Melanoma Stem Cells: The Dark Seed of Melanoma
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ABSTRACT

Cells with stem-cell markers and features have recently been identified in melanoma tissues and cell lines. Melanoma stem-like cells possess many traits of tumor-initiating or tumor stem cells including self-renewal capacity, high tumorigenicity, and differentiation into various mesenchymal lineages, including melanocytic cells. Four subpopulations of melanoma-initiating cells have been distinguished: CD20⁺, CD133⁺, label-retaining or slow-cycling cells, and side-population cells with high efflux activities. Whether these are distinct or overlapping populations is currently under investigation. Ongoing studies are dissecting and characterizing the hierarchy of these subpopulations within a malignant lesion. Understanding these and the dynamics of clonal dominance will aid in the development of novel therapeutic strategies.

J Clin Oncol 26:2890-2894. © 2008 by American Society of Clinical Oncology

INTRODUCTION

Melanoma development has classically been perceived as a stepwise process in which mature melanocytes in the epidermis progressively acquire genetic mutations in oncogenes or tumor-suppressor genes that provide survival and growth advantages allowing uninhibited proliferation, ultimately leading to metastatic melanoma (Fig 1A). Yet clinically, only 26% of melanomas observed evolve from nevi, and less than 50% are associated with dysplastic nevi. This indicates that the majority of melanomas arise from normal-appearing skin and not from dysplastic nevi, suggesting that melanoma development may not follow the classical linear mode of progression (Fig 1). Thus, although the apparent target of transformation is differentiated melanocytes, melanomas may also be derived from transformed melanocytic stem or progenitor cells (Fig 1B). Yet on the basis of clinical and pathological evidence, melanocyte stem cells the in hair follicles of mice and humans are not the targets of transformation, suggesting that an alternative source of melanocyte-producing cells may be hit. Preliminary studies from our laboratory indicate the existence of a neural-crest stem cell with differentiation potential for melanocytes in the dermis. Whether these cells migrate through the basement membrane to disburse among the basal-layer keratinocytes, and whether they are more prone to transformation than mature melanocytes, remains to be determined.

Cancer stem cells, also known as tumor-initiating cells, have been defined in a variety of human and mouse tumors through known and novel stem-cell markers, as well as stringent self-renewal and tumorigenicity assays. Expression of stem-cell markers and the isolation of stem-like cells has recently been described in melanoma. Here, we briefly summarize our current knowledge of melanoma stem cells. To date, four subpopulations including, CD20⁺, CD133⁺, label-retaining cells, and side-population (SP) cells (Fig 2) have been identified in melanoma tissues and cell that possess stem-cell characteristics, defined as self-renewal capacity, differentiation potential, and high tumorigenicity.

MELANOMA SPHERES

Utilizing an embryonic stem cell–based media, our laboratory has propagated cells from both metastatic melanomas specimens and cell lines that harbor properties of cancer stem cells. Melanoma cells capable of surviving and proliferating in this selective media grew as nonadherent spheres, termed melanoma spheres, similar to the mammospheres and neurospheres generated from breast and brain cancer, respectively. Distinct from their bipolar, spindle-shaped adherent counterparts grown in conventional media, melanoma spheres could differentiate along multiple mesenchymal lineages, including adipocytes, chondrocytes, and osteoblasts. Spheres were more than 10-fold more tumorigenic than adherent cells, could be reisolated after in vivo transplantation, and could persist despite serial cloning, demonstrating self-renewal capacity.

CD20

Although maintaining expression of melanoma-associated markers MCAM and β3 integrin, indicative of their melanocytic origin, a subpopulation of...
Melanoma spheres was found to consistently express CD20, a cell surface marker normally associated with B cells, that was completely absent in the corresponding adherent cells grown in conventional media. CD20+ sphere cells could rapidly proliferate, reform spheres, and differentiate into adipogenic, chondrogenic, and osteogenic lineages. Moreover, CD20+ cells could be identified in melanoma specimens. The frequency of CD20 expression among metastatic melanomas and whether its expression in melanomas can be...
correlated with clinical outcome are currently under investigation. Ongoing studies are also in progress to assess whether elimination of CD20^+ cells from melanomas can abolish tumorigenic potential.

Highly specific anti-CD20 treatments that currently exist may be used to distinctly target these cells. Rituximab, a highly effective anti-CD20 monoclonal antibody, has been successfully used to treat non-Hodgkin’s lymphomas and B-cell chronic lymphocytic leukemias. The high efficacy of rituximab has led to the development of other anti-CD20 radioimmunoconjugates including ibritumomab tiuxetan and tositumomab, approved for use in relapsed or refractory B-cell lymphomas. Thus, with such highly selective and effective treatments to CD20 readily available, it is possible to target CD20-expressing melanoma stem cells.

