A Tumorigenic Subpopulation with Stem Cell Properties in Melanomas

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Abstract

Recent studies suggest that cancer can arise from a cancer stem cell (CSC), a tumor-initiating cell that has properties similar to those of stem cells. CSCs have been identified in several malignancies, including those of blood, brain, and breast. Here, we test whether stem cell–like populations exist in human melanomas. In ~20% of the metastatic melanomas cultured in growth medium suitable for human embryonic stem cells, we found a subpopulation of cells propagating as nonadherent spheres, whereas in standard medium, adherent monolayer cultures were established. Individual cells from melanoma spheres (melanoma spheroid cells) could differentiate under appropriate conditions into multiple cell lineages, such as melanocytic, adipocytic, osteocytic, and chondrocytic lineages, which recapitulates the plasticity of neural crest stem cells. Multipotent melanoma spheroid cells persisted after in vitro serial cloning and transplantation in vivo, indicating their ability to self-renew. Furthermore, they were more tumorigenic than adherent cells when grafted to mice. We identified similar multipotent spheroid cells in melanoma cell lines and found that the stem cell population was enriched in a CD20+ fraction of melanoma cells. Based on these findings, we propose that melanomas can contain a subpopulation of stem cells that contribute to heterogeneity and tumorigenesis. Targeting this population may lead to effective treatments for melanomas. (Cancer Res 2005; 65(20): 9328-37)

Introduction

Due to their resistance to current therapies, melanomas remain a significant cause of mortality in Caucasians. Tumors consist of heterogeneous populations whose biological properties remain poorly characterized. Melanomas are believed to arise from a mature, differentiated melanocyte. However, mounting evidence suggests that cancer may in fact arise from a transformed stem cell, which is able to self-renew, differentiate into diverse progenies, and drive continuous growth (1). Cancer stem cells (CSC) have been identified in leukemias, and tumors of the breast and brain by tumor initiation or stem cell (CSC), a tumor-initiating cell that has properties similar to those of stem cells. CSCs have been identified in several malignancies, including those of blood, brain, and breast. Here, we test whether stem cell–like populations exist in human melanomas. In ~20% of the metastatic melanomas cultured in growth medium suitable for human embryonic stem cells, we found a subpopulation of cells propagating as nonadherent spheres, whereas in standard medium, adherent monolayer cultures were established. Individual cells from melanoma spheres (melanoma spheroid cells) could differentiate under appropriate conditions into multiple cell lineages, such as melanocytic, adipocytic, osteocytic, and chondrocytic lineages, which recapitulates the plasticity of neural crest stem cells. Multipotent melanoma spheroid cells persisted after in vitro serial cloning and transplantation in vivo, indicating their ability to self-renew. Furthermore, they were more tumorigenic than adherent cells when grafted to mice. We identified similar multipotent spheroid cells in melanoma cell lines and found that the stem cell population was enriched in a CD20+ fraction of melanoma cells. Based on these findings, we propose that melanomas can contain a subpopulation of stem cells that contribute to heterogeneity and tumorigenesis. Targeting this population may lead to effective treatments for melanomas. (Cancer Res 2005; 65(20): 9328-37)

Materials and Methods

Primary culture, propagation, and separation of melanoma cells. Metastatic melanomas were obtained in accordance with consent procedures approved by the Internal Review Boards of the University of Pennsylvania School of Medicine and the Wistar Institute. They were obtained within 1 to 2 hours after surgical removal. Tumors were rinsed, trimmed to remove connective tissues, and subjected to enzymatic dissociation in 1 ng/mL collagenase IV (Invitrogen, Carlsbad, CA) in DMEM for 4 to 6 hours at 4°C. Single cells were washed with HBSS, resuspended in culture medium, and plated onto noncoated flasks.

