Genomic and Morphological Analysis of Human Neuroblastoma Cell Growth in Three-Dimensional Matrices

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Introduction: Recent efforts in biomedical research have focused on simulation of three-dimensional (3D) *in vivo* environments with *in vitro* cell culture systems. Our hypothesis is that the phenotype and gene expression of neurons grown in three-dimensional systems are different from their two-dimensional counterparts. We have used oligonucleotide microarrays to compare the gene expression patterns of neurons grown in 3D versus 2D culture environments, and have identified hundreds of genes that were differentially regulated in 3D cultures when compared to 2D cultures. In this study, we focused our efforts on nine genes relevant to the cell cytoskeleton and extracellular matrix and confirmed the microarray data using the real time RT-PCR technique. We studied neuronal gene expression and morphology in two distinct matrices (collagen I and Matrigel) and characterized physical properties of the matrices.

Methods: For 3D culture, human neuroblastoma SHSY5Y cells were suspended in reconstituted bovine collagen I gel in Dulbecco's Modified Eagle's Media (DMEM) titrated to pH 7.4 (PureCol; Inamed) or in Matrigel (BD Biosciences). For 2D culture, glass coverslips were coated with either collagen I or Matrigel prior to cell seeding. Cells were grown for 24 hours and either recovered for real time RT-PCR or fixed for visualization. RNA was isolated with the RNAeasy Mini Kit (Qiagen), analyzed for degradation and DNA contamination, and used as a template for real-time RT-PCR experiments. Reactions were carried out using an Applied Biosystems 7300 Real Time PCR system (Qiagen). Fold changes in gene expression (3D versus 2D) were calculated relative to the level of GAPDH mRNA. GAPDH, a housekeeping gene, was chosen because microarray analysis indicated that its expression levels did not change when cells were plated on 2D versus 3D. Neurons were visualized by phase contrast microscopy at 200X in 2D using a Nikon Eclipse TE2000-S microscope and Zeiss Axiovert microscope for 3D samples. Images and z-stacks were captured using a Hamamatsu Orca-ER camera. Phalloidin staining of F-actin and fluorescent cell tracking with PKH67 were performed on all samples to visualize structures using a Leica TCS SP2 AOBS spectral confocal microscope. Voxx software was used for image analysis. Neurite lengths were determined from phase contrast images using the measurement tool in OpenLab 4.0.2 (Improvision) for 2D samples and Zeiss Axiovert (Zeiss) for 3D samples. The longest neurite of at least 30 neurons was recorded. Significance at $p<0.05$ was determined using an unpaired Student's t-test. Gels were visualized by confocal reflectance microscopy. The matrices were also examined under scanning electron microscopy (SEM) using a Hitachi S-2700 scanning electron microscope using an acceleration voltage of 3kV. Samples were fixed with Karnovsky's fixative, rinsed in cacodylate buffer, washed with dH_2O, dehydrated with ethanol, and dried with hexamethyldisilazane (HMDS). Mechanical analysis of the gels was performed using a DMA 7e Dynamic Mechanical Analyzer (Perkin Elmer). The storage modulus and loss modulus of the hydrogels were measured for 2-4 mm thick hydrogel samples subjected to static force of 11 mN at 2.6 Hz.

Results/Discussion: Genes encoding for the cytoskeleton proteins filaminA and actinin1, and the extracellular matrix proteins fibronectin1 and collagen III were down-regulated in cells cultured in both 3D matrices (collagen I and Matrigel) relative to the 2D cultures. The genes encoding for the cytoskeleton proteins actin filament capping protein (CAPZA2) and neurofilament, and the extracellular matrix protein microfribillar associated protein 3 (MFAP3) were up-regulated in cells cultured in both 3D matrices. Neurons grown in 3D collagen I showed significantly longer neurites (47.22 ± 2.56 μm) than neurons in 2D collagen I (25.97 ± 1.33 μm). This trend was reversed for neurons grown in Matrigel, where neurites grown in 2D were significantly longer (33.67 ± 2.77 μm) than 3D (28.75 ± 2.77 μm). Neurite widths were not significantly different for all samples tested. Cells grown on both 2D substrates and stained with phalloidin exhibited a flat, spread morphology with actin staining strongest at the edges. Cells in 3D were rounded with uniform actin staining. We hypothesized that morphological differences between cells grown in collagen I and Matrigel were due to differences in the porosity, stiffness, and structure of the matrices. Confocal reflectance and SEM of the matrices revealed that the 3D collagen I matrix had a highly porous, fibrillar structure while 3D Matrigel was dense and non-fibrillar. Matrigel was also shown to have a lower shear modulus by DMA analysis suggesting that it was stiffer than the collagen matrix.

Conclusions: In a complex 3D environment, changes in gene expression and physical matrix parameters were each correlated with morphological differences in cultured neurons. The physical environment of the cells played an important role in local structural changes as seen by the marked differences in neurite length. In 3D cultures, differences in neurite growth between two types of 3D matrices reflected differences in physical properties of the matrices, but reflected no corresponding changes in gene expression. Interestingly, genes that regulate cell spreading through filopodial dynamics were differentially regulated in 3D independent of the chemical composition of the matrix. These results suggest that while gene expression in 3D environments plays an important role in regulating cell spreading, the physical properties of the specific 3D matrix have a strong influence on neurite growth.

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