Neuroblastoma Cells Isolated from Bone Marrow Metastases Contain a Naturally Enriched Tumor-Initiating Cell

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Abstract

Neuroblastoma is a heterogeneous pediatric tumor thought to arise from the embryonic neural crest. Identification of the cell responsible for propagating neuroblastomas is essential to understanding this often recurrent, rapidly progressing disease. We have isolated and characterized putative tumor-initiating cells from 16 tumors and bone marrow metastases from patients in all neuroblastoma risk groups. Dissociated cells from tumors or bone marrow grew as spheres in conditions used to culture neural crest stem cells, were capable of self-renewal, and exhibited chromosomal aberrations typical of neuroblastoma. Primary spheres from all tumor risk groups differentiated under neurogenic conditions to form neurons. Tumor spheres from low-risk tumors frequently formed large neuronal networks, whereas those from high-risk tumors rarely did. As few as 10 passaged tumor sphere cells from aggressive neuroblastoma injected orthotopically into severe combined immunodeficient/Beige mice formed large neuroblastoma tumors that metastasized to liver, spleen, contralateral adrenal and kidney, and lung. Furthermore, highly tumorigenic tumor spheres were isolated from the bone marrow of patients in clinical remission, suggesting that this population of cells may predict clinical behavior and serve as a biomarker for minimal residual disease in high-risk patients. Our data indicate that high-risk neuroblastoma contains a cell with cancer stem cell properties that is enriched in tumor-initiating capacity. These cells may serve as a model system to identify the molecular determinants of neuroblastoma and to develop new therapeutic strategies for this tumor. [Cancer Res 2007;67(23):11234–43]

Introduction

Neuroblastoma is an embryonal tumor of the autonomic nervous system and the most common extracranial solid tumor of childhood. Although treatment regimens for many childhood cancers have radically improved patient outcome over the past three decades, survival rates have failed to improve for neuroblasta. Patients with a high-risk neuroblastoma have a long-term survival of <40% (1). Growing evidence exists to support the hypothesis that a reservoir of tumor cells within a tumor that share similar properties to normal stem cells drives tumorigenesis and results in aberrantly programmed and differentiated cells (2). This subpopulation of cells has been termed cancer stem cells or tumor-initiating cells (TIC) and have been described in adult leukemia (3), breast cancer (4), pediatric brain tumors (5, 6), melanoma (7), ependymomas (8), colon cancer (9, 10), and head and neck squamous cell carcinoma (11) based on their abilities to self-renew, differentiate into the cellular lineages observed in the tumors from which they were derived, and serially propagate tumors in vivo.

The clinical presentation and treatment response of neuroblastoma suggests that a TIC likely exists in neuroblastoma tumors. Neuroblastoma tumors exhibit a wide range of differentiated phenotypes from largely undifferentiated tumors in patients that have a poor prognosis, to those containing neuronal, neuroendocrine, and Schwannian cell types, which, depending on the degree of differentiation, may have a better prognosis (12). Neuroblastoma tumors are thus composed of a heterogeneous population of tumorigenic cells with respect to their ability to proliferate and differentiate and may contain a TIC that is responsible for this diversity. In vitro studies in immortalized neuroblastoma cell lines have shown that activation of distinct signal transduction pathways can induce neuroblastoma cell lines to generate cells with evidence of neuronal (13), chromaffin (14), or Schwannian (15) phenotypes, further supporting the existence of a cell with stem cell properties in neuroblastoma.

Metastasis in high-risk neuroblastoma patients occurs in the bone marrow, bone, and lymph nodes. The bone marrow is the most common metastatic site, occurring in 70% of the cases (16), and cytologic and histologic screening of bone marrow samples for neuroblastoma cells is an important aspect for the initial staging and assessment of response to chemotherapy (17). One prediction of the cancer stem cell hypothesis is that cancer stem cells are inherently resistant to chemotherapy, and that they can remain in the patient after therapy has been completed, contributing to relapse and poor survival. Indeed, in acute myelogenous leukemia, a higher percentage of the leukemia stem cell population (CD34⁺CD38⁻) correlates with poorer survival (18). We therefore asked whether a cancer stem cell or an enriched population of TICs resides in the bone marrow of relapsed neuroblastoma patients. We found that cells with properties of cancer stem cells could be isolated from pretreatment and posttreatment neuroblastoma tumors, from the bone marrow of patients who had relapsed after multiple courses of chemotherapy, and from the bone marrow of
patients in remission. Neuroblastoma TICs expressed neural crest progenitor markers, self-renewed, and differentiated into neurons, characteristics of the primary neuroblastoma. Neuroblastoma TICs isolated from the bone marrow of high-risk patients formed fatal neuroblastoma tumors in severe combined immunodeficient (SCID)/Beige mice with as few as 10 cells. The identification of an experimentally defined population of neuroblastoma cells from bone marrow that is greatly enriched in tumor-initiating capacity will enhance our understanding of the molecular and cellular basis of neuroblastoma, the ability to detect minimal residual disease, and to develop effective therapeutics.