For growing melanoma cells, we used two types of media: (a) mouse embryonic fibroblast (MEF)-conditioned human embryonic stem cell (hESC) medium (25, 26). Before use, we mixed MEF conditioned with fresh hESC medium at a 3:1 ratio and supplemented with basic fibroblast growth factor (bFGF) at 4 ng/mL. (b) Mel 2% melanoma growth medium, which was used to establish permanent melanoma cell lines (27). It consisted of MCDDB 153 medium (Sigma, St. Louis, MO; 4 parts), L15 medium (Invitrogen; 1 part), 2% FCS, 5 μg/mL insulin (Sigma), 15 μg/mL bovine pituitary extract (Cambrex, East Rutherford, NJ), 1.68 mmol/L calcium chloride, and 5 μg/mL epidermal growth factor (EGF, Sigma). Established melanoma cell lines WM115 and WM1299A were cultured in Mel 2% medium without pituitary extract and EGF (27). EBV-transformed B-cell lines from melanoma patients were kindly provided by Dr. D. Herlyn of the Wistar Institute and cultured in RPMI 1640 with 10% FCS (28).

To isolate melanoma cells from heterogeneous primary cultures, individual cells derived from mechanically or enzymatically dissociated primary spheres were cloned by limiting dilution assay in hESC medium. Although one cell showed limited potential for proliferation, new spheroid cells were generated from two single primary cells. For subculture, spheroid cells were dissociated and replated every 7 to 10 days at a clonal density of 1,000 cells/mL (29).
Evaluation of tumorigenicity and histologic staining. Tumorigenicity was determined by s.c. injecting single cells into the right flank of severe combined immunodeficient (SCID) mice at 1.7 × 10^3 cells per mouse. To compare tumorigenicity of melanoma sphere cells and their adherent counterparts with limiting amount of cells, mice were treated with 200 μg cyclophosphamide monohydrate (Cytoxan, Sigma), which further suppressed the immune response. Four days later, 2 × 10^3 tumor cells were injected. H&E, melanin, and hematoxylin staining were done on 5-μm paraffin-embedded sections following standard protocols.

Flow cytometry and fluorescence-activated cell sorting. Adhesive cells were removed with 0.02% EDTA in HBSS. Cells were washed, suspended in buffer [0.1% bovine serum albumin (BSA), 0.1% NaCl in PBS], and incubated with primary antibodies for 60 minutes at 4°C with constant agitation. Cells were washed twice with buffer then incubated with Alexa Fluor 488–conjugated goat anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) for 60 minutes when unconjugated primary antibodies were used. Suspended sphere cells were dissociated mechanically into single cells and stained. Approximately 5 × 10^5 cells were analyzed in an EPICS XL instrument (Beckman-Coulter, Inc., Miami, FL). Monoclonal antibodies (mAb) against CD3 (PE conjugated), CD4 (PE–), CD8 (PE–), CD20 (FITC–), MAC1, CD117, and CD26 were purchased from BD Pharmingen (San Diego, CA). We purchased mAbs against CD45 (PeliCluster, Amsterdam, The Netherlands), CD33 (PeliCluster), CD133 (Miltenyi Biotech, Auburn, CA), CD31 (Dako, Carpinteria, CA), neural Probes). Staining was observed by fluorescence microscopy.

RNA isolation, reverse transcription-PCR, and Western blotting. RNA isolation, reverse transcription-PCR (RT-PCR), and Western blot analyses were done using primers and antibodies against dopachrome tautomerase (DCT/DCT), MITF/MITF, and TYR as described (33, 34). TYR primers were 5′-GCAACGTCCTATATCC (sense) and 3′-GAGGAGTG-CTGCTTTTCT (antisense). A mAb against β-actin (Sigma) was used as a control.

Statistical analysis. To assess the statistical significance of differences, a one-sided Student's t test was used. Ps <0.05 were considered significant, as indicated by asterisks.

Results

Tumor cells form nonadherent spheres in mouse embryonic fibroblast-conditioned human embryonic stem cell medium. Seventeen metastatic melanoma lesions were used in this study, including 15 lymph nodes, one brain, and one parotid. To isolate a putative stem cell population from metastatic melanoma lesions, we used growth medium for totipotent hESCs and standard fibroblast–conditioned human embryonic stem cell medium. Mel 2%, to culture dissociated single tumor cells (Fig. 1A, a). Within 14 days, Mel 2% yielded adherent, spindle, or epithelioid melanocytic cells from all specimens (Fig. 1A, b). In three metastatic lesions (~20%), hESC medium supported cells growing as nonadhesive spheres (Fig. 1A, c). Spheres derived from lesions WM3517 and WM3539 were pigmented, as were their adhesive cultures established in Mel 2% (data not shown). Spheres of WM3523 lesion were nonpigmented, whereas the adherent monolayer cultures grown in Mel 2% from the same specimen were pigmented (Fig. 1A, d).

