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 neuroblasto-
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, 3.3 mM HEPES, 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, 3.3 mM HEPES, 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, 3.3 mM HEPES, 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, 3.3 mM HEPES, 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, 3.3 mM HEPES, 10% fetal bovine serum (FBS; HyClone) to inhibit enzyme activity.

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, 3.3 mM HEPES, 10% fetal bovine serum (FBS; HyClone), 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 μg/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-βIII-tubulin monoclonal (1:500; TuJ1 clone; Covance); anti-neurofilament-M polyclonal (NF-M; 1:200; Chemicon; s100β) monoclonal (1:1,000; Sigma); anti-glial fibrillary acidic protein (GFAP) polyclonal (1:200; DAKO); galactocerebroside C (GalC) polyclonal (1:200; Chemicon); anti-neurofascin monoclonal (1:400; Chemicon); anti-neurofilament monoclonal (1:400; Chemicon); and anti-filamin monoclonal (1:400; 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.

Cyto genetic 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 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 (Taconic) were performed as described (22). Mice were housed in pathogen-free conditions and cared for in accordance with the NIH Animal and Care 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 × 10⁵/ml, 3 × 10⁶/ml, 3 × 10⁷/ml, or 3 × 10⁸/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 10⁶, 10⁷, 10⁸, and 10⁹). 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 10⁴ cells from in vitro passaged primary neuroblastoma cells were harvested and resuspended in proliferation medium, frozen 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 cm³.

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 antibody and secondary staining with the Alexa 488–conjugated goat anti-goat (1:1,000) and Alexa 555–conjugated goat-anti-rabbit (1:1,000; Molecular Probes). 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 FACS Calibur four-color analyzer. Monoclonal antibodies against human CD133/1 (biotin-conjugated) and CD271 (p75 neurotrophin

11 http://biosun1.harvard.edu/complab/dchip

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For the Fisher’s exact test with procedure FREQ.

**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. Isotyping for human CD24 (phycoerythrin) was performed using the Fisher’s exact test with procedure FREQ. Statistical significance was established at P < 0.05.

<table>
<thead>
<tr>
<th>Sample name</th>
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<td>Tumor</td>
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</tr>
<tr>
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</tr>
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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 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 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
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 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
spheres contained chromosomal aberrations consistent with neuroblastoma, SNP analysis was performed on two pri-
mary 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 amplifica-
tion 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). Interestsingly, 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 chro-
osomal 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 chromo-
somal 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 106 cells (Fig. 4A), whereas much larger
tumors formed in animals injected with 103 to 105 (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. 4B).
High-risk neuroblastoma tumor 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
doze 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 102
injected cells was significantly different as determined by Wilcoxon
statistics test (P = 0.0047). These results indicate that neuroblas-
toma 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.

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

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**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 105 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).

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). 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 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, β3-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.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 progression and relapse of neuroblastoma.

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