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Multiple Myeloma: A Paradigm for Translation of the Cancer Stem Cell Hypothesis

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Abstract

Despite recent advances in drug development, multiple myeloma (MM) remains incurable for the majority of patients due to relapse and disease progression. The cancer stem cell (CSC) hypothesis may provide an explanation for these clinical findings. It suggests that the long-term proliferative potential responsible for disease initiation, maintenance, and relapse is contained within specific subpopulations of biologically distinct tumor cells. Data in MM suggest that CSCs represent a rare cell population phenotypically resembling normal memory B cells. Compared to MM plasma cells, MM CSCs also appear to be relatively resistant to a wide variety of standard anti-cancer agents suggesting they may persist following treatment and mediate tumor re-growth and relapse. A unique property CSCs share with their normal counterparts is the potential for self-renewal that likely maintains the malignant clone over time. The development of therapeutic strategies targeting the signaling elements contributing to cancer cell self-renewal has been limited primarily because the cellular processes involved are poorly understood. However, it is common that the signaling pathway components regulating normal stem cell self-renewal are aberrantly activated in human cancers and may serve as potential therapeutic targets. One class of shared regulatory pathways are those active during normal embryonic patterning and organ formation such as Hedgehog (Hh), Notch and Wntless (Wnt), and emerging data suggest that these may play a role in CSCs. Here we review the identification and characterization of MM CSCs, the role of Hh in MM, and issues to be considered during the early clinical testing of CSC targeting agents.

Keywords

Multiple myeloma; Cancer stem cells; Hedgehog; Developmental pathways

INTRODUCTION

Multiple myeloma (MM) is the second most common hematologic malignancy in the United States with over 20,000 new cases and 10,000 deaths each year [1]. It is pathologically characterized by the expansion of clonal plasma cells within the bone marrow (BM) as well as the production of excessive serum monoclonal immunoglobulin (M protein). Clinically, MM affects a wide range of physiologic systems and common manifestations include osteolytic bone disease, immunodeficiency, anemia and renal insufficiency. A wide variety of clinical approaches have been developed for the treatment of symptomatic MM patients [2]. The combination of the cytotoxic alkylator melphalan and the corticosteroid prednisone (MP) represents the oldest and best-established chemotherapeutic regimen [3]. Treatment with MP results in disease responses indicated by reduction in the serum M protein levels or

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the frequency of bone marrow plasmacytosis in approximately 50 to 60% of newly diagnosed patients. Despite the ability of MP to improve symptoms, median survival following treatment with MP is limited and ranges from 2 to 3 years.

Subsequent studies have examined combining multiple cytotoxic agents, but these approaches have provided little additional improvements in overall survival [4]. Dose intensification with standard cytotoxic chemotherapeutic agents, primarily melphalan, and autologous stem cell transplantation (autoSCT) produces significantly higher complete remission rates, but the overall survival benefit of autoSCT has remained unclear [5,6]. In a subset of patients, allogeneic bone marrow transplantation (alloBMT) may be curative through the immune-mediated graft versus MM effect, but this procedure carries a high rate of transplant related mortality and most patients are ineligible due to advanced age or co-morbidities [7,8]. Recently several novel compounds, such as the immunomodulatory agent thalidomide, its analog lenalidomide, and the proteasome-inhibitor bortezomib have been approved for use in MM [9–11]. These drugs can improve response rates and time to disease progression and have significantly improved the care of MM patients [12]. However, there is little data regarding the impact of these agents on overall survival, and in most advanced patients they appear incapable of producing long-term remissions. The failure of most of these therapeutic strategies suggests that MM cells may be biologically heterogeneous and the existence of a subset of tumor cells both resistant to treatment and possessing the growth potential to mediate disease relapse.