To characterize the cells obtained in stem cell medium, we tested for expression of cell surface markers. Spheres derived from WM3523, WM3517, and WM3539 were negative for endothelial markers VWF, CD31, CD34, and VEGFR2; stem cell markers SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and CD133; and neural markers GAP-43 and CD56/NCAM (data not shown). Consistent with their pigmented phenotypes, both WM517 and WM3539 spheres contained homogeneous melanoma cells expressing melanoma-associated cell surface markers CSPG (98.20% and 99.00%, respectively), β3 integrin (98.70% and 99.40%), and MCAM (99.90% and 99.20%, respectively). However, only a subpopulation of WM3523 spheres expressed melanoma markers GD2 (21.30%), CSPG (27.10%), β3 integrin (62.00%), MCAM (47.70%), and p70NGFR (26.00%; Fig. 1B). In contrast, adherent monolayer
WM3523 cells established in Mel 2% medium were more homogenously positive for GD2 (78.40%), CSPG (96.80%), β3 integrin (98.60%), MCAM (98.40%), and p70NGFR (94.80%). Tumor-infiltrating B cells isolated from melanoma lesions were included as negative controls. Only a small fraction of the B cells expressed typical melanoma markers (Fig. 1B).

Both WM3517 and WM3539 spheres were negative for hematopoietic markers (data not shown), whereas WM3523 spheres contained hematopoietic cells. Very few of WM3523 primary spheroid cells expressed CD3 (0.99%), CD4 (1.21%), and CD8 (1.04%), but a significant population of WM3523 spheroid cells expressed CD45 (38.90%) and CD20 (41.70%; Fig. 1C). Whereas B cells uniformly expressed CD45 (99.80%) and CD20 (98.10%), adhesive melanoma cells isolated from the same lesion were homogeneously negative for all hematopoietic markers tested (Fig. 1C). These results suggest that the WM3523 spheres contained both melanoma and hematopoietic cells.

**Sphere formation of melanoma cells isolated from heterogeneous populations.** To ensure the purity of cell population, melanoma cells were clonally isolated from mixed cultures as described in Materials and Methods. All 14 clones of WM3523 were pigmented and were able to produce proliferating melanoma spheres (Fig. 2A). The spheres were grown for >8 months by continuous passage. A minor population, ranging from 5% to 10%, grew adherent and subsequently differentiated into small, oval melanocytic cells with short dendrites (data not shown), whereas the major population grew as melanoma spheres. Flow cytometry analyses of five clones showed that spheroid cells homogeneously expressed melanoma markers CSPG (95.30%), β3 integrin (96.00%), and MCAM (97.50%; Fig. 2B). A significant fraction of cells expressed E-cadherin (89.60%) but not N-cadherin (2.54%). Hematopoietic markers CD3 (0.76%) and CD4 (0.90%) remained negative, with a small portion expressing CD8 (1.22%), CD45 (1.10%), and CD20 (3.34%; Fig. 2B). The CD20-positive subpopulation coexpressed melanoma-associated β3 integrin and was enriched by FACS (Fig. 2C-D). This indicates that a subpopulation of melanoma spheroid cells express the hematopoietic marker CD20.