Materials and Methods
Primary culture of tumor spheres from tumors and bone marrow aspirates. Tumor samples and bone marrow aspirates were obtained from consenting patients, as approved by SickKids Hospital’s Research Ethics Board (protocol 1000006069). Bone marrow aspirates were filtered through a 40-μm cell strainer and tumor cells were collected by inverting and washing the filter with HBSS (Invitrogen), whereas tumor samples were collected and cut into 2 to 3 mm2 pieces. All samples were then enzymatically dissociated with Liberase Blendzyme 1 (0.62 Wunsch units/mL; Roche) in HBSS for 15 to 45 min at 37°C followed by the addition of 10% fetal bovine serum (FBS; HyClone) to inhibit enzyme activity. Tumor cells were then triturated in medium and the suspension filtered through a 70-μm cell strainer. Dissociated cells were pelleted and resuspended in DMEM-F12, 3:1 (Invitrogen) containing 100 units/mL penicillin/streptomycin, 2% B27 supplement (Life Technologies), 40 ng/mL basic fibroblast growth factor 2 (bFGF), and 20 ng/mL epidermal growth factor (EGF; both from Collaborative Research; proliferation medium), and cultured in 12.5 or 25 cm2 flasks in a 37°C, 5% CO2 tissue culture incubator. Cells were fed fresh proliferation medium weekly. Tumor spheres were passaged by mechanical dissociation and split 1:6 with 50% fresh proliferation medium and 50% conditioned medium from the initial flask.

Tumor sphere growth and growth curves. The self-renewal capacity of tumor spheres was determined to be the limit of sphere formation in liquid culture. Growth curves were established by mechanically dissociating passaged tumor spheres, plating 8.3 × 103 single cells in 12.5 cm2 flasks and assessing cell number 3, 5, and 7 days after plating. The results represent the mean cell count from duplicate flasks in three independent experiments.

Neurogenic differentiation of tumor spheres. Five to 10 tumor spheres were plated onto poly-lysine/laminin–coated eight-well chamber slides (Nalge Nunc), expanded in medium containing DMEM-F12, 3:1, 10 ng/mL bFGF, and 15% FBS for 5 to 10 days and cultured in DMEM-F12, 3:1 containing 100 units/mL penicillin/streptomycin, 2% B27 supplement (Life Technologies), 40 ng/mL basic fibroblast growth factor 2 (bFGF), and 20 ng/mL epidermal growth factor (EGF; both from Collaborative Research; proliferation medium), and cultured in 12.5 or 25 cm2 flasks in a 37°C, 5% CO2 tissue culture incubator. Cells were fed fresh proliferation medium weekly. Tumor spheres were passaged by mechanical dissociation and split 1:6 with 50% fresh proliferation medium and 50% conditioned medium from the initial flask.

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Immunocytochemistry and quantification. Tumor spheres or differentiated cells were immobilized on coated glass slides using the Shandon cytosip system (Thermo) as described by Toma and colleagues (19, 20). Immunocytochemical analysis was performed using the following primary antibodies: NB84 monoclonal (1:50; Novocastra); anti–tyrosine hydroxylase polyclonal (TH; 1:150; Chemicon); anti–β3-tubulin monoclonal (1:500; TuJ1 clone; Covance); anti–neurofilament-M monoclonal (NF-M; 1:200; Chemicon); s100α monoclonal (1:1,000; Sigma); anti–glial fibrillary acidic protein (GFAP) polyclonal (1:200; DAKO); galactocerebroside C (GalC) polyclonal (1:200; Chemicon); anti–nestin monoclonal (1:400; Chemicon); anti–nestin polyclonal (1:400; Chemicon); and anti–fibronectin polyclonal (1:1,000; Sigma). The following secondary antibodies were used: Alexa 488–conjugated goat-anti-mouse (1:1,000) and Alexa 555–conjugated goat-anti-rabbit (1:1,000; Molecular Probes).

Differentiation was quantified by calculating the percentage of spheres that formed neuronal networks for individual patient samples.

Cytogenetic analysis of tumor spheres. Tumor sphere DNA was extracted from fresh or frozen primary cell pellets using the Qiagen blood and tissue DNA isolation kit (Qiagen) and subjected to 100K single nucleotide polymorphism (SNP) array. When no matched normal sample was available, peripheral blood mononuclear DNA from normal individuals was used as controls.