CANCER STEM CELLS IN MULTIPLE MYELOMA

The first studies examining the functional heterogeneity of MM cells were undertaken using a mouse plasma cell tumor model [13]. Specific strains of mice were known to develop autoimmunity following the intraperitoneal injection of pristane, a naturally occurring saturated terpenoid alkane. In some instances affected mice also developed plasmacytomas reminiscent of human MM that could be serially propagated in naïve recipients. Initial studies with one of these murine plasma cell tumors, Adj. PC-5, demonstrated that the frequency of tumor cells capable of clonogenic growth was low both *in vivo* using a spleen colony forming assay as well as *in vitro* measured by colony formation in semi-solid media [13,14]. Qualitatively similar results were also obtained in studies of human MM. Using *in vivo* labeling studies, the vast majority of tumor cells were found to be quiescent, and *in vitro* only a minority of cells from primary MM specimens could form colonies [15–17]. These data suggest that only a minority of cells is capable of clonogenic growth in both mouse and human plasma cell tumors.

Since normal plasma cells are terminally differentiated, we examined whether MM plasma cells are clonogenic. During normal plasma cell development surface expression of CD138 (syndecan-1) is restricted to terminally differentiated plasma cells, and this antigen is present at high levels on MM plasma cells in virtually all patients [18,19]. We isolated cells from clinical BM specimens based on this surface marker and found that CD138⁺ cells could not form tumor colonies in semi-solid media [20]. In contrast, cells lacking CD138 (CD138[−]) formed mature tumor colonies that expressed the identical immunoglobulin light chain isotype as each patient's original tumor. Moreover, these tumor colonies continued to form colonies upon serially replating suggesting that they were self-renewing. We also investigated the growth potential of MM *in vivo* using immunodeficient nonobese diabetic, severe combined immunodeficient (NOD/SCID) mice [20]. Similar to the *in vitro* results, CD138⁺ cells failed to engraft animals following intravenous injection, but CD138[−] cells produced disease marked by presence of immunoglobulin light chain restricted plasma cells in the bone marrow and M protein in the blood that was identical to the original patient specimen [20].

These studies suggested that the capacity for long-term proliferation might be restricted to CD138[−] cells in MM rather than the bulk CD138⁺ plasma cells. However, they did not provide more detailed information regarding the phenotype of the MM tumor cell capable of clonogenic growth. Normal plasma cells arise from the differentiation of B cells and several groups had previously investigated the potential that B cells are involved in the disease. The unique immunoglobulin produced by MM plasma cells provides a tumor-specific marker that can identify clonal relationships among various cell types. Using oligonucleotide probes specific for the immunoglobulin rearrangement encoding the M protein, clonotypic cells that phenotypically resemble B cells rather than plasma cells could be identified in the bone marrow and blood of the majority of MM patients [21–23]. Overall, these clonotypic B cells were rare and found at a frequency of 0.001–0.1% of all tumor cells and 0.2–0.8% of total B-cells in primary clinical specimens [24–28].

Further insights into the nature of these clonotypic B cells were provided by reports analyzing the specific immunoglobulin gene rearrangements expressed in human MM. These studies identified extensive somatic hypermutation in addition to recombination of the variable (V), diversity (D), and joining (J) genes within the immunoglobulin locus that suggested MM arises from a germinal center or post-germinal center B cell [22]. Moreover, these gene sequences were unaltered during the entire course of an individual patient's disease further implicating a post-germinal center cell, namely memory B cells or plasmablasts, in which further somatic hypermutation does not normally occur.