**Self-renewal and melanocytic differentiation of melanoma spheroid cells.** After separating WM3523 melanoma spheres into single cells and reseeding at a clonal density of 1,000 cells/mL, they remained in stem cell growth medium as individual cells without aggregate formation for 5 hours (Fig. 3A, a). New spheres developed from individual cells 4 days after seeding (Fig. 3A, b). When dissociated spheroid cells were treated with melanocyte differentiation medium, a large proportion of cells adhered to flasks precoated with fibronectin (Fig. 3A, c). Within 4 days, they differentiated into melanocytic cells (Fig. 3A, d), whose pellets displayed increased pigmentation (Fig. 3A, e, right) when compared with pellets from parental cells (Fig. 3A, e, left). The capacity for melanocytic differentiation of melanoma spheroid cells persisted in long-term cultures for up to 8 months with no significant decrease in efficiency. Melanoma spheroid cells in stem cell growth medium maintained their growth potential; whereas under differentiation...
Mesenchymal differentiation of melanoma spheroid cells.

Melanocytes are derived from the neural crest during embryonic development. The transient neural crest consists of pluripotent stem cells that give rise to a wide array of lineages, including neurosecretory cells, peripheral neurons, glia, and the cephalic mesenchyme (bone and cartilage; ref. 35). To determine whether WM3523 melanoma spheroid cells can differentiate similarly to neural crest stem cells, we examined neural and mesenchymal differentiation. The cells failed to differentiate into neural lineages in defined medium containing 60% DMEM, 40% MCDB 201, 0.05 μmol/L dexamethasone, 1× ITS, 1 mg/mL LA-BSA, 10−4 mol/L L-ascorbic acid, and 100 ng/mL bFGF (ref. 36; data not shown). On the other hand, WM3523 melanoma spheroid cells readily differentiated into mesenchymal lineages with varying efficiencies: adipogenic (31.0%), chondrogenic (18.6%), and osteogenic (5.6%; Fig. 4A-B). Few or no undifferentiated melanoma spheroid cells stained for Oil Red O (5.6%), type II collagen (1.6%), or alkaline phosphatase (0.0%). Adherent monolayer melanoma cells established in Mel 2% showed significant potential for adipogenesis only (4.0%; Fig. 4B). These results suggest that multipotent stem cells are enriched in melanoma spheroid populations isolated in stem cell growth medium.

To verify the common origin of melanocytic lineages and differentiated mesenchymal cells, we double stained with melanocyte-associated transcription factors MITF or SOX10 and mesenchymal markers Oil Red O or alkaline phosphatase (Fig. 4C). In adipogenic medium, differentiated melanoma cells containing multiple MITF- or SOX10-positive nuclei also displayed Oil Red O staining (Fig. 4C). In osteogenic medium, differentiated melanoma cells expressing alkaline phosphatase also exhibited diffuse MITF or SOX10 expression. These data indicate a common origin for differentiated mesenchymal cell lineages and melanocytic cells, and that mesenchymal differentiation is not due to contaminating non-tumor stem cells.

The percentage of differentiated mesenchymal cell types varied considerably among clones. Of six tested, clones 4, 6, and 11 could differentiate into melanogenic, adipogenic, chondrogenic, and osteogenic lineages. Other clones were either tripotent (melanogenic, adipogenic, and chondrogenic lineages), bipotent (melanogenic and adipogenic), or unipotent (melanogenic). The mesenchymal differentiation capacity of melanoma spheroid cells persisted in long-term cultures up to 8 months, albeit with decreased efficiency (particularly in osteogenic differentiation). Melanoma spheroid cell lines WM3517 and WM3539 showed differentiation capacity for the melanocytic lineage only (data not shown).

Multipotent melanoma spheroid cells derived from established melanoma cell lines. We explored whether a stem cell population could be isolated from long-established melanoma cell lines. A total of 18 cell lines were tested in stem cell culture conditions using hESC medium. Spheroid phenotypes developed in WM115 and WM239A, a pair of primary and metastatic melanoma...
Multipotent melanoma spheroid cells are capable of forming tumors in vivo and self-renewing after transplantation. We examined the tumorigenic capacity of WM115 and WM3523 spheroid cells by s.c. injection in SCID mice. All mice injected with WM115 spheroid cells (n = 5), or WM3523 spheroid cells (clone 4, n = 5; clone 6, n = 3) developed tumors (Fig. 6A). The majority of tumors were observed between 28 and 40 days. Tumors consisted of large cells with abundant eosinophilic cytoplasm, oval to irregular nuclei, and dominant nucleoli. Most tumors also contained giant tumor cells (data not shown). Their melanocytic origin was confirmed by positive staining for melanin and not for hemosiderin (Fig. 6A; data not shown). These results suggest that melanoma spheroid cells isolated in stem cell medium are tumorigenic.

