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

Loen M. Hansford,1 Amy E. McKee,2 Libo Zhang,2 Rani E. George,10 J. Ted Gerstle,3 Paul S. Thorner,4,7 Kristen M. Smith,1,4 A. Thomas Look,10 Herman Yeger,1 Freda D. Miller,5,6 Meredith S. Irwin,1,6 Carol J. Thiele,1 and David R. Kaplan1,8

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 neuroblas-

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: David R. Kaplan, Cell Biology Program, Hospital for Sick Children, Room 12-314, Floor 12, Toronto Medical Discovery Tower, 101 College Street, Toronto, Ontario, Canada M5G 1L7. Phone: 416-813-7654, Fax: 416-813-2212 E-mail: dkaplan@sickkids.ca.

©2007 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-07-0718

Cancer Res 2007; 67: (23). December 1, 2007 11234 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2007 American Association for Cancer Research.
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 consented 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), 1% N2 supplement (Life Technologies), 10 ng/mL bFGF, and 15% FBS for 5 to 10 days and cultured in neurobasal medium. Expanded in medium containing DMEM-F12, 3:1, 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 each week. Tumor spheres were passaged by mechanical dissociation and split 1:6 with 50% fresh proliferation medium each week. Tumor spheres was determined to be the limit of sphere formation in liquid culture.

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 × 10² 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-d-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 neurobasal medium (Invitrogen) containing 2% B27 supplement, 1% FBS, 1% N2 supplement (Life Technologies), 16 ug/mL nerve growth factor (Cedarlane), and 8 ng/μL NT3 (Peprotech; differentiation medium) for an additional 14 days. Half the medium was replaced with fresh differentiation medium every other day for the course of the experiment.

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-β III-tubulin monoclonal (1:500; Tuj1 clone; Covance); anti–neurofilament-M monoclonal (NF-M; 1:200; Chemicon); s100B monoclonal (1:1,000; Sigma); anti–glial fibrillary acidic protein (GFAP) polyclonal (1:200; DAKO); galactocerebroside C (GaC) polyclonal (1:200; Chemicon); anti-nestin monoclonal (1:400; Chemicon); anti-nestin polyclonal (1:400; Chemicon); and anti-fibronectin polyclonal (1:1000; 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.

Cytogetic 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).11 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 <1.2 copies. Homozygous loss was defined as an inferred copy number of <0.3 in at least two consecutive SNPs.

In vivo assays of tumorigenicity and immunohistochemistry. In vivo tumor assays using 4- to 5-week-old female SCID/Beige mice (Tacomic) were performed as described (22). Mice were housed in pathogen-free conditions and cared for in accordance with the NIH Animal Care and Use Committee (protocol PB-023). In vitro passaged primary neuroblastoma sphere-forming cells (passages 4–5) were harvested and brought to final cell densities of 3 × 10⁶/mL, 3 × 10⁶/mL, 3 × 10⁷/mL, or 3 × 10⁷/mL in HBSS for both orthotopic adrenal and heterotopic s.c. injections. Cells were kept at 4°C until ready for injection and mixed 1:3 with basement membrane extract (Trevigen) just before injection (final cell doses 10⁵, 10⁶, 10⁶, and 10⁷). Orthotopic and heterotopic injections were performed as previously described (22). Animals were monitored thrice weekly for evidence of tumor formation by palpation and associated morbidity. All mice that were sacrificed underwent complete necropsy examination and tissues fixed in 10% formalin for 24 h before paraffin embedding and staining with H&E. The end points evaluated were the percent tumor take (i.e., the percentage of animals that developed primary tumors) and tumor latency (i.e., the time from tumor cell injection to tumor-associated morbidity).