Interestingly, studies in both acute myeloid leukemia and brain tumors demonstrated that CSCs may phenotypically resemble their normal counterparts [29,30]. These results suggested that some human cancers may arise within normal adult tissue-specific stem cells that already harbor the capacity for self-renewal. Unlike most cell lineages in the human body in which stem cells are restricted to the least differentiated cell types, both memory B and T cells are thought to possess self-renewal potential that allows antigen-specific immunity to be maintained over the lifetime of an individual despite being committed cell types without multi-lineage differentiation potential [31–33]. Therefore, we hypothesized that MM may arise from self-renewing memory B cells that maintain the capacity to differentiate into mature tumor cells. We further studied the CD138[−] cell fraction and found that CD19⁺CD27⁺ memory B cells within the peripheral blood of MM patients were clonotypic and shared the identical immunoglobulin gene rearrangements as the malignant plasma cells similar to previously reported studies [28,34]. Moreover, when injected into NOD/SCID mice, the memory B cells produced symptomatic disease and CD138⁺ mature MM plasma cells within the bone marrow similar to previous studies using CD138[−] cells [34]. Finally, these CD19⁺CD27⁺ could be also be re-isolated from affected animals and continued to produce disease in secondary recipients demonstrating that these cells fulfill the functional requirements for CSCs as they were capable of both self-renewal and differentiation that recapitulated the original tumor [35].

POTENTIAL ROLE OF MULTIPLE MYELOMA STEM CELLS IN TREATMENT FAILURES

In order to generate disease relapse, MM CSCs must persist following treatment. We compared the relative sensitivity of MM plasma cells and CSCs to a variety of agents including dexamethasone, cyclophosphamide, lenalidomide and bortezomib [34]. Each of these drugs is capable of producing clinical responses detected as decreased levels of serum M protein and the relative frequency of plasma cells in the BM, and we found that all were effective at inhibiting CD138⁺ plasma cells. In contrast, CD138[−] MM CSCs were relatively resistant to all of these agents. Normal stem cells are broadly drug resistance due to several concurrent processes including high expression of intracellular detoxifying enzymes and

membrane bound drug exporters. Interestingly, these properties form the basis for unique flow cytometric assays, such as the ALDEFLUOR and side population assays that can enrich for normal adult stem cells from a number of tissues [36,37]. We found that MM CSCs from primary clinical specimens could be identified using these assays, suggesting that some mechanisms of drug resistance were shared between MM CSCs and normal stem cells. Several studies have demonstrated that normal stem cells are also highly quiescent and this property may mediate resistance to cytotoxic agents that require active cell cycling or decrease the expression of cellular components inhibited by target approaches [38]. We also found that MM CSCs were largely quiescent, suggesting an additional mechanism mediating resistance to conventional therapeutics [34]. Given the inability of standard agents to significantly improve long-term clinical outcomes, several novel therapeutic strategies are under development to target MM CSCs (Table 1).

DEVELOPMENTAL PATHWAYS IN MYELOMA CANCER STEM CELLS

Self-renewal is a defining functional property of both normal and cancer stem cells. Normal stem cells rely on this property to maintain the homeostatic production of differentiated effectors and mediate tissue regeneration following injury. In CSCs, the inhibition of self-renewal may ultimately result in exhaustion of the malignant clone and improve long-term clinical outcomes. The processes that regulate the self-renewal of CSCs are poorly understood, but similarities between normal and cancer stem cells, such as distinguishing phenotypic features (in some instances) and potential mechanisms promoting drug resistance, suggest that shared cellular pathways may mediate CSC self-renewal. Developmental pathways, such as Hh, Wnt and Notch, are evolutionarily highly conserved and required for embryonic axial patterning and organ formation by dictating essential cell fate decisions that include migration and differentiation. These signaling pathways are generally silenced in most adult tissues, but may be transiently activated in tissue specific stem cells following injury. Moreover, aberrant activity of each of these pathways can also be found in a wide variety of human cancers [42,43].

Emerging data suggests the Hh, Wnt, and Notch pathways can all regulate CSCs in human malignancies. However, only the Hh pathway has been implicated in the pathobiology of MM CSCs, and we will focus on this pathway in this review. Hh signal transduction involves binding of one of the three mammalian orthologs of *Drosophilla* hedgehog, Sonic (SHh), Indian (IHh) and Desert (DHh) Hh ligands, to the cell surface receptor patched (PTCH), a twelve-transmembrane protein that normally inhibits the seven-transmembrane protein smoothened (SMO) and renders the pathway inactive in the absence of ligand [44]. Ligand binding inactivates PTCH, which in turn, de-represses SMO and modulates the activities of the three GLI proteins that act as transcriptional regulators and represent the downstream effectors of Hh signaling. GLI1 acts as positive effector of Hh signaling by inducing the transcription of target genes such as the cell cycle regulator cyclin D1 [45]. GLI3 acts primarily as a negative effector of Hh signaling and represses transcription of Hh target genes, whereas GLI2 can act in either a positive or negative manner depending on both post-transcriptional and post-translational modifications [46].

