Biomaterials xxx (2013) 1–10

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Neuroglial differentiation of adult enteric neuronal progenitor cells as a function of extracellular matrix composition

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ARTICLE INFO

Article history:
Received 26 March 2013
Accepted 11 May 2013
Available online xxx

Keywords:
Neural stem cell
Neural tissue engineering
Extracellular matrix
Adult stem cell
Enteric glia

ABSTRACT

Enteric neuronal progenitor cells are neural crest-derived stem cells that can be isolated from fetal, postnatal and adult gut. Neural stem cell transplantation is an emerging therapeutic paradigm to replace dysfunctional or lost enteric neurons in several aganglionic disorders of the GI tract. The impetus to identify an appropriate microenvironment for enteric neuronal progenitor cells derives from the need to improve survival and phenotypic stability following implantation. Extracellular matrix composition can modulate stem cell fate and direct differentiation. Adult mammalian myenteric ganglia *in vivo* are surrounded by a matrix composed primarily of Collagen IV, Laminin and a Heparan sulfate proteoglycan. In these studies, adult mammalian enteric neuronal progenitor cells isolated from full thickness rabbit intestines were induced to differentiate when cultured on various combinations of neural ECM substrates. Neuronal and glial differentiation was studied as a function of ECM composition on coated glass coverslips. Poly-lysine coated coverslips (control) supported extensive glial differentiation but very minimal neuronal differentiation. Individual culture substrata (Laminin, Collagen I and Collagen IV) were conducive for both neuronal and glial differentiation. The addition of laminin or heparan sulfate to collagen substrates improved neuronal differentiation, significantly increased neurite lengths, branching and initiation of neuronal network formation. Glial differentiation was extensive on control poly lysine coated coverslips. Addition of laminin or heparan sulfate to composite collagen substrates significantly reduced glial immunofluorescence. Various neural ECM components were evaluated individually and in combination to study their effect of neuroglial differentiation of adult enteric neuronal progenitor cells. Our results indicate that specific ECM substrates that include type IV Collagen, laminin and heparan sulfate support and maintain neuronal and glial differentiation to different extents. Here, we identify a matrix composition optimized to tissue engineer transplantable innervated GI smooth muscle constructs to remedy aganglionic disorders.

1. Introduction

Gastrointestinal motor function is intimately controlled by the intramural enteric nervous system. It is a complex interplay between the smooth muscle of the muscularis externa and the two enteric neuronal plexi [1]. Aganglionosis of varying lengths of distal gut is the central pathology in Hirschsprung's disease [2]. Enteric neuropathy is also secondary to several other disorders (diabetes, Parkinson's disease, inflammation) resulting in gastrointestinal dysfunction [3,4]. Neural stem cell therapy is an emerging therapeutic paradigm that ideally aims to reinstate neuronal function and thus gastrointestinal motor function by repopulating the enteric plexi. The research is driven by two significant findings: i) neuroglial progenitor cells can be isolated from adult mammalian gut, including ganglionated colon of Hirschsprung's patients [5,6]; and ii) progenitor cells can be induced to differentiate into several neuronal subtypes and glia characteristic of the ENS upon transplantation into explant cultures of aganglionic/aneural gut [5–9], or *in vivo* into distal colo-rectums of adult rodents [10,11]. However, phenotypic stability, long-term survival, and post-transplant fate all remain to be optimized while moving forward with neural stem cell transplantation for clinical use [12,13]. In order to provide trophic support and a permissive microenvironment, a more fundamental understanding of factors that affect and maintain differentiation of enteric neuronal progenitor cells is required. The studies described here focus on in vitro differentiation of adult mammalian enteric neuronal progenitor cells, particularly related

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0142-9612/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.biomaterials.2013.05.023

to the effect of varying extracellular matrix composition of culture substrata.