CD133

CD133, a transmembrane glycoprotein also known as prominin-1, is normally expressed on undifferentiated cells including endothelial progenitor cells, hematopoietic stem cells, fetal brainstem cells, and prostate epithelial cells, but has also been exploited to identify and purify cancer stem cells from various solid tumors including brain, prostate, and pancreatic cancers. Thus, despite its unknown function in stem-cell biology, CD133 appears to mark both normal and cancer stem cells. In addition, expression of CD133 has been identified in melanomas and primary human melanocytes. Frank et al. concluded that 0.2% to 0.5% of primary human-cultured melanocytes and 0.5% to 2% of G3361 melanoma cells comprise CD133 cells, demonstrating that a distinct subset of melanocytic cells are marked by the CD133 progenitor phenotype. Klein et al. observed significant increases in the expression of stem-cell markers CD133, CD166, and nestin in primary and metastatic melanomas compared with banal nevi. Increased melanoma aggressiveness corresponded with greater expression of these markers, suggesting that during melanoma progression, stem-cell markers become more evident due to the increased dysregulation of stem-/progenitor-cell function and proliferation. Similarly, Mozani et al. determined that less than 1% of cells within metastatic melanomas express CD133. Moreover, only the CD133^+ cells collected from these biopsies induced tumors in nonobese diabetic/severe combined immunodeficient mice, whereas the CD133^− fraction failed to regenerate tumors, indicating that only a small percentage of cells are capable of recapitulating tumor growth. Surprisingly, long-term cultured cell lines, such as WM115, expressed CD133 at nearly 100%, whereas only a subpopulation of these cells coexpressed adenosine triphosphate–binding cassette (ABC) G2, a transporter commonly associated with stem cell lines, such as WM115, expressed CD133 at nearly 100%, whereas only a fraction of SP cells express ABCG2, suggesting that other transporters such as ABCB1, ABCG1, or ABCA2 may be involved in mediating the SP phenotype.

Likely, the SP is not solely composed of melanoma tumor-initiating cells. Despite the coexpression of ABCG2 with CD133^+ melanoma cells, it is not known whether this transporter contributes to SP, nor has it been determined whether ABCG2^+/CD133^+ melanoma cells are tumor-initiating cells. Thus, further in-depth analysis is required to determine the constituents of the SP and to assess the role of these transporters in melanoma establishment and resistance.

ABCB5, a novel energy-dependent drug efflux transporter highly similar to ABCB1, was identified and characterized by Doxorubicin drug resistance in melanoma, yet is speculated to have an even broader range of chemotherapeutic activity. ABCB5 marked 11% of primary melanocytic, 3% of melanoma cells, and 13% of cisplatin-resistant melanoma cells. Moreover, ABCB5^− cells highly coexpressed CD133 and could enrich for CD133^− cells, indicating that ABCB5^−/CD133^+ may mark melanoma stem cells. More recently, this ABC-transporter alone has been shown to mark melanoma cells, harvested from fresh specimens as well as from melanoma cell lines, that are capable of initiating tumors in xenotransplantation models. Whether this transporter also contributes to the SP phenotype is not yet known.

SP

SP is a widely utilized flow cytometric technique based on the heightened efflux capacity of stem cells to exclude fluorescent dyes. This technique, pioneered by Goodell et al. and found to enrich for hematopoietic stem cells, has been adapted to identify and isolate stem/progenitor cells from multiple tissues including umbilical cord blood, skeletal muscle, mammary glands, lung, liver, epidermis, forebrain, testis, heart, kidney, and prostate. Additionally, cancers such as neuroblastoma, breast, and lung, and tumor cell lines including melanomas, have also been shown to harbor SP with stem-cell characteristics. Melanoma SP cells have been characterized as small in size and less proliferative than their larger, non-SP counterparts. Despite the slower rate of proliferation, SP cells stained positive for the neural-crest stem-cell marker nestin and retained a greater capacity than non-SP cells to expand in vitro, giving rise to larger cells. Although this study did not further assess the capacity of SP cells to give rise to both SP and non-SP cells, nor whether size was linked to tumorigenicity, it does suggest that a hierarchy exists within melanoma, in which heterogeneous populations compete with each other for dominance.

The heightened efflux capacity of the SP is mediated primarily through the expression of the ABC transporters, a superfamily of proteins with more than 30 members that translocates both hydrophilic and hydrophobic molecules across the cell membrane, thought to provide protection from harmful exogenous substances. The major representative of the SP phenotype is the ABC transporter ABCG2, which has also been shown to be coexpressed on a subpopulation of CD133^+ melanoma cells. However, whether ABCG2 expression coincides with SP has not been examined, nor have the properties of ABCG2^+ melanoma cells been explored. Contrary to this study, however, others have found that ABCG2 is not expressed in melanoma specimens. It should be noted that, in some systems, only a fraction of SP cells express ABCG2, suggesting that other transporters such as ABCB1, ABCG1, or ABCA2 may be involved in mediating the SP phenotype.