An alternative in vivo assay was used to establish secondary and tertiary tumor engraftment because of latency to tumor formation in our orthotopic adrenal model of tumorigenicity. Four- to 5-week-old nonobese diabetic (NOD)/SCID mice (Charles River Laboratories) were housed in pathogen-free conditions and cared for in accordance with SickKids Hospital’s Animal Care Committee. Fifty spheres or 10⁶ cells from in vitro passaged primary neuroblastoma cells were harvested and resuspended in proliferation medium immediately before injection, the cell suspension was mixed 1:1 with Matrigel basement membrane matrix (BD Biosciences), and mice were given an injection s.c. into the inguinal fat pad. Tumor growth was measured weekly in two dimensions using a digital caliper, and mice were sacrificed when the tumor size reached 0.5 mm³. Formalin-fixed, paraffin-embedded tumor samples were prepared for immunohistochemical analysis by standard protocols and stained for TH (1:150) and NB84 (1:50), the latter using trypsin antigen retrieval as described by the manufacturer. Sections were incubated with a biotinylated anti-goat, mouse, rabbit secondary antibody (1:500; DAKO), and tertiary staining with peroxidase-conjugated streptavidin (1:500; DAKO) was performed. The immune complex was visualized using 3,3'-diaminobenzidine (DAKO) as a chromogen and hematoxylin as a counterstain.

Flow cytometry and fluorescence-activated cell sorting. If unconjugated primary antibodies were used, cultured primary cells were initially incubated with human IgG (1:56) followed by incubation with the primary antibody and secondary staining with the Alexa 488–conjugated goat anti-mouse secondary antibody (Molecular Probes, Invitrogen). If conjugated primary antibodies were used, cultured primary cells were incubated in the primary antibody only. All cells were then fixed in 2% paraformaldehyde. Approximately 10⁵ cells were stained and analyzed on a Becton Dickinson FACSCalibur four-color analyzer. Monoclonal antibodies against human CD133/1 (biotin-conjugated) and CD271 (p75 neurotrophin receptor, mouse monoclonal) were used to establish secondary and tertiary tumor engraftment because of latency to tumor formation in our orthotopic adrenal model of tumorigenicity. Four- to 5-week-old nonobese diabetic (NOD)/SCID mice (Charles River Laboratories) were housed in pathogen-free conditions and cared for in accordance with SickKids Hospital’s Animal Care Committee. Fifty spheres or 10⁶ cells from in vitro passaged primary neuroblastoma cells were harvested and resuspended in proliferation medium immediately before injection, the cell suspension was mixed 1:1 with Matrigel basement membrane matrix (BD Biosciences), and mice were given an injection s.c. into the inguinal fat pad. Tumor growth was measured weekly in two dimensions using a digital caliper, and mice were sacrificed when the tumor size reached 0.5 mm³.