The Affymetrix 100K SNP array (Affymetrix) was used according to the methods described by the manufacturer as described in Supplementary Fig. S1. Copy number change was measured based on comparing the hybridization intensity between normal and tumor samples using the dCHIP software, with a sliding window of three SNPs as previously described (21). Copy number gain was defined as between 2.8 and 5 copies (alleles). Amplification was defined as an inferred copy number of >5 in three consecutive SNPs. Copy number loss was defined as an inferred copy number of <0.3 in at least two consecutive SNPs.
receptor; FITC-conjugated) were purchased from Miltenyi Biotec; the monoclonal antibody against human CD56 (phycoerythrin conjugated) was purchased from DAKO; the monoclonal antibody against human NB84 (FITC conjugated) was purchased from Novocastra; the monoclonal antibodies against human CD24 (phycoerythrin conjugated), CD29 (phycoerythrin Cy5-conjugated), CD81 (APC-conjugated), CD34, CD44, CD45, CD20, and CD117, and rat monoclonal antibody CD49f (phycoerythrin conjugated) were purchased from BD Biosciences. Isotype-matched antibodies (BD Biosciences) were used as controls.

Fluorescence-activated cell sorting (FACS) was done on a DAKO Cytomation MoFlo nine-color cell sorter. Sorting was performed on cells stained with purified monoclonal CD34 and Alexa 488-conjugated goat anti-mouse secondary antibody followed by phycoerythrin-conjugated monoclonal CD24 antibody. FlowJo version 6.3 (Tree Star, Inc.) was used to analyze flow and FACS data.

**Statistical analysis of data.** The statistical analyses of our orthotopic adrenal studies were performed with the SAS software version 8.02 (SAS Institute, Inc.). The time to morbidity analysis was performed using a Kaplan-Meier analysis with the procedure LIFETEST to accommodate for censored times and the Wilcoxon statistics test was performed for the two interested comparisons. The $\chi^2$ analysis for the limit of self-renewal was performed using the Fisher’s exact test with procedure FREQ. Statistical significance was established at $P < 0.05$.

## Results

**Neuroblastoma cells from tumors and bone marrow aspirates form nonadherent spheres when grown in serum-free conditions.** Sixteen tumor samples were used in the study including nine high-risk and seven low-risk neuroblastoma tumors and bone marrow metastases (Table 1). Two of the high-risk patients were in clinical remission at the time of sample collection. Acutely dissociated tumor cells were cultured as spheres in serum-free medium containing bFGF and EGF, conditions used for neural crest stem cell growth (19, 20). Within 2 to 8 weeks (median time 2 weeks), we observed primary sphere formation in our cultures as previously described for brain tumor TICs (5, 6). The two high-risk remission bone marrow patient samples (NB61 and NB67) took the longest time to form spheres at 8 weeks after dissociation. Tumor samples that remained as single cells after dissociation and several months of culture were excluded from this study. These samples included those from low-risk tumors as well as from tumors from patients after treatment with multiple chemotherapeutic regimes (data not shown). Upon passaging, the majority of primary spheres from high-risk tumors (Fig. 1A) and bone marrow samples (Fig. 1A) formed secondary spheres. A single high-risk neuroblastoma tumor sample became adherent when primary tumor spheres were passaged (Fig. 1A). In contrast to tumor spheres from high-risk samples, primary tumor spheres from low-risk neuroblastoma samples (Fig. 1A) tended to form adherent cultures when passaged.

We first determined whether the tumor spheres expressed markers of neuroblastoma and neural crest progenitors by immunostaining for NB84, a commonly used clinical neuroblastoma marker, TH, a catecholamine biosynthetic pathway marker that is a unique feature of neuroblastoma (23), and fibronectin and nestin, which are expressed in neural crest progenitors (24–26). All primary cell lines propagated as spheres or as adherent cells expressed NB84, TH, fibronectin, and nestin (Fig. 1B). These data indicate that primary cells from neuroblastoma tumors and bone marrow aspirates that express neuroblastoma and neural crest progenitor cell markers can be isolated and propagated using neural crest stem cell culture conditions.

