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Induced differentiation inhibits sphere formation in neuroblastoma

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ABSTRACT

Neuroblastoma arises from the neural crest, the precursor cells of the sympathoadrenal axis, and differentiation status is a key prognostic factor used for clinical risk group stratification and treatment strategies. Neuroblastoma tumor-initiating cells have been successfully isolated from patient tumor samples and bone marrow using sphere culture, which is well established to promote growth of neural crest stem cells. However, accurate quantification of sphere-forming frequency of commonly used neuroblastoma cell lines has not been reported. Here, we show that *MYCN*-amplified neuroblastoma cell lines form spheres more frequently than non-*MYCN*-amplified cell lines. We also show that sphere formation is directly sensitive to cellular differentiation status. 13-*cis*-retinoic acid is a clinically used differentiating agent that induces a neuronal phenotype in neuroblastoma cells. Induced differentiation nearly completely blocked sphere formation. Furthermore, sphere formation was specifically FGF-responsive and did not respond to increasing doses of EGF. Taken together, these data suggest that sphere formation is an accurate method of quantifying the stemness phenotype in neuroblastoma.

1. Introduction

Neuroblastoma arises from the neural crest, the precursor cells of the sympathoadrenal axis, and is the most common extracranial solid tumor in children [1]. High-risk disease accounts for the vast majority of deaths and is characterized by *MYCN* gene amplification, lack of tumor stroma, and poor or undifferentiated tumor cell histology [1]. Identifying those tumor cell subsets with undifferentiated features and developing a better understanding of the molecular mechanisms that maintain this cellular state are key challenges in the process of developing novel therapeutics to alter the devastating clinical course of high-risk neuroblastoma [2].

Cancer stem cells are tumor cells that demonstrate stem cell features and that can be prospectively identified by cell surface marker expression [3,4]. Tumor-initiating cells are those subsets that form *in vivo* xenografts at higher frequency than control or parental cell populations, and must be able to faithfully recapitulate the genetic and histologic features of the parental tumor [3]. Neuroblastoma tumor-initiating cells have been successfully isolated from patient tumor samples and bone marrow using sphere culture [5,6], which is well established to promote growth of neural crest stem cells [7]. However, the mechanisms that are responsible for sphere formation remain poorly understood.

A major limitation of current studies involving neuroblastoma sphere formation has been the lack of a reliable quantification method that can be easily repeated. The predominant approach is to count the number of spheres that are over a certain size after a period of incubation [8] and to report the number of spheres formed as a percentage of cells plated. This method is highly dependent on the number of cells plated and culture area, for which there are no standard parameters. In this study, we apply limiting dilution analysis to quantitatively assess the frequency of intrinsic sphere-forming ability for a variety of neuroblastoma cell lines, and use induced differentiation with retinoic acid to validate this approach. Retinoic acid is a clinical therapeutic that is used as differentiating maintenance therapy after induction and consolidation chemotherapy in children with high-risk neuroblastoma [9]. Retinoic acid is well established to induce differentiation *in vitro* across multiple neuroblastoma cell lines [10–12] and is also shown to regulate stem cell differentiation [13]. We further show that limiting dilution analysis can be used to assess the contribution of signaling pathways to sphere formation.

Abbreviations: RA, 13-*cis*-retinoic acid; SFF, sphere-forming frequency.

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2. Materials and methods

2.1. Adherent cell culture and sphere culture conditions

Human neuroblastoma cell lines SK-N-AS, SK-N-SH, BE(2)-C and LAN-1, were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured on tissue-culture treated flasks and plates in RMPI 1640 media (Cellgro Mediatech, Herndon, VA) supplemented with fetal bovine serum at 10% v/v (Sigma-Aldrich, St. Louis, MO) in a humidified atmosphere of 5% CO_2 at 37 °C. Spheres were cultured in a 1:1 mixture of DMEM and Ham's F12, with B27 supplement (50×), penicillin/streptomycin (5% v/v), epidermal growth factor (EGF, 20 ng/ml) and fibroblast growth factor (FGF, 40 ng/ml) on polystyrene-coated, ultra-low attachment plates (Corning Inc., Corning, NY).

2.2. Quantification of sphere formation

Cells were trypsinized with 0.25% trypsin (Cellgro Mediatech, Herndon, VA) for 1–5 min at 37 °C and the reaction was quenched with 10% FBS-containing RPMI 1640 media. Cells were stained with Trypan Blue to determine viability and manually counted in 8 replicates on a light microscope. Separate aliquots of 5×10^5 cells constituted each biological replicate for analysis, and were centrifuged at 1000 rpm × 5 min and supernatant removed. Cells were resuspended in 500 μl of sphere media as described above. Two 10× dilutions followed by seven 2× dilutions were serially performed. Cells were plated on polystyrene-coated, ultra-low attachment 96-well plates (Corning Inc., Corning, NY) at final cell doses of 500, 250, 125, 62, 31, 16, 8 and 4 cells/well in 100 μl of sphere media in replicates of 6 per dose. After 4 d incubation in a humidified atmosphere of 5% CO_2 at 37 °C, each well was scored as "positive" or "negative" for sphere formation, determined by the presence of ≥1 sphere consisting of ≥10 cells. The Eliza-Hall online calculator was used to generate a point estimate of 1/x cells with sphere-forming frequency (SFF) from the tested population [14]. Each biological replicate was repeated for a minimum of 6 replicates per group on different days to limit the potential impact of non-experimental variability such as cell counts or aliquots on the final estimates of sphere-forming frequency, and values were averaged and analyzed by Student's *t*-test or one-way ANOVA as appropriate.