11 http://biosun1.harvard.edu/complab/dchip
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 χ2 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>
<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>
</tr>
</thead>
<tbody>
<tr>
<td>NB04</td>
<td>F</td>
<td>Greater</td>
<td>High</td>
<td>Relapse bone marrow</td>
</tr>
<tr>
<td>NB05b</td>
<td>M</td>
<td>Greater</td>
<td>High</td>
<td>Tumor</td>
</tr>
<tr>
<td>NB07</td>
<td>F</td>
<td>Greater</td>
<td>Low</td>
<td>Tumor</td>
</tr>
<tr>
<td>NB08</td>
<td>M</td>
<td>Greater</td>
<td>High</td>
<td>Relapse bone marrow</td>
</tr>
<tr>
<td>NB12</td>
<td>F</td>
<td>Greater</td>
<td>High</td>
<td>Relapse bone marrow</td>
</tr>
<tr>
<td>NB13</td>
<td>M</td>
<td>Greater</td>
<td>Low</td>
<td>Tumor</td>
</tr>
<tr>
<td>NB14</td>
<td>F</td>
<td>Less</td>
<td>Low</td>
<td>Tumor</td>
</tr>
<tr>
<td>NB15</td>
<td>F</td>
<td>Greater</td>
<td>Low</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>NB17</td>
<td>F</td>
<td>Greater</td>
<td>Low</td>
<td>Tumor</td>
</tr>
<tr>
<td>NB19</td>
<td>M</td>
<td>Greater</td>
<td>High</td>
<td>Tumor</td>
</tr>
<tr>
<td>NB20</td>
<td>F</td>
<td>Greater</td>
<td>Low</td>
<td>Tumor</td>
</tr>
<tr>
<td>NB24</td>
<td>M</td>
<td>Less</td>
<td>Low*</td>
<td>Liver metastasis</td>
</tr>
<tr>
<td>NB25</td>
<td>M</td>
<td>Greater</td>
<td>High</td>
<td>Relapse bone marrow</td>
</tr>
<tr>
<td>NB32</td>
<td>F</td>
<td>Less</td>
<td>High</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>NB61</td>
<td>M</td>
<td>Greater</td>
<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* MTCN-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 passaged 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 passaged 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 $\chi^2$ 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 passing (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 $\alpha$-SMA, 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 $\alpha$-SMA, 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 \(1.45 \pm 1.45\%\)) when compared with low-risk tumor spheres \((n = 5\); overall average \(39.57 \pm 17.37\%\)), 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 \(10^2\) cells (Fig. 4A), whereas much larger tumors formed in animals injected with \(10^3\) 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 spherelines 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.
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 CD56<sup>very bright</sup> (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<sup>−</sup> (~0.24%) and CD34<sup>−</sup> (~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<sup>−</sup>/CD34<sup>−</sup> double sort (total, CD24<sup>+/CD34</sup><sup>−</sup>, CD24<sup>−</sup>/CD34<sup>+</sup>, CD24<sup>−</sup>/CD34<sup>+</sup>, and CD24<sup>−</sup>/CD34<sup>−</sup> cell populations; NB12, passage 33). Although all cellular fractions formed tumors in...
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).

Figure 5. High-risk neuroblastoma tumor-derived sphere cells from bone marrow metastases can be enriched for tumorigenic potential by sorting for the cell surface markers CD24 and CD34. A, flow cytometry analysis of high-risk neuroblastoma tumor-derived sphere cells. Cells were highly positive for the clinical neuroblastoma marker NB84 and for the neural crest progenitor marker CD271/p75, and CD133/1 expression was not detected (top). A small fraction (~0.24%) of neuroblastoma tumor-derived sphere cells from two different patients (NB12 and NB25) stained positive for the metastatic marker CD24 (middle). Similarly, a small fraction (~3.04%) of high-risk neuroblastoma tumor-derived sphere cells from two different patients (NB12 and NB25) stained positive for the progenitor cell marker CD34 (bottom). B, small numbers (%) of brightly positive CD24+ and CD34+ cells were observed in neuroblastoma tumor spheres by immunocytochemistry. Very small numbers (~0.55%) of cells costained brightly positive CD24+/CD34+ in the high-risk neuroblastoma tumor-derived spheres (NB12) by immunocytochemistry. C, suprarenal implantation of the CD24+/CD34+ cells from high-risk neuroblastoma tumor-derived spheres (NB12) into immunocompromised mice resulted in a statistically significant acceleration in tumor-associated morbidity compared with the unselected tumor-derived sphere cells or those expressing CD24 or CD34 alone (P < 0.01; statistical analysis using ANOVA with Bonferroni and Tukey tests). Scale bar, 50 μm.
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). Neuroblastosomas 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 tumors 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 GaC. 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.12 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: The 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. McKee and C.J. Thiele are supported by the Intramural Research Program in the Center for Cancer Research at the National Cancer Institute, Bethesda, MD.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

14. Beiske K, Ambros PF, Burchill SA, Cheung IY, Swerts
16. Beiske K, Ambros PF, Burchill SA, Cheung IY, Swerts
Neuroblastoma Cells Isolated from Bone Marrow Metastases Contain a Naturally Enriched Tumor-Initiating Cell

Loen M. Hansford, Amy E. McKee, Libo Zhang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/23/11234

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2007/11/21/67.23.11234.DC1

Cited articles
This article cites 47 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/23/11234.full.html#ref-list-1

Citing articles
This article has been cited by 19 HighWire-hosted articles. Access the articles at:
/content/67/23/11234.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.