**Tumor spheres from high-risk neuroblastoma self-renew to a greater extent than tumor spheres from low-risk neuroblastoma.** Self-renewal is a fundamental feature of either normal or tumor-derived stem cells and can be assessed by serial passage (6, 27). The self-renewal capacity of primary tumor spheres was

### Table 1. Summary of patient population

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sex</th>
<th>Age at diagnosis (18 mo)</th>
<th>Tumor risk group</th>
<th>Specimen type</th>
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<tr>
<td>NB04</td>
<td>F</td>
<td>Greater</td>
<td>High</td>
<td>Relapse bone marrow</td>
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<td>Greater</td>
<td>High</td>
<td>Tumor</td>
</tr>
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<td>Tumor</td>
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<td>Relapse bone marrow</td>
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<td>Relapse bone marrow</td>
</tr>
<tr>
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<td>Low</td>
<td>Tumor</td>
</tr>
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<td>Tumor</td>
</tr>
<tr>
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<td>Low</td>
<td>Bone marrow</td>
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<td>Greater</td>
<td>Low</td>
<td>Tumor</td>
</tr>
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<td>High</td>
<td>Tumor</td>
</tr>
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</tr>
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</tr>
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<td>High</td>
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<td>High</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>NB67 ‡</td>
<td>F</td>
<td>Greater</td>
<td>High</td>
<td>Bone marrow</td>
</tr>
</tbody>
</table>

*NOTE: NB05b and NB19; NB12 and NB67; NB15 and NB20 are samples from the same patient.

*Unable to determine whether patient was low or intermediate risk.

†Borderline MYCN-amplified, patient died.

‡Remission patient.
evaluated in liquid culture. Cells were mechanically dissociated and replated in liquid culture containing 50% conditioned medium. This process was repeated until the sphere-forming cell populations were depleted. Primary spheres from low-risk neuroblastoma were capable of being passed 0 to 1 times (median 0 passages) and high-risk neuroblastoma samples formed spheres 1 to 15 times (median 6 passages) when passaged in liquid culture. NB12 has been passed 34 times, but the cytogenetics of these cells is not normal (data not shown). Four high-risk neuroblastoma samples have remained in liquid culture without exhaustion. Self-renewal was expressed as the number of passages in liquid culture until the limit of self-renewal for an individual tumor sample (Fig. 2A).

Growth curves for dissociated cells from high-risk tumor spheres (NB12, passage 7) showed that they proliferated logarithmically in liquid passage and retained their proliferative capacity after extended passage (Fig. 2B). Statistical analysis was performed between the low-risk and high-risk groups using a Fisher’s exact test to compare the self-renewal limit between groups. Statistical significance was obtained between these groups (P < 0.05). The morphology of secondary and subsequently passaged tumor spheres was identical to that of primary spheres, and all cells retained expression of the neuroblastoma markers NB84 and TH with passaging (data not shown). Thus, primary cells from high-risk neuroblastoma self-renew to a greater extent than their low-risk counterparts.

Tumor spheres from high-risk neuroblastoma exhibit much less differentiation potential than tumor spheres from low-risk neuroblastoma. Because neuroblastoma is a tumor thought to be of embryonic neural crest origin, we used the conditions used to differentiate skin-derived precursor cells (SKP), a normal neural crest–derived human progenitor cell, to test the differentiation potential of our primary tumor spheres (19). After culture in differentiation medium for 2 weeks, immunocytochemistry was performed on tumor spheres using antibodies to the sympathetic neuron markers TH, βIII-tubulin, and NFM; the progenitor marker nestin; and the glial cell markers s100β, GFAP, and GalC. Cells from both low- and high-risk neuroblastoma tumor spheres retained expression of the neuroblastoma markers NB84 and TH under these conditions (Fig. 3A). Tumor spheres from all neuroblastoma tumor clinical risk phenotypes expressed the neuronal marker βIII-tubulin and continued to express nestin (Fig. 3B). Although the neurogenic conditions under which we cultured our tumor spheres were not designed to be permissive for differentiation of cells along glial lineages, we observed rare, spontaneous Schwann-like cells that immunostained with the glial lineage markers s100β, GFAP, and GalC, in high-risk neuroblastoma bone marrow and tumor

Figure 1. Phenotype of cultured primary neuroblastoma tumors of different disease phenotypes grown in serum-free culture conditions containing bFGF and EGF. Sphere-like clusters formed in the majority of tumor phenotypes after dissociation. A, after passaging, high-risk tumor spheres from primary tumors and bone marrow (BM) aspirates were capable of reforming and growing as spheres, with the exception of a single high-risk tumor sample that acquired adherent growth characteristics upon passaging. Tumors from low-risk neuroblastoma tumors acquired adherent growth characteristics upon passaging. Scale bar, 100 μm. B, undifferentiated primary neuroblastoma tumor spheres from both low- and high-risk groups were immunostained for characteristic markers of neuroblastoma (NB84 and TH) and characteristic SKP/neural crest progenitor markers (fibronectin and nestin). Scale bar, 50 μm.
spheres (data not shown). Schwann-like cells were not observed in tumor spheres from low-risk neuroblastoma patients.

