Comparison of the Side Populations in Pretreatment and Postrelapse Neuroblastoma Cell Lines

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Abstract
Cancer stem–like cells have been identified in both primary tumors and in cell lines and seem to have a high degree of inherent resistance to traditional chemotherapeutic agents. Relapsed cancers including neuroblastoma are generally chemotherapy-resistant and carry a very poor prognosis. We investigated the side populations of three pairs of neuroblastoma cell lines derived from single patients at the time of their initial presentation and then at relapse after multimodality therapy. We found that the size of the side populations in the relapsed cell lines was significantly increased compared with its paired pretreatment cell line. In addition, these side population cells showed increased proliferation and were significantly more efficient at forming colonies in soft agar than their prerelapse pair. Gene expression analysis of the stem cell genes NANOG and POU5F1 (Oct3/4) showed increased expression in the unsorted relapsed cell lines compared with pretreatment lines as well as in the side populations of the relapsed versus prerelapse cell line pairs. The increased size, proliferative ability, and colony-forming efficiency of the side populations of the postrelapse cell lines demonstrated in this study suggest that a population of stemlike cells is not being efficiently targeted by conventional therapy and implies that strategies to specifically target the stem cell fraction of neuroblastomas are needed to improve outcomes in this devastating childhood disease.

Introduction
Neuroblastoma is the most common extracranial solid tumor of childhood, accounting for 15% of pediatric cancer fatalities annually. Despite advances in pediatric cancer therapy, cure rates for high-risk neuroblastoma continue to be poor, with a less than 40% long-term survival rate despite intensive treatment including chemoradiotherapy and bone marrow transplantation. On recurrence, neuroblastoma carries a high level of therapy resistance and an extremely poor prognosis with an almost universally fatal outcome [1–3].

Most cancers have been shown to contain a subpopulation of cells that exhibit stem cell–like properties. This observation has led to the formulation of the cancer stem cell hypothesis that states that tumors contain a small population of cells that have the capacity to self-renew and to differentiate, thus giving rise to the heterogeneous tumor phenotype. Although evidence suggests the existence of cancer stem cells, the hypothesis remains controversial, particularly with regard to solid tumors [4–6]. The significance of the cancer stem cell hypothesis is that reports have shown that the putative cancer stem cells have increased chemoresistance and are likely responsible for clinical relapse [7]. Thus, eradicating tumors may be difficult because conventional treatments target the bulk of the tumor cells, leaving behind the cancer stem–like cells, which, like their normal counterparts, maintain the tumor tissue. According to this hypothesis, identifying and eliminating cancer stem cells will be necessary to develop more effective cancer treatments.

The cancer stem cell hypothesis was originally proposed and has been most thoroughly studied in hematological malignancies [8,9]. However,
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mounting evidence supports the existence of cancer stem–like cells in solid tumors as well [10–19].

Identification of cancer stem–like cells has been performed using specific differentially expressed markers and side population (SP) analysis. In acute myelogenous leukemia (AML), CD34+ and CD38+ cells are highly enriched for tumor-initiating potential [8,9]. CD133, the defining member of the novel pentaspan transmembrane glycoprotein family, has proven to be useful in the identification of cancer stem cells in brain and colon cancers [13,14,16,17], and CD44high/CD24low/Lin− cells have been shown to isolate stemlike cells from human breast cancers [11]. Because selectively expressed markers have not been identified for many cancers, researchers have also used the differential ability of certain cells to exclude DNA binding dyes such as Hoechst 33342 and DyeCycle Violet to identify subpopulations enriched for cells with stemlike characteristics. Cells with the capacity to efflux these dyes were first identified in mouse bone marrow and were called SP cells because they fell to the “side” of the positively stained cells in flow cytometry analysis plots [20]. Since this original discovery, SP cells have been identified in a wide variety of normal tissues [21–24], tumors, and cell lines [7,25–32]. The mechanism regulating this efflux seems to be conferred, in part, through the expression of ATP binding cassette protein (ABC) transporters [33].