We then isolated and cultured melanoma cells from tumors grown in mice. Typical melanoma spheres were formed within 2 to 3 weeks in stem cell medium (Fig. 6A). These tumor cells could be stained by a specific monoclonal antibody against human nuclei, confirming their human origin (data not shown). Melanogenic, adipogenic, chondrogenic, and osteogenic differentiation of these spheroid cells were observed under appropriate differentiation conditions (data not shown). These results indicate that a stem cell population exists in melanomas after in vivo transplantation. Therefore, a self-renewing stem cell population...
persists not only in long-term cultures but also in tumors transplanted in vivo.

To address whether multipotent melanoma spheroid cells differed in tumorigenicity from adherent monolayer melanoma cells, we implanted melanoma spheroid cells or adherent monolayer cells into Cytoxan-treated SCID mice at \(2 \times 10^5\) cells per mouse, an amount that usually does not induce tumors. After \(\sim 35\) days, mice transplanted with spheroid cells had developed pigmented tumors, whereas tumors were not observed in mice injected with adherent cells. When mice were sacrificed \(70\) days after injection, three animals in the spheroid group \((n = 5)\) had small to large tumors compared with only one small tumor in one of the adherent groups \((n = 5)\). Total tumor weight and volume from the spheroid group were significantly larger than the adherent group (Fig. 6B). This suggests that melanoma spheroid cells isolated in hESC culture conditions are more tumorigenic.

**Multipotent stem cells are enriched in the CD20^+ fraction of melanoma spheres.** We consistently observed CD20^+ populations in WM3523 and WM115 melanoma spheroid cells but not in the corresponding adherent populations. Individual CD20^+ tumor cells could be detected by immunohistochemistry in \(\sim 20\%\) of metastatic melanoma lesions, supporting our in vitro observations (data not shown). We did cell sorting to determine whether the stem cell population was within CD20^+ fractions. Both positive and negative fractions from WM3523 and WM115 proliferated extensively after sorting. Their phenotypes were confirmed by flow cytometry (data not shown). The CD20^- fractions of WM3523 cells formed larger spheres compared with the CD20^+ fraction. In WM115 cells, only the CD20^+ fraction proliferated as spheres, whereas the CD20^- fraction remained as single cells (data not shown). These data suggest that the stem cell population may reside within the CD20^+ fraction.

The CD20^- or CD20^+ fractions of WM3523 and WM115 were then subjected to differentiation. Whereas WM115 CD20^- fraction remained in suspension, the other three populations adhered to substrate, indicating their differentiation potentials (data not shown). Although similar percentages showed immunoreactivity for MITF in both CD20^- and CD20^+ fractions from both spheroid cell lines after melanocytic differentiation, only CD20^- fractions showed substantial potential for mesenchymal differentiation (Fig. 6C-D). These data confirm that multipotent stem cells are enriched in the CD20^- fraction of melanoma spheroid cells isolated from fresh tumor lesions and established cell lines.
Discussion

We report a subpopulation of melanoma cells with characteristics of primitive progenitors for melanocytes, neural crest cells, that give rise to a broad range of cell types. When cultured in medium used for hESCs, melanoma cells proliferated as nonadherent spheres. When initially isolated from the lesion, the spheres contained both melanoma and hematopoietic cells. After cloning, a subpopulation of melanoma cells could be isolated that maintained stem cell characteristics; they were able to self-renew and differentiate into melanogenic, adipogenic, chondrogenic, and osteogenic lineages. These melanoma spheroid cells were also found in established cell lines, suggesting that a subpopulation of melanoma cells in long-term culture can maintain stem cell properties.