Differentiation assays were performed on whole tumor spheres under neuronal conditions, and differentiation potential was determined by calculating the percentage of tumor spheres that gave rise to TH, βIII-tubulin, or NFM-positive neurons, either as individual neurons or large neuronal networks. Large neuronal networks were defined as spheres with many axonal projections, as observed in Fig. 3B (top, βIII-tubulin). High-risk neuroblastoma tumor spheres (n = 5) showed limited differentiation potential (overall average 1.45 ± 1.45%) when compared with low-risk tumor spheres (n = 5, overall average 39.57 ± 17.37%), with respect to their ability to form large neuronal networks (Fig. 3C). Tumor spheres derived from the bone marrow of four high-risk neuroblastoma patients failed to form neurons or large neuronal networks under any conditions (NB12, NB25, NB61, and NB67; data not shown). We observed extensive neuronal differentiation in low-risk patient sphere cells and little or no differentiation in high-risk patient sphere cells, suggesting that the capacity of neuroblastoma tumor sphere cells to differentiate reflects the clinical phenotype of neuroblastoma tumors.

**Tumor spheres contain chromosomal aberrations observed in neuroblastoma tumors.** To confirm that high-risk tumor sphere lines contained chromosomal aberrations consistent with neuroblastoma, SNP analysis was performed on two primary cell lines from high-risk neuroblastoma; one from a bone marrow metastasis (NB12) and the other from a tumor (NB19). The primary sphere line NB12, passage 3, showed amplification of chromosomal regions in 1p, 7q, 10q, 11q, 15q, and 17q when using a copy number cutoff of >8 (Supplementary Table S1), as previously observed in neuroblastoma tumors (28–30). Gain of 17q is found in over 75% of neuroblastoma samples (31). Interestingly, amplification of 7q and 11q chromosomal regions has not been described in the absence of MYCN amplification (28). However, our sample NB12 contained both 7q and 11q amplifications in the absence of MYCN amplification. Thus, the NB12 primary sphere line contained amplifications of chromosomal regions consistent with neuroblastoma, even upon extended passage. A second primary sphere line (NB19, passage 6) did not contain amplifications at a copy number cutoff of 8 as used in our analysis, but did show amplifications in chromosomal regions 1p, 7q, 11q, and 17q at a cutoff of 5 or 6, as previously observed in neuroblastoma (data not shown; refs. 28–31). These data show that primary sphere cells isolated from high-risk neuroblastoma tumors and bone marrow aspirates contain multiple chromosomal amplifications consistent with those found in neuroblastoma.

As few as 10 cells from bone marrow–derived tumor spheres can form tumors in vivo. Cancer stem cells are defined, in part, as cells with the potential to form tumors upon transplantation into mice and to recapitulate the phenotype of the original tumor (2). We used an orthotopic adrenal murine model for assessing tumor propagation in our cells, because neuroblastoma tumors most frequently arise in the adrenal medulla. Ten to one hundred thousand dissociated high-risk neuroblastoma tumor sphere cells (NB12) were injected into the adrenal fat pads of immunocompromised mice, and the mice assessed for palpable tumors or tumor-associated morbidity. As early as 3 weeks after tumor implantation, microtumors were observed in one of four animals injected with 10^3 cells (Fig. 4A), whereas much larger tumors formed in animals injected with 10^4 to 10^5 (Fig. 4A). By day 48, four of four animals injected with 10 neuroblastoma tumor cells had metastatic tumors resulting in tumor-associated morbidity (Supplementary Tables S2 and S3). These tumors contained cells resembling immature neuroblasts with small refractile cell bodies and a high nuclear to cytoplasmic ratio. Moreover, tumor cells stained positive for the neuroblastoma markers NB84, TH, and the progenitor marker nestin (Fig. 4A; arrowheads). High-risk neuroblastoma tumor sphere cells metastasized to distant sites, including the liver, spleen, contralateral adrenal gland and kidney, and lung, and invaded local organs (Fig. 4B). The time to morbidity decreased according to the cell dose the animals received (Fig. 4C), and all animals that exhibited metastatic disease succumbed to tumors (Supplementary Tables S2 and S3, respectively). The time to morbidity comparing 10 to 10^2 injected cells was significantly different as determined by Wilcoxon statistics test (P = 0.0047). These results indicate that neuroblastoma sphere-forming cells have a high tumorigenic potential.
forming neuroblastoma tumors that metastasize with as few as 10 cells. Tumors were also observed using tumor spheres isolated from two bone marrow aspirates (NB61 and NB67) from high-risk patients who had shown no morphologic evidence of disease on histologic examination of the bone marrow smear (data not shown). Mice injected orthotopically with NB12 and NB67, primary sphere lines from the same patient, exhibited small primary tumors and widely metastatic disease. These animals died as a consequence of tachypnea and metastatic disease, in contrast to animals injected with NB61 sphere cells, which died as a result of tumor-associated morbidity.

