β1-integrin Cytoskeleton Signaling Regulates Sensory Neurons Response to Matrix Dimensionality

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Statement of Purpose: While great strides have been made in developing new biomaterials focused on nerve repair[1], researchers' efforts have been limited by a poor understanding of how neurons interact with their three-dimensional (3D) environment[2]. Neuronal signaling controls neurite outgrowth, and recent studies with non-neuronal cells demonstrated that signaling pathways are dramatically altered when cells are placed in a 3D matrix, with 3D culture environments being a better representation of in vivo systems than 2D[3]. To determine whether 3D culture alters signalling in neurons, we are currently culturing sensory neurons from mouse Dorsal Root Ganglia (DRG) in 2D and 3D collagen. We examined and quantified the outgrowth of neuronal projections and the expression levels of β1-integrin, focal adhesion kinase (FAK) and the phosphorylation of FAK. Our work has established that DRG neurons sense the dimensionality of their environment and respond to the different arrangement of external signals by modulating neuron morphology, neurite extension and branching[4]. Most significantly, in 3D, sensory neurons adapt features on a timescale that recapitulates the in vivo process, establishing that a simple 3D environment alone can provide optimized conditions for normal development to occur[4]. In this study we show that DRG neuronal signaling pathways are altered in 3D as a response to the adhesion of the neurons to their surrounding matrix. This study delineates key signaling mechanisms in 3D neuronal culture to provide a biological basis for testing the potential of new 3D peripheral nerve repair therapies.

Table 1. Morphological analysis of DRG neurons in 2D and 3D culture[4]

2 days in culture	**2D**	**3D**	**in vivo**
Neurite length	180 μm	220 μm	up to 1mm
Branches	≤1	≥2	several
% Bipolar	**60**	22	~20
% Unipolar	33	**73**	**~80**

Methods: DRG neurons were isolated from E13.5 mice according to established methods[5]. Dissociated neurons were seeded onto 2D collagen coated coverslips (~7 μg/cm^2) at 1.5x10^4 cells/cm^2 and within 3D collagen (1 mg/ml) gels (20 μl) at 5x10^5 cells/ml and cultured for 2 days and then fixed in 4% formalin. Cells were maintained in serum free medium (DMEM supplemented with 1X N2, 100 U/ml of Penicillin/Streptomycin, 20 mM of L-Glutamine and 50 ng/ml of NGF; Invitrogen). For analysis of integrin signaling molecules, the samples were incubated with primary antibodies directed against β1 integrin, FAK, FAKpY861 and FAKpY397 (BD Biosciences, Santa Cruz) following incubation with the appropriate fluorescently conjugated secondary antibodies (Jackson Immunoresearch, Alexa) and subsequent confocal imaging (Leica SP5). We have analyzed the immunofluorescent signals for differences in reactivity (present vs absent), location (whole cell or soma, neurites, growth cones) and signal morphology (diffuse vs punctate).

Results: Immunocytochemical studies in 2D and 3D collagen indicate that β1-integrin signaling is altered in 2D vs 3D (representative data, Figs 1,2). This is demonstrated by the clustered staining in 2D where larger signaling complexes (point contacts) are seen on the cell's surface of attachment. In 3D gels, integrin signaling molecules are much more diffuse throughout the neuron in response to the cell adhesion to a surrounding 3D matrix; this makes sense because there is a larger surface area for contact, better mimicking the in vivo environment. We note that in the 3D matrix adhesions formed by the sensory neurons, FAK phosphorylation is dramatically altered at Y397 which was demonstrated by the absence or weak immunoreactivity of this molecule in 3D (Fig 2). This major difference suggests that FAK phosphorylation at this site is required for the regulation of neuronal viability and process outgrowth in 2D culture, whereas this response is altered in 3D. Therefore, this pathway seems to play an important role in how neurons sense and respond to their environment dimensionality. This result supports findings from previous studies with non-neuronal cells where Y397-autophosphorylation of FAK is lost when the cells are cultured in 3D collagen gels[3]. This is the first time signaling differences are established in neuronal cells in different presentations of the same substrate.

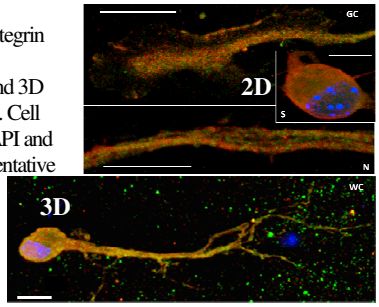

Fig 1. DRG neurons β1-integrin (red) and FAK (green) immunoreactivity in 2D and 3D culture (scale bars, 10 μm). Cell nuclei are labeled with DAPI and are shown in blue. Representative images of whole cells (WC), soma (S), neurites (N) and growth cones (GC) are shown.

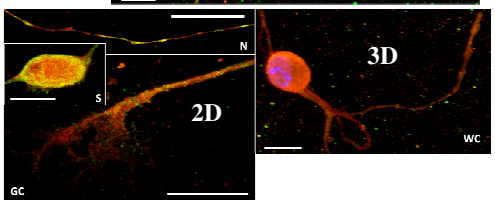

Fig 2. DRG neurons β1-integrin (red) and FAKpY397 (green) immuno - reactivity in 2D and 3D culture (scale bars, 10 μm). Cell nuclei are labeled with DAPI and are shown in blue. Representative images of whole cells (WC), soma (S), neurites (N) and growth cones (GC) are shown.

Conclusions: The goal of this work was to demonstrate that 3D culture alters neuronal behavior by modulating neuronal signaling. Our work so far has demonstrated that β1-integrin signaling pathways are altered when sensory neurons are cultured in 2D and 3D collagen matrices. Ongoing work focuses on the inhibition of β1 integrin and FAKpY397 to analyze the specific roles of these molecules in the process of cell recognition and adaptation to a 3D microenvironment. We will apply these findings to improve the design of synthetic gel scaffolds for nerve repair.

References: 1. Schmidt CE., Leach, JB. Annual Review of Biomed. Eng. 2003, 5:293-347. **2.** Leach JB. Encyclopedia of Biomed. Eng, Wiley, 2006, 6: 3568-3578. **3.** Cukierman, E. et al Science 2001, 294: 1708-1712. **4.** Ribeiro, A. et al, PLoS One, 2010 (submitted). **5.** Banker, G, K Goslin. *Culturing Nerve Cells*. 1998, 2nd ed. MIT Press.

Funding: NIH-NINDS R01NS065205 (JBL), Henry-Luce Foundation (JBL), Wyeth Fellowship at UMBC (AR)