2.3. Statistical analysis

All results are shown as mean ± SEM. Data were analyzed with Student's *t*-test or one-way ANOVA with Tukey correction for two or ≥3 group experiments, respectively. In all instances, a p value < 0.05 was considered significant.

3. Results

3.1. Sphere formation in neuroblastoma cells correlates with MYCN status

All human neuroblastoma cell lines tested formed spheres after variable periods in culture, indicating a general capacity for neuroblastoma cells to proliferate in conditions that favor the growth of neural crest stem cells [7]. To assess the basal SFF of neuroblastoma cells, four human neuroblastoma cell lines (SK-N-AS, SK-N-SH, BE(2)-C, LAN-1) were cultured in sphere culture conditions for 4 d (Fig. 1A). After 4 d, formed spheres had a tightly packed morphology even when plated at low cell density (Fig. 1B). The two cell lines that contain only a single copy of the *MYCN* oncogene demonstrated a low "background" rate of sphere formation (SK-N-AS: 0.7 ± 0.09%, SK-N-SH: 1.7 ± 0.18%, Fig. 1C). Cell lines with *MYCN* amplification, however, showed high SFF at 4 d of incubation (BE(2)-C: 4.3 ± 0.41%, LAN-1: 6.1 ± 0.74%, Fig. 1C). BE(2)-C and LAN-1 sphere formation was significantly higher than SK-N-AS or SK-N-SH ($p < 0.001$). These data suggest that sphere formation is correlated with other known parameters of cell behavior, as the highly aggressive cells lines are able to form spheres at a much higher rate compared to less aggressive, slower growing, *MYCN*-single copy cell lines [15].

3.2. 13-cis-Retinoic acid induces cellular differentiation and blocks sphere formation

Sphere culture is known to promote proliferation of neural crest stem cells [7]. We therefore hypothesized that sphere formation in neuroblastoma would be sensitive to the differentiation status of the cells being exposed to the sphere stimulus. The differences in SFF between cell lines (Fig. 1C) suggests this, as BE(2)-C are I-type cells that retain the ability to differentiate along the neuronal or the Schwannian cell lineages after retinoic acid or bromodeoxyuridine stimulation, respectively [16]. SK-N-AS and SK-N-SH cells are both neuronal-type [15], indicating a more differentiated basal state. To directly test our hypothesis, we used 13-*cis*-retinoic acid (RA), an agent that is utilized clinically as a differentiating maintenance therapy after induction and consolidation chemotherapy and radiation [9]. Incubation of BE(2)-C cells with RA (5 μM) for 4 d in sphere culture conditions nearly completely blocked sphere formation (Fig. 2A). Sphere formation could be rescued by simultaneous co-treatment with the pan-retinoic acid receptor inhibitor BMS493 (1 μM, Fig. 2A).

To further test the impact of differentiation status on sphere formation, we pre-treated BE(2)-C cells with RA (5 μM) for 24 h or 7 d prior to exposure to the sphere culture conditions. Seven days of RA treatment, but not 24 h, was able to induce neurite outgrowth and elongation of cell bodies indicating differentiation along a neuronal lineage (Fig. 2B) [10–12]. Pre-treatment with RA for only 24 h had no effect on sphere formation compared to vehicle-treated controls (Fig. 2C). After successful differentiation with 7 d of RA, however, sphere formation was dramatically reduced (4.3 ± 1.3% vs. 1.4 ± 0.8%, $p < 0.001$, Fig. 2C). Taken together, these data suggest that sphere formation is critically regulated by differentiation status. In addition to inducing differentiation, RA could be exerting a direct effect on sphere formation given its central role in regulating differentiation-related behaviors, and we next sought to address this possibility directly.

3.3. FGF, but not EGF, promotes sphere formation

There are three main factors that are fundamental to sphere growth: serum-free media, FGF and EGF supplementation and non-attachment. We hypothesized that activation of downstream signaling pathways in response to FGF and EGF stimuli could be a site of potential regulation by RA. To test this hypothesis, sphere formation was assessed in response to each individual growth factor. Increasing doses of FGF led to increased SFF (Fig. 3B, $p < 0.05$ for 60 or 80 ng/ml vs. 20 ng/ml). Increasing doses of EGF had no effect on SFF over the same dose range (Fig. 3A, p = NS). These data suggest that sphere formation is a phenotypic response to activated signaling pathways rather than a fixed property of a subset of cells in a population. FGF signaling via the FGF receptor 1 is predominantly transduced through the MAPK signaling pathway [17]. PD98059 is a specific MAPK inhibitor and was able to inhibit SFF (Fig. 3C). These data suggest that activation of canonical downstream FGF signaling via the MAPK pathway is critical for sphere formation.

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