To determine whether the tumors could be serially passaged, we followed secondary and tertiary tumor formation in NOD/SCID mice. For these experiments, small sections of tumors that arose from injection of sphere-forming cells from five high-risk patients, four bone marrow metastasis (NB12, NB25, NB61, and NB67) and the other a pretreatment tumor (NB05b), were taken at sacrifice and reimplanted into mice. Secondary tumor formation was observed with all samples and the tumor morphology was similar to their respective primary tumors (data not shown). Secondary tumors that arose were reimplanted, resulting in tertiary tumors whose morphology was similar to the primary tumor (Fig. 4D). These data show that 10 unsorted high-risk neuroblastoma sphere cells reproducibly formed neuroblastoma and could be serially passaged in immunocompromised mice, both of which are hallmarks of cancer stem cells.

Tumor-initiating ability of high-risk tumor spheres can be enriched by cell sorting for the cell surface markers CD24 and CD34. Tumor spheres from high-risk neuroblastoma were examined by flow cytometry to identify subpopulations enriched

![Figure 3](image_url)

Figure 3. High-risk neuroblastoma tumor-derived sphere cells exhibit limited differentiation potential when compared with tumor spheres derived from low-risk neuroblastoma tumors. A and B, tumor spheres were differentiated under neurogenic conditions and immunostained with a variety of neuronal markers. Differentiation was observed in both low- and high-risk tumor spheres as assessed by the neuronal marker βIII-tubulin (B), and retained expression of the neuroblastoma markers NB84 (A), TH (A), and nestin (B). B and C, spheres from low-risk tumors differentiated into large nestin-positive or βIII-tubulin–positive neuronal networks (B, top), whereas spheres from high-risk tumors did not (B, bottom). C, low-risk tumor-derived spheres displayed a greater differentiation potential in their ability to form neuronal networks when compared with high-risk neuroblastoma tumor-derived spheres (NB15 and NB20 are bone marrow– and tumor-derived spheres, respectively, from the same low-risk patient). Scale bar, 50 μm.
for tumor-initiating capacity. Cells from bone marrow–derived high-risk neuroblastoma tumor spheres (NB12) expressed NB84 and the neural crest marker CD271 (p75 neurotrophin receptor; Fig. 5A, top). A large proportion of these cells were also positive for the melanoma TIC enrichment marker CD20 (ref. 7; data not shown), and thus could not account for a rare neuroblastoma TIC, so these were not used as candidate unique identifiers in neuroblastoma. We did not detect expression of CD133/1, the marker of brain and colon cancer TICs (refs. 5, 10; Fig. 5A, top). No Hoechst 33342 “side population” was observed in these cells (data not shown).

Previous reports have used a combination of CD45, a pan-hematopoietic cell marker, as a negative selector and CD56very bright (N-CAM) as a positive selector to detect neuroblastoma cells in bone marrow metastases (32–35). In contrast to published reports, the cells from bone marrow–derived high-risk neuroblastoma tumor spheres were positive for CD45 and lacked N-CAM expression (data not shown). The glycoprotein CD24 was investigated as a candidate unique identifier for the TIC in our neuroblastoma tumor spheres as this antigen has been shown to be expressed on renal cell carcinomas, small-cell lung carcinomas, and neuroblastomas (36, 37). The hematopoietic progenitor marker CD34 was investigated as a potential unique identifier of the TIC in our neuroblastoma tumor spheres because several studies suggested that a small number of neuroblastoma cells in the bone marrow expressed CD34 and might be present in sufficient numbers in autologous bone marrow transplants to cause relapse (38–41). We observed the presence of a small fraction of CD24− (~ 0.24%) and CD34− (~ 3.04%) cells in our high-risk neuroblastoma tumor spheres derived from bone marrow aspirates (Fig. 5A, middle and bottom), respectively, that were otherwise absent in neuroblastoma cell lines or tumor spheres from ganglioneuroma, a more differentiated, benign, and clinically nonaggressive subtype of neuroblastoma (data not shown). A subpopulation of cells within tumor spheres from high-risk tumors expressed CD24 and CD34 (Fig. 5B) and a small proportion of cells (~0.55%) costained for these markers (Fig. 5B). We tested the enrichment capacity of these markers for tumor formation by orthotopic adrenal injection of each population of a CD24+/CD34− double sort (total, CD24+/CD34−, CD24+/CD34−, CD24−/CD34−, and CD24−/CD34− cell populations; NB12, passage 33). Although all cellular fractions formed tumors in