The extracellular matrix (ECM) plays an enormous role in dictating stem cell fate. ECM composition, structure and mechanical properties can all modulate progenitor cell differentiation [14,15]. The adult mammalian myenteric plexus is surrounded by an extracellular matrix primarily composed of Collagen IV, Laminin and a heparan sulfate proteoglycan, with enteric glia always in direct contact with the ECM. Enteric neurons also come in direct contact with this ECM, though much less frequently than glia [16,17]. Laminin, fibronectin and proteoglycans are expressed within the embryonic gut to aid its colonization by vagal neural crest cells. Collagen IV is distributed in the developing nervous system along the neural crest. Additionally, laminin is implicated in both the central and peripheral nervous system in promoting neural cell adhesion and axonal outgrowth [18]. Heparan sulfate is required for GDNF signaling in the gut, and has been known to stabilize and influence neuronal differentiation in vitro [19–21].

In this paper, we describe the effect of components of neural ECM on the differentiation of gut-derived neuronal progenitor cells of neural-crest lineage in vitro. Two timepoints were defined to identify early and late differentiation events – day 5 (early) and day 15 (late) based on previous experiments [11]. Immunohistochemistry for βIII Tubulin (neuron specific microtubule) and GFAP (Glial fibrillary acidic protein) was used to identify differentiated neurons and glia on coated culture substrata. The principal objective of this study was to identify the effect of extracellular matrix composition on the differentiation of adult enteric neuronal progenitor cells in vitro.

2. Materials and methods

2.1. Reagents

All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless specified otherwise. Primary and fluorophore conjugated secondary antibodies were purchased from Abcam (Cambridge, MA). Rat tail type I Collagen and natural mouse type IV Collagen were purchased from BD Biosciences (Bedford, MA) and Laminin was from Invitrogen (Carlsbad, CA). Heparan sulfate was purchased from Celsus Labs (Cincinnati, OH).

2.2. Isolation and culture of rabbit enteric neuronal progenitor cells and rabbit intestinal smooth muscle cells

New Zealand white rabbits were euthanized using ketamine/xylazine. Intestinal smooth muscle cells were isolated and cultured using previously described protocols [22]. For the isolation of enteric neuronal progenitor cells, 5 cm^2 biopsies were dissected from the jejunum, and retrieved in Hank's Buffered Salt Solution (HBSS) with 2X antibiotics/antimycotics and 1X gentamicin sulfate. Luminal content was cleaned and tissues were washed extensively with HBSS. Enteric neuronal progenitor cells were isolated from these tissues using a collagenase/dispase digestion method, reported by Almond et al. [6]. Cells were plated on to bacterial petri dishes in neuronal growth media (Neurobasal + 1X N2 supplement + 1X antibiotics) following filtration through a 40 μm mesh.

2.3. Immunohistochemical characterization of rabbit enteric neurospheres

In order to characterize the initial phenotype of rabbit enteric neurospheres in culture, neurospheres were harvested by centrifugation at 1000 g for 10 min in microfuge tubes. The growth media was gently aspirated, and neurospheres were fixed with 3.7% neutral buffered formaldehyde and blocked with 10% horse serum. Primary antibodies for p75 (Millipore, Billerica MA), Sox2 and Nestin were incubated for 30 min at room temperature. Unbound antibody was washed using phosphate buffered saline (PBS), and appropriate fluorophore-conjugated secondary antibodies were incubated for an additional 30 min. Neurospheres were mounted using Prolong Gold antifade mounting medium (Invitrogen, Carlsbad CA), and visualized using an inverted Nikon TiE fluorescent microscope.

2.4. Differentiation of rabbit enteric neurosphere differentiation as a function of extracellular matrix composition

22 × 11 mm coverslips were washed in Neutrad (Decon Labs, King of Prussia PA) and rinsed extensively in deionized water. Coverslips were sterilized by 70% ethanol, and subsequent UV exposure for 45 min. Coverslips were coated with poly-L-lysine (pLL; 1 mg/ml), pLL + 10 μg/cm^2 type I Collagen, pLL + 10 μg/cm^2 type IV Collagen or pLL + 10 μg/cm^2 Laminin. Composite coatings included:

i) 5 μg/cm^2 Collagen I + 5 μg/cm^2 type IV Collagen;
ii) 5 μg/cm^2 Collagen I + 5 or 10 μg/cm^2 Laminin;
iii) 5 μg/cm^2 Collagen IV + 5 or 10 μg/cm^2 Laminin;
iv) 5 μg/cm^2 Collagen I + 5 μg/cm^2 Collagen IV + 0.1 μg/cm^2 Heparan Sulfate (HS);
v) 5 μg/cm^2 Collagen I + 5 μg/cm^2 Collagen IV + 5 μg/cm^2 Laminin + 0.1 μg/cm^2 HS.