Stem cells are typically undifferentiated, infrequently dividing cells that normally remain quiescent until their participation is required.
during tissue regeneration. Many have exploited this property to identify stem cells via label-retaining experiments. Through pulse-chase experiments with a DNA-traceable agent such as BrdU, which is rapidly titrated out as cells divide, only those cells that have infrequently divided retain BrdU. These slow-cycling, label-retaining cells have in many instances defined the location of stem cells from tissues as diverse as skin and lung.\textsuperscript{2,3,20} Others have utilized this approach to prospectively seek out cancer stem cells within tissues.\textsuperscript{7} Meanwhile, others have adapted this technique to in vitro tissue culture systems.\textsuperscript{21} Although the ability of cells to retain label is indicative of slow-cycling stem cells, this method is not full proof given that recent evidence suggests that not all stem cells can be identified on the basis of BrdU label retention.\textsuperscript{22} It may, however, provide important clues regarding tumor heterogeneity (Fig 2). This type of adaptation has been applied to in vitro cultured melanoma cells utilizing the fluorescent dye CTO to segregate and monitor the proliferation of phenotypically different cells. In preliminary studies, we have identified a label-retaining melanoma cell population that can self-renew and differentiate, and that are highly tumorigenic, fulfilling the basic stem-cell criteria. Such cells also have high efflux activities. These combined properties make label-retaining cells key in the search for drug-resistant cells that are not only resistant to chemotherapeutic agents but that may also be unaffected by drugs that block proliferation pathways.

CONCLUSIONS AND PERSPECTIVES

The biologic and molecular characterization of melanoma stem cells is still in the early phase. The origin of melanoma stem cells has yet to be determined. Whether melanoma stem cells are derived from melanocyte stem cells, melanocyte progenitors, or more mature melanocytes that have de-differentiated remains unclear at this point. Also, whether any of the four subpopulations isolated from cultures and (in part) from specimens overlap or are indeed the true tumor-initiating cells requires further characterization. With the exception of the label-retaining cells, all subpopulations have been detected in freshly isolated tumors, yet how these correlate with clinical outcome remains to be determined. Although each subpopulation can self-renew, it is not known yet whether any of these subpopulations will exhaust their self-renewal potential over time. Extensive genomewide gene expression analyses suggest that each population has a distinct profile, with the SP having the least “stemness” (unpublished data), confirming data in other malignancies. Melanoma sphere cells, in contrast to other malignancies, can be readily isolated and cultured from patient lesions at a success rate of more than 80% (unpublished data); however it is unclear at this point which subpopulation is being lost in culture. Thus, titration studies are ongoing in the nonobese diabetic/severe combined immunodeficiency mouse model to determine the minimum number of cells from which population is required for tumor formation. Further classification of cell-surface molecules specific to melanoma stem cells, such as CD20 and CD133, will allow for purification and characterization of these cells from the bulk tumor population. For clinical applications, melanoma cancer stem-cell surface molecules would allow their diagnostic identification in melanoma biopsies and may be used as prognostic indicators. Furthermore, they may pose as targets for antibody-based therapies that would selectively eliminate these cells. Knowledge of the mechanisms important for survival and maintenance of melanoma stem cells will benefit in the development of strategies aimed at destroying these cells. Pathways and signaling molecules involved in stem-cell homeostasis may be used to determine whether and how they contribute to de-differentiation and transformation to melanoma stem cells and what the consequences of blocking these pathways are. Many of the overactive signaling pathways and mutations identified to date through gene expression profiling are largely markers of heterogeneous tumor cells. However, because cancer stem cells typically constitute only a minority of the total tumor population,\textsuperscript{9} the gene expression profiles of melanoma stem cells are in all probability lost to the bulk of the tumor population. As a result, most treatment strategies are currently aimed at the bulk tumor population. Thus, combinations of markers will be required to define the ultimate stem cells because the current populations most likely represent a variety of cells and not true stem cells.

The notorious resistance of melanoma cells against all major chemotherapeutic drugs strongly suggests that this tumor type possesses major properties for drug resistance. Whether this drug resistance resides in cells with high ABC transporter activities or in the nonproliferative label-retaining cells remains to be determined. Clinically, it can be predicted that melanomas need to be treated with dual or even triple agents. Treatment strategies targeting overactive signaling pathways should have a major immediate impact on the large, rapidly proliferating population of melanomas cells, resulting in tumor debulking. Yet, without targeting the minor hidden fraction of perhaps quiescent or drug-resistant melanoma stem cells, the goal of curing this disease may be unattainable. Despite initial remissive responses, failure to abolish self-renewing cancer stem cells sets the scene for melanoma regrowth. Thus, multimodal treatment strategies that combine therapeutic agents aimed at melanoma stem cells simultaneously with regimens intended for the bulk tumor population should effectively eliminate melanomas and reduce the risk of relapse.

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