In neuroblastoma, cancer stem–like cells have been found in both primary tumor specimens and established cell lines and comprise 4% to 37% of the total population [7]. Previous studies measuring the expression of CD133 and CD117 have suggested a two-fold difference in the number of cancer stem cells in metastatic versus versus localized tumors (33% vs 14%) and in progressive tumors versus tumors in which remission was achieved (35% vs 18%) [34]. Additional studies have shown that many neuroblastoma cells express numerous different primitive neural markers including CD34, ABCG2, and nestin [7,35–37]. Results of SP analysis have been complex, with one report showing that 65% of primary tumors contain a stemlike SP, whereas another failed to identify a SP in neuroblastoma cells isolated from bone marrow metastases [7,15], suggesting that the significance of the SP in neuroblastoma remains unclear and warrants further evaluation.

Given the cancer stem cell hypothesis, it would seem logical that the proportion of SP cells within a particular cancer cell line would correlate with its tumorigenicity; however, to date, there have been no reports demonstrating this in cell lines. Here, we show for the first time that neuroblastoma cell lines have a stable SP and that the SP in postrelapse cell lines is both increased in size and shows increased tumor-initiating potential at 5 μg per 5 × 10⁶ cells as previously described [39], and the SP was identified on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). The location of the SP on the flow histogram was confirmed using the ABCG2 inhibitor fumitremorgin C (Sigma, St Louis, MO) during initial analyses.

Flow Cytometry—Cell Sorting

Vybrant DyeCycle Violet stain (Invitrogen) was added to the cell suspension at 10 μM per 5 × 10⁶ cells as previously described [39]. After identification of the SP, the SP and non-SP cells were sorted using the BD FACS Aria Flow Cytometer (BD Biosciences). The SP cells and non-SP cells were then used for gene expression analysis as previously described and for in vitro growth, colony-forming, and cell proliferation assays.

Materials and Methods

Cell Lines

We used three paired neuroblastoma cell lines for in vitro investigations. These were a gift from the laboratory of Dr C. Patrick Reynolds from the Children’s Hospital of Los Angeles. These include SMS-KCN and SMS-KCNR, SMS-KAN and SMS-KANR, and CHLA-122 and CHLA-136. Each pair of cell lines was derived from the same patient at the time of initial surgery and again at the time of relapse after chemotherapy. All cell lines were derived from original tumors and have not been otherwise modified. Cell lines were cultured in either RPMI 1640 or Iscove medium supplemented with fetal bovine serum as previously described [38]. All three pairs were used for SP percentage analysis; SMS-KCN and SMS-KCNR and CHLA-122 and CHLA-136 were used for all subsequent experiments.

Flow Cytometry—Analysis

Hoechst 33422 (Invitrogen, Carlsbad, CA) was added to the solution of cells at 5 μg per 5 × 10⁶ cells as previously described [39], and the SP was identified on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). The location of the SP on the flow histogram was confirmed using the ABCG2 inhibitor fumitremorgin C (Sigma, St Louis, MO) during initial analyses.

In Vitro Growth Assay

Equal numbers (2.5 × 10⁶) of sorted SP and non-SP cells were placed in growth medium and were allowed to proliferate under the same conditions. Samples were counted using the Beckman Coulter Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Inc, Brea, CA) every third to fifth day to estimate the cell population. After 4 weeks, cell subpopulations were counted and reanalyzed by flow cytometry.

Colony-Forming Assay

Equal numbers (2000) of sorted cells were suspended in growth medium with 3% agarose and were then plated in six-well plates. After 7 days, the number of colonies per 4x high-power field was counted in 20 random fields to determine the mean colony-forming units of each sample.