Aberrant Hh signaling has been implicated in the pathogenesis of several human cancers. Germline loss of function mutations in *PTCH* produce ligand-independent pathway activation and results in Gorlin syndrome that is characterized by craniofacial and limb defects and a marked predisposition to cancers of the central nervous system (medulloblastoma), skin (basal cell carcinoma) and skeletal muscle (rhabdomyosarcoma) [42]. Acquired mutations of *PTCH* and *SMO* leading to increased pathway activity have also been identified in a significant percentage of sporadic basal cell carcinomas and medulloblastomas [47,48]. Aberrant pathway activation has also been described in various

solid tumors including carcinomas of the lung, prostate, stomach and pancreas [49–51]. However, few, if any mutations in Hh pathway components have been described in these tumors, and increased signaling is thought to be mediated by increased expression of the positive pathway regulator SMO and/or increased production of activating ligand. These findings suggest that in most commonly occurring epithelial tumors, Hh signaling remains ligand-dependent and autocrine in nature. More recently, Hh signaling has also been found to mediate the interaction between tumor cells and the supporting stromal elements [52,53]. In this paracrine model, the tumor cells secrete Hh ligands which subsequently activate the surrounding stroma cells to secrete undefined factors supporting tumor growth.

In MM, key Hh pathway components including *PTCH*, *SMO* and *GLI1* were found to be over-expressed in both human MM cell lines and primary clinical specimens compared to normal plasma cells and B cells [39]. Moreover, Hh pathway activity was significantly elevated in the CD138⁺ cells isolated from MM cell lines and could be further increased in response to exogenous SHh ligand or inhibited by the naturally occurring SMO antagonist cyclopamine or the Hh ligand neutralizing antibody 5E1 [39]. Functionally, treatment with SHh ligand resulted in the expansion of CD138⁺ MM CSCs without further differentiation. In contrast, the inhibition of Hh pathway activity with cyclopamine induced the loss of MM CSCs which was accompanied by the induction of plasma cell differentiation and inhibition of clonogenic growth potential [39]. These results suggest that ligand-driven Hh signaling is required for MM CSC self-renewal and maintenance of their relatively undifferentiated state. In addition to MM, Hh pathway activity has been implicated in regulating CSCs in other diseases, notably chronic myeloid leukemia, brain tumors and breast carcinoma [54–57]. Interestingly, we found that CD138⁺ plasma cells isolated from human MM cell lines highly expressed Hh ligand, whereas little ligand expression was noted in primary clinical specimens [39]. These results suggest that activation of Hh signaling in MM CSCs may be primarily mediated by ligand expression by stromal elements within the BM early in the course of disease, but with clinical progression differentiated tumor cells may express ligand that results in the formation of MM CSC niches. In MM, the role of Hh ligands produced by stromal cells has also been studied and found to enhance the survival of mature plasma cells [58]. Therefore, effective inhibition of Hh signaling may impact both MM CSCs and plasma cells.