Figure 4. High-risk tumor-derived spheres from a bone marrow metastasis form tumors in SCID/Beige mice with as few as 10 cells. Tumor spheres derived from a high-risk neuroblastoma bone marrow aspirate (NB12) were dissociated and injected orthotopically into the adrenal fat pads of SCID/Beige mice. A, H&E staining shows microtumors formed 3 wk after injection of 100 unselected cells, whereas large tumor masses formed from implantation of 104 cells in the same period. Tumors stained positive immunohistochemically for the neuroblastoma markers NB84 and TH and for the neural progenitor cell marker nestin (arrowheads). *, fat lobules. B, H&E staining shows micrometastasis to the liver (left) and invasion of the adjacent kidney (right). C, the time to morbidity decreased when comparing 10 to 104 injected tumor-derived sphere cells implanted as depicted in the Kaplan-Meier survival curves (P = 0.0047). D, H&E staining shows that the morphology of tertiary tumors (right) was similar to the primary tumor from which the serially passaged tumor-derived sphere cells arose (left).
immunocompromised mice, CD24+/CD34+ cells formed tumors in half the time of all other cellular fractions (19.0 ± 0.0 days compared with 34.0 ± 0.72 days; Fig. 5C), suggesting that the CD24+/CD34+ enriches the growth-forming potential of these bone marrow-derived neuroblastoma tumor spheres. This was supported by statistical analysis using ANOVA with Bonferroni and Tukey tests. The CD24+/CD34+ group was statistically different from all other groups (P < 0.01).
Discussion

Cells enriched in tumor-initiating capacity have recently been identified in a number of solid tumors. In this report, we used culture conditions that support the proliferation and self-renewal of neural crest stem cells to enrich and characterize TICs for neuroblastoma. We show that cells from all risk groups of neuroblastoma can be propagated as neurospheres in minimal medium with bFGF and EGF. Our characterization of these cells support the following conclusions: (a) sphere-forming cells from high-risk tumors expressed markers of neural crest stem cells and clinical markers of neuroblastoma; (b) cells from high-risk tumors self-renewed at a higher frequency than those from low-risk tumors; (c) cells from all tumor risk groups were capable of differentiating into neurons, but only cells from low-risk neuroblastoma tumors formed complex neural networks; (d) cells from high-risk tumors formed metastatic tumors in a murine xenograft model with as few as 10 cells and could be serially passaged in immunocompromised mice; and (e) tumor formation and growth was accelerated by enriching for cells expressing the cell surface markers CD24 and CD34. Isolation of neuroblastoma cells using neural crest stem cell conditions provides for the first time an expandable source of cells from low-risk tumors and from bone marrow metastases from high-risk tumors.

Are the neuroblastoma cells we have isolated from bone marrow metastases cancer stem cells or TICs? These cells indeed have a number of properties of cancer stem cells. They were isolated as sphere-forming cells in medium that supports stem cell growth, without going through the crisis stage that typically accompanies the generation of immortalized cell lines. Similar to other cancer stem cells, neuroblastoma sphere-forming cells from all tumor risk groups were capable of self-renewal and differentiation into the cell types observed in neuroblastoma including neurons, and formed tumors that could be serially passaged in immunocompromised mice and that recapitulated the original phenotype of the tumor. Also, similar to other cancer stem cell populations, the frequency of TICs we found was much higher than established cell lines derived from neuroblastoma. In our case, as few as 10 cells from dissociated spheres from high-risk tumors were capable of neuroblastoma formation in SCID/Beige mice, which is 5 logs fewer than the amount of cells from immortalized neuroblastoma cell lines that have been reported to form tumors at between $1 \times 10^6$ and $4 \times 10^7$ injected cells (22, 42, 43). Neuroblastoma bone marrow TICs do not seem to contain a hierarchical organization of cells whereby a subpopulation of tumor cells expressing a distinct cell surface marker contains all of the tumorigenic potential, as has been reported for some cancer stem cells, including leukemia, colon, and brain. We do not yet know whether this is a fundamental biological difference between developmental tumors such as neuroblastoma compared with adult tumors or whether this is an inherent property associated with metastatic disease sites. Recently, however, Kelly and colleagues showed that as few as 10 unsorted cells from human acute myeloid leukemia formed tumors in NOD/scid mice (44), and based on this finding, suggested that tumors need not contain a rare or even hierarchical tumorigenic subpopulation. We therefore suggest that neuroblastoma cells that metastasize to the bone marrow are highly enriched in TIC potential compared with the tumor from which they arose, and that no hierarchical organization exists for neuroblastoma in this niche. Our data provide the first example of primary human solid tumor TICs forming tumors with as few as 10 unsorted cells, and suggest that relative to other solid tumor cells, a high proportion of cells in high-risk neuroblastoma bone marrow metastases are tumorigenic.