Real-time Reverse Transcription–Polymerase Chain Reaction

We measured gene expression levels of six genes: ABC transporter genes ABCA3, ABCB1, ABCG1, and ABCG2 and the stem cell pluripotency transcription factors NANOG and POU5F1. 18S Ribosomal RNA was used as an endogenous control. Total RNA was extracted using the TRIzol Reagent (Molecular Research Center, Inc, Cincinnati, OH). Gene expression was measured using a one-step real-time polymerase chain reaction on the ABI 7500 Real-Time PCR System (Applied Biosystems, Inc, Carlsbad, CA). Relative differences between samples were determined using the ΔΔCt method according to the manufacturer’s instructions.

Statistical Analysis

Comparison between two means was performed using the Student’s t test. P < .05 was considered statistically significant. All statistical analyses were performed using Prism 5.0 and/or InStat 3.1a software (GraphPad Software, Inc, La Jolla, CA).

Results

Postrelapse Neuroblastoma Cell Lines Contain a Stably Increased SP

We performed SP analysis on the three pairs of case-controlled prerelapse and postrelapse neuroblastoma cell lines. In each case, we found
that the postrelapse cell lines maintained a significantly increased SP compared with its paired pretreatment cell line: SMS-KANR 13.5% ± 1.0% versus SMS-KAN 9.6% ± 0.7% (P < .01), SMS-KCNR 11.1% ± 1.5% versus SMS-KCN 6.7% ± 0.9% (P < .01), and CHLA-136 3.2% ± 0.8% versus CHLA-122 2.0% ± 0.4% (P < .01; Figure 1). For several months and multiple repeated experiments, the percentage of cells in the SP remained relatively stable for each cell line. This consistency was found both during analysis on the BD LSRII and during sorting on the BD FACS Aria Flow Cytometer. These results were also consistent regardless of whether cells were cultured in serum-free medium or in medium supplemented with fetal bovine serum.

**SP Cells Show Increased Proliferation Rate and Colony-Forming Ability**

To determine whether the non-SP and SP cells show inherent differences in growth potential, we regrew cells after sorting. We found that only the SP cells could both proliferate and recapitulate the original cell line. During a 4-week period, the non-SP cells showed minimal expansion. In contrast, during the same 4-week period, the total number of the SP cells increased in all cell lines, ranging from a doubling to a 19-fold increase (Figure 2). Not only did the SP cells proliferate but also, after several weeks, they morphologically resembled the original cell line from which they were derived. These cells were reanalyzed to determine the percentage of SP versus non-SP cells after 4 weeks. The non-SP cells remained without any evidence of a SP in all cases, whereas the SP cells differentiated to include both SP and non-SP populations. The SMS-KCN SP differentiated to 2% SP and 98% non-SP, whereas the SMS-KCNR SP had 11% SP and 89% non-SP, which was similar to the original cell line composition (data not shown).

We also investigated the ability of the sorted SP and non-SP cells to form colonies in soft agar. After 1 week, the sorted SP cells from the cell
Increased Expression of Stem Cell–Related Genes in Postrelapse Cell Lines

We sought to determine whether the postrelapse cells had increased expression of genes related to stem cell function. We measured the expression of two critical stem cell regulatory genes, *NANOG* and *POU5F1* (previously known as *Oct3/4*), in our three cell line pairs. In each case, we found significantly increased gene expression levels in the postrelapse line compared with its paired prerelease line. This expression difference was 1.6- to 2.9-fold for *NANOG* and nearly 2-fold for *POU5F1* (Figure 5, A and C). We also examined the expression in the SP cells compared with that in the non-SP cells and found that these genes were consistently increased in the SP cells compared with the non-SP fraction. Postrelapse SP cells showed a 1.5- to 8-fold increased expression of both genes compared with the prerelease SP cells from their paired cell line (Figure 5, B and D).