Our studies reveal that melanoma lesions can contain a subpopulation with stem cell properties and a fraction of more differentiated tumor cells. The adherent population established in vitro under standard conditions displays spindle to epithelioid morphology. In general, these cells reflect the stage of tumor progression from which they were initially derived, and those cells from metastatic lesions are tumorigenic in mice (27). They are characterized for their expression of melanoma-associated antigens such as MCAM or β3 integrin, which facilitate invasion and metastasis (16, 17). The second population, described for the first time here, can differentiate and self-renew. This population is characterized by its ability to form spheres. Sphere formation was initially observed in cultured neural stem cells (38). Cells within neural spheres have stem cell properties that manifest as self-renewal and multilineage differentiation potential (39). Recently, sphere formation was found when stem cells from a variety of normal and tumor tissues were isolated (3, 5, 40, 41), suggesting that sphere formation may be a common growth characteristic of stem cells.

It is not surprising that melanoma spheroid cells are capable of mesenchymal differentiation, because mesenchymal and melanocytic cells may originate from the same embryonic tissue, the neural crest (35). Differentiation of human melanomas into mesenchymal lineages has also been described in fat and osteocartilaginous differentiation (20–24). Differentiating cells were...
often multinucleated, their cultures could not be maintained for extended periods of time, and their differentiation was irreversible. Furthermore, melanoma spheroid cells gave rise to cells that expressed both melanocytic and mesenchymal markers. These data confirmed that the mesenchymal progenitors are not derived from contaminating normal mesenchymal stem cells but rather are a property of the tumor itself.

In sphere-forming melanoma cell lines, WM3517 and WM3539, spheroid cells were able to undergo only melanogenic differentiation, suggesting they might arise from a cell at a later stage of differentiation, such as a lineage-committed melanocyte precursor cell. Multipotent WM3523 melanoma spheroid cells had a stable capacity for self-renewal over prolonged culture periods. Clones of WM3523 spheroid cells showed differences in their stability of differentiation potential. Over time in culture, some clones could no longer differentiate into all four cell lineages but only into three, two, or one suggesting the control mechanisms for each differentiation lineage is unique.

Neoplastic melanocytic cells frequently exhibit characteristics of other neural crest derivatives both in vitro and in vivo. Differentiation of melanoma cells into neural lineages has been well shown (12–14); however, in this study, melanoma spheroid cells failed to differentiate into neural lineages. The differentiation medium contains bFGF, which has been used to induce neural differentiation of hESCs and bone marrow-derived stem cells (36, 42, 43). In this case, the plasticity of melanoma spheroid cells was limited.

Multipotent melanoma spheroid cells isolated from fresh tumors persist in culture for a long time (8 months) when passaged at clonal densities and as tumor xenografts. Similar multipotent melanoma spheroid cells can also be isolated from established cell lines. Importantly, multipotent melanoma spheroid cells can be serially recloned from a minimum of two cells during 8 months in culture. We have provided evidence that this subpopulation of melanoma cells is able to self-renew and meet other criteria of stem cells. Moreover, melanoma spheroid cells have differentiation potential similar to neural crest stem cells, supporting the notion that melanomas may arise from a transformed melanocyte stem cell. Recent studies suggest that neural crest stem cells persist in the skin in adult animals and are capable of differentiating into

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**Figure 6.** Tumorigenic capacity of melanoma spheroid cells in vivo and CD20-positive fraction harboring stem cell populations. A, tumors developed in mice injected with WM115 (top) and WM3523 (bottom) melanoma spheroid cells, respectively. Tumors showed typical melanoma morphology (H&E). Fontana-Masson staining confirmed the presence of variable melanin pigment in tumors (melanin). Spheroid cultures were then derived from mouse tumors (cultured). B, tumorigenic capacity of WM3523 spheroid (clone 4) and adherent cells were compared as detailed in Materials and Methods. Total tumor weight and volume from each group (open columns, spheroid; filled columns, adherent). Columns, means; bars, ± SEM. C-D, CD20+ fractions in both WM115 and WM3523 (clone 4) spheroid cells retained potentials for adipogenic, chondrogenic, and osteogenic differentiation, whereas their CD20- fractions rarely differentiated into the above lineages. Differentiated cells (arrows). In melanocytic differentiation, both nuclear (arrowheads) and diffuse (arrows) MITF-positive cells were observed.


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