What is the significance of finding highly tumorigenic cells in the bone marrow from relapsed neuroblastoma patients? The detection of tumor cells in the bone marrow is a prerequisite for correct risk stratification and monitoring of the response of neuroblastoma patients to therapy (17). Neuroblastomas in the bone marrow typically appear as clumps of cells closely adhering to each other, expressing neuroblastoma, neuronal, or neural crest markers such as NB84, CD56, TH, and GD2 (35, 45–47). We isolated primary sphere-forming lines by disaggregating these cells and allowing them to form spheres in medium that supports neural crest stem cells. The primary cell lines from the spheres expressed the neural crest markers nestin, vimentin, and fibronectin and the neuroblastoma markers NB84 and TH. However, they differed from the markers expressed by cells in the aggregates of bone marrow from neuroblastoma patients in that they expressed the hematopoietic marker CD45. The expression of both neural and mesenchymal markers on primary neuroblastoma sphere-forming lines, which also exhibit chromosomal aberrations characteristic of neuroblastoma confirming their origin, suggests that metastatic cells can adapt characteristics of the cells in the particular niche they reside. The expression in neuroblastoma TICs of CD34, a marker of hematopoietic stem and progenitor cells that has been used as a positive selection marker in the preparation of autologous peripheral stem cell products for transplantation, may have clinical significance. There are reports that neuroblastoma cells, like our sphere-forming cells from the bone marrow of high-risk tumors, may express CD34 (38, 39), suggesting that patients who relapse after transplantation with CD34+ selected bone marrow or stem cells may have residual TICs expressing this antigen. Finding highly tumorigenic cells in the bone marrow from relapsed neuroblastoma patients may be important for developing residual disease treatment strategies and understanding how metastatic neuroblastoma cells adapt to their microenvironment.

There are several potential applications of our findings. First, we can readily detect highly tumorigenic cells in the bone marrow of patients at risk of relapse. We have isolated neuroblastoma TICs from the bone marrow of patients in relapse as well as in remission with a high chance of relapse, suggesting that this population may predict clinical behavior and thus, may serve as a biomarker for minimal residual disease. In this regard, as few as 10 neuroblastoma tumor sphere cells isolated from a patient showing no morphologic evidence of disease (NB61) formed tumors in immunocompromised mice; this patient clinically relapsed ~6.5 months after the tumor spheres were isolated. These data highlight the potential of our assay for testing treatment efficacy in patients with high-risk neuroblastoma and in detecting minimal residual disease in these patients. By comparing the biology of neuroblastoma TICs isolated from newly diagnosed patients to TICs isolated from stable relapse and remission patients, we may better understand the cellular and molecular events leading to relapse and disease progression in neuroblastoma. We do not know, however, whether this population will predict relapse or poor survival. This analysis awaits clinical follow-up. Second, neuroblastoma TICs could be used to understand the developmental origins of neuroblastoma by comparing their biology to a normal human counterpart such as skin-derived precursor cells, which like neuroblastoma are of neural crest origin (19). In this regard, undifferentiated and differentiated neuroblastoma TICs and SKPs share expression of many neural and progenitor markers, including...
vimentin, fibronectin, TH, β3I-tubulin, NFM, NB84, nestin, S100β, GFAP, and GalC. Third, we propose the use for neuroblastoma TICs for drug discovery, as we believe such cells are likely to be integral in the treatment failure of patients with high-risk neuroblastoma. We have begun to use the sphere-forming cells from high-risk neuroblastoma in high-throughput screens to identify known drugs that induce the death of these cells but not nontransformed pediatric neural precursor cells, and have identified both known chemotherapeutic agents and new classes of anticancer agents that selectively kill neuroblastoma TICs.13 Thus, targeting TICs from individual patients may eventually prove to be effective for treating neuroblastoma. Because we believe neuroblastoma TICs are responsible for the progression and treatment failure of patients with neuroblastoma, targeted treatments in conjunction with conventional chemotherapy may be effective at preventing residual disease, which leads to relapse and progression of neuroblastoma.

Acknowledgments


Grant support: Operating grants from the James Birrell Neuroblastoma Research Fund and the Hospital for Sick Children Foundation, National Cancer Institute of Canada; and fellowships from the National Cancer Institute of Canada (Terry Fox Foundation; L.M. Hansford), Canadian Institutes of Health Research (K.M. Smith), and the James Birrell Neuroblastoma Research Fund/Canadian Institutes of Health Research (L. Zhang). F.D. Miller, M.S. Irwin, and D.R. Kaplan are recipients of Canada Research Chairs. A.E. McKe and C.J. Thiele are supported by the Intramural Research Program in the Center for Cancer Research at the National Cancer Institute, Bethesda, MD.

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References

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