TARGETING THE HEDGEHOG SIGNALING PATHWAY

The development of inhibitors of Hh signaling has emerged as an active area of clinical investigation. The first clinical trial examining the anti-cancer activity of Hh inhibition used the specific agent GDC-0449 that was originally identified in a chemical compound screen as a small molecule inhibitor of SMO, similar to the mechanism of action of cyclopamine [59]. Given the high frequency of mutations resulting in increased Hh pathway activity in basal cell carcinomas of the skin, a phase I trial with GDC-0449 was initiated in patients with advanced disease. Of a total of 33 patients with locally advanced or metastatic basal cell carcinoma, 55% demonstrated clinical responses. Moreover, a case report described tumor responses in a single patient with medulloblastoma treated with GDC-0449 [60]. These reports provide important evidence for the clinical relevance of Hh pathway activity in human cancers and the therapeutic potential of pathway inhibition. However, further investigations exploring the possible reasons for the lack of initial activity in a subset of patients despite uniformly high expression levels of *GLI1* mRNA as a surrogate of elevated Hh pathway activity and drug levels well beyond the concentrations required for pathway inhibition *in vitro* are needed to fully understand the clinical situations most likely to benefit from SMO antagonism.

The majority of human cancers thought to have aberrant Hh pathway activity, including MM, differ from basal cell carcinoma and medulloblastoma since they have not been found to harbor activating mutations in pathway components. It is unclear whether these tumors are fully dependent on Hh pathway activity for their growth and survival. Moreover, multiple models involving the role of Hh signaling in regulating CSCs or mediating the interactions between tumor and stromal cells further complicates the clinical challenges of developing Hh pathway inhibitors. An apparently acquired mutation in SMO was identified following fatal disease progression in the medulloblastoma patient treated with GDC-0449 [60]. Therefore, it is possible that other, distinct, compounds targeting SMO or inhibiting other components of the signaling pathway, such as the GLI transcription factors themselves may provide useful alternative targeting strategies [61,62].

TRANSLATING THE CANCER STEM CELL HYPOTHESIS TO CLINICAL TRIALS

Identification of CSC targeting approaches, such as Hh pathway inhibition in MM, represents the initial steps in clinically testing the CSC hypothesis. In many diseases, CSCs appear to represent a minority of tumor cells which presents a challenge in early clinical trials. Most early testing of novel agents, including GDC-0449, relies on standard response criteria that reflect changes in overall tumor burden to demonstrate clinical activity. However, it is possible that even full eradication of CSCs may not produce immediately detectable reductions in tumor bulk [63]. Moreover, as in the case of MM CSCs, the inhibition of self-renewal may result in enhanced tumor cell differentiation that is clinically interpreted as disease progression. Therefore, it is likely that novel clinical trial designs or evaluation strategies will be needed to evaluate CSC inhibition.

Since effective CSC targeting should result in the prolongation of disease responses, the use of CSC targeting agents following induction therapy either with autoSCT or emerging regimens that incorporate novel agents with standard cytotoxic chemotherapy in MM may be able to most definitively demonstrate clinical efficacy [64,65]. The quantification of CSCs may also provide novel biomarker strategies that can act as reliable surrogates of long-term clinical outcomes and endpoints during early clinical testing. In MM, it is possible that serially measuring relative CSC frequency in patients undergoing treatment using either *in vitro* or *in vivo* clonogenic assays or phenotypic flow cytometric assays may accurately reflect anti-CSC targeting and provide initial evidence of activity.

CONCLUSIONS

The emerging CSC hypothesis has provided a means to explain several clinical dilemmas in MM, such as the discrepancy between response rates and overall survival commonly seen in many clinical trials [66]. Despite data suggesting that MM CSCs are phenotypically and functionally distinct from the plasma cells that form the vast majority of tumor cells, the clinical relevance of CSCs in any disease has not been firmly established. However, further characterization of MM CSCs has provided potential therapeutic targets such as the Hh signaling pathway that can now be clinically tested. In addition, the development of novel biomarker strategies may allow the efficacy of novel CSC-targeting agents to be rapidly evaluated. Therefore, several components required to clinically test the CSC concept are being assembled in MM, and these early attempts may serve as a paradigm for other diseases.

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Table 1

Emerging MM CSC Targeting Strategies

Class	Agent	Reference
Hedgehog inhibitor	Cyclopamine	[39]
Telomerase inhibitor	GRN163L	[40]
Differentiation induction	Interferon + Interleukin-6	[41]
B cell targeting	Rituximab	[34]