Uncoated glass coverslips were seeded with rabbit colonic smooth muscle cells, and allowed to reach confluence. Rabbit enteric neurospheres were harvested and treated with Accutase to obtain a mixture of single cells as well as small neurospheres. 10,000 neuronal progenitor cells were harvested and plated on to coated coverslips. To stimulate differentiation induced via soluble smooth muscle factors, each plate was shared by one confluent smooth muscle coverslip along with a coated coverslip containing adhered neurospheres. Enteric neurospheres were allowed to differentiate for a period of fifteen days, with a supplementation of neuronal differentiation medium every 2 days (Neurobasal-A medium + 1X B27 supplement + 2% fetal calf serum + 1X antibiotics).

2.5. Immunohistochemical analysis of neuronal and glial differentiation of rabbit enteric neurospheres on coated coverslips

Neuronal and glial differentiation was analyzed at two time points – Day 5 and Day 15 post initiation of differentiation. Medium was aspirated and cells on coverslips were fixed with 3.7 neutral buffered formaldehyde. Cells were permeabilized with 0.15% Triton-X 100 and blocked with 10% horse serum. βIII Tubulin was used to stain neuronal cells, and glial fibrillary acidic protein (GFAP) was used to stain glial cells. Primary antibodies were incubated for 1 h at room temperature and unbound antibody was washed with PBS. Fluorophore conjugated secondary antibodies (FITC-anti mouse and TRITC-anti rabbit) were used to visualize fluorescence using an inverted Nikon TiE fluorescent microscope. Staining with FITC-conjugated secondary antibody without the primary antibody was used as a negative control. Confluent smooth muscle coverslips were stained with neuronal or glial markers to avoid a false positive staining while identifying differentiated neurons or glia.

2.6. Data analysis

Neurite lengths were measured from individual 10X micrographs obtained at the same amplifier gain and exposure. Neurites were identified primarily by expression of immunoreactivity for βIII Tubulin concurrently with neuronal morphology. Up to five sequential fields of view were measured on each coverslip starting from one edge to the other, covering the area of the coverslip. All cells were measured on each coverslip, covering the entire area of the neuronal coverslip. Number of neurites measured for each coverslip coating varied between 20 and 50 readings and is indicated next to a reported measurement. The length of the longest neurite from each cell was measured using NIH Image J using the freeform tool. Neurite lengths between coatings were compared using one way ANOVA, with Bonferroni post-test to identify a significant difference ($p < 0.05$) in neurite lengths by varying culture substrata. GFAP immunofluorescence was quantified using the Nikon Elements imaging software. Mean red (TRITC) fluorescence was calculated from 10X micrographs, using a constant rectangular area tool that covered 100% of the field of view. Multiple (at least 5) sequential fields of view at the same magnification were chosen for each sample to obtain mean fluorescence. Mean red fluorescence indicated the presence of GFAP expressing cells. One way ANOVA with Bonferroni post-test was used to identify a significant difference in red fluorescent intensity between coated culture substrata. GraphPad Prism 5.1 for Windows (San Diego, CA) was used to perform statistical analysis. All statistics are from experiments between 3 and 5 individual sets, with multiple micrographs within each set. Reported numbers are mean ± standard error of the mean.

3. Results

3.1. Initial phenotype of rabbit enteric neurospheres

Upon digestion of rabbit jejunal biopsies with collagenase/dispase, near single cell suspensions were obtained by filtration through 70 μm and 40 μm meshes. Single cells were approximately 7 μm in diameter. These cells were plated in non-adherent culture dishes. Over the course of two weeks post plating, rabbit enteric neuronal progenitor cells aggregated and proliferated in culture and formed floating spherical structures, called enteric neurospheres (Fig. 1A). Average neurospheres were 98.2 ± 8.3 μm ($n = 34$) two weeks post plating. The neurospheres continued to

Please cite this article in press as: Raghavan S, et al., Neuroglial differentiation of adult enteric neuronal progenitor cells as a function of extracellular matrix composition, Biomaterials (2013), http://dx.doi.org/10.1016/j.biomaterials.2013.05.023