**Discussion**

Relapsed neuroblastoma remains one of the greatest challenges in pediatric oncology today; despite decades of research, relapsed high-risk neuroblastoma remains almost universally fatal. The cancer stem cell hypothesis suggests that the difficulty in permanently eradicating tumors may be because conventional treatment regimens preferentially eliminate the bulk of the progeny cells while leaving the cancer stem cells intact. SP analysis has been used successfully in many different cancers to identify a subpopulation of cells that exhibit stemlike properties and that are enriched for tumor-initiating cells. This study presents data describing the SP in several neuroblastoma cell lines and suggests that these SPs are enriched for a population of cells that exhibit stemlike characteristics. Importantly, it is the first study to present and evaluate the SP properties of an *in vitro* pretreatment versus postrelapse model using single-patient derived paired cell lines.

Using this resource, we have shown that the size of the SP increased significantly between the pretreatment and postrelapse cell lines and that these cell lines maintain a relatively constant ratio of SP versus the higher proportion of SP cells found in postrelapse cell lines and the currently prevalent hypothesis that cancer stem cells may be responsible for cancer relapse. We measured the expression of two critical stem cell regulatory genes, *NANOG* and *POU5F1* (previously known as *Oct3/4*), in our three cell line pairs. In each case, we found significantly increased gene expression levels in the postrelapse line compared with its paired prerelease line. This expression difference was 1.6- to 2.9-fold for *NANOG* and nearly 2-fold for *POU5F1* (Figure 5, A and C). We also examined the expression in the SP cells compared with that in the non-SP cells and found that these genes were consistently increased in the SP cells compared with the non-SP fraction. Postrelapse SP cells showed a 1.5- to 8-fold increased expression of both genes compared with the prerelease SP cells from their paired cell line (Figure 5, B and D).

**Figure 3.** Results of soft agar colony-forming assays. SP cells have significantly increased colony-forming ability compared with the non-SP cells in every case. *P* < .01 for all four cell lines tested. Results shown are the means of at least three experiments.

**Figure 4.** Comparison of growth and colony formation of prerelease versus postrelapse SP cells. (A and B) Increased proliferative ability of the postrelapse SP cells from the SMS-KCNR (A) and CHLA-136 (B) cell lines compared with their paired prerelease cell lines, respectively. (C) Postrelapse SP cells show increased colony formation efficiency compared with the prerelease SP cells from their paired prerelease cell lines. Results shown are means of at least three experiments. *P* < .01.
non-SP cells over time. In addition, when reanalyzed after several weeks in culture, the SP cells had phenotypically recapitulated the original cell line in every case, whereas the non-SP cells showed little proliferative ability and had not regenerated a SP. These findings, combined with the data showing the SP cells’ increased capacity to form colonies in soft agar, represent what is to our knowledge the first study to show a correlation between the percentage of SP cells within a cell line and its tumorigenicity. Studies to confirm these findings in vivo using xenograft models are planned.

NANOG and POU5F1, along with SOX2, have been shown to be the critical regulators of embryonic stem cell maintenance [40] and have recently been shown to be overexpressed in several different cancers [41,42]. Neither gene has been previously characterized in neuroblastoma. We found that the messenger RNA expressions of NANOG and POU5F1 as well as that of the ABCG2 transporter were significantly increased in the postrelapse cell lines compared with its paired pretreatment cell lines. Both NANOG and POU5F1 also showed increased expression in the postrelapse SP cells compared with the prerelapse SP cells, suggesting that the pathways that control stem cell maintenance and expansion may play an important role in neuroblastoma relapse. Given that the messenger RNA expression of these stem cell regulators is increased in the relapsed cell lines and in the SP cell subpopulation, it seems logical that possible mechanisms for the increase in the size of the SPs include dysregulation of these pathways.

This work represents the first study to characterize the SPs in paired pretreatment and relapsed neuroblastoma cell lines. Our study supports the hypothesis that failure to eradicate the underlying stem cell population in neuroblastoma contributes to its relapse and suggests that novel agents that specifically target neuroblastoma cancer stem cells are needed if we are to improve outcomes in high-risk neuroblastoma.

References