompatibility of the amyloid fibrils was tested using MTT assay. Finally, the amyloid fibrils of kassinin and Sub P were coated on glass coverslip as reported previously.

Adhesion of hMSC on Amyloid Fibrils

For studying the attachment of stem cells on amyloid fibril surface, bone marrow derived human mesenchymal stem cells (hMSCs) that were purchased from Stempeutics, India and were maintained in KnockOut DMEM (GIBCO) with 10% FBS and 2 mM Glutamax (GIBCO) and 0.25% pencillin and streptomycin. The hMSCs were cultured and maintained at 37°C in a 5% CO₂ incubator. The cell monolayers were then trypsinized and pelleted by centrifugation at 1500 rpm for 3 mins. These cells were then plated at a density of 1 x 10⁴ on kassinin and collagen coated glass coverslips. After 24 hrs of plating the cells, the medium was removed and the cells were treated with DMEM with 10 µM RA and incubated for 48 hrs at 37°C. Afterwards the cells were imaged and fixed with 4% PFA for further analysis. These cells were immunostained with β III tubulin using the protocol described previously and the neurite extension was calculated using image J software (NIH, Version 1.47).

Statistical Analysis

The statistical significance was either determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison post hoc test, **P value for each plot is mentioned in the corresponding legend.

Acknowledgements: Authors wish to acknowledge CRNTS and IRCC (IIT Bombay) for electron microscopy. Authors also wish to acknowledge DBT (BT/PR9797/NNT/28/774/2014), Government of India for financial support.

2016, 291, 5278.
64, 29.
2015, 5, 9228.