Molecular Pathways

CANCER STEM CELLS AND CHEMOSENSITIVITY

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Abstract

Cancer lethality is mainly due to the onset of distant metastases and refractoriness to chemotherapy. Thus, the development of molecular targeted agents able to restore or increase chemosensitivity will provide valuable therapeutic options for cancer patients. Growing evidence indicates that a cellular subpopulation with stem cell-like features, commonly referred to as cancer stem cells (CSCs), is critical for tumor generation and maintenance. Recent advances in stem cells biology are revealing that this cellular fraction shares many properties with normal adult stem cells and represents the prominent tumorigenic population able to propagate the parental tumor in animal models. CSCs seem to be protected against widely used chemotherapeutic agents by means of different mechanisms such as marked proficiency in DNA damage repair, high expression of ATP-binding cassette drug transporters and activation of PI3K/AKT and Wnt pathways. Moreover, microenvironmental stimuli such as those involved in epithelial-mesenchymal transition and hypoxia indirectly contribute to chemoresistance by inducing in cancer cells a stem-like phenotype. Understanding how CSCs overcome chemotherapy-induced death stimuli, and integrating such knowledge into clinical research methodology, has become a priority in the process of identifying innovative therapeutic strategies aimed at improving the outcome of cancer patients.
Background

Adult stem cells are a rare and long-living cellular fraction that ensures tissue homeostasis by replacing senescent or damaged cells (1). The stem cell fate is regulated within specialized micro-architectonic structures, named niches, responding to both local and systemic conditions (2). When required, stem cells divide asymmetrically generating a slow-cycling daughter cell that retains the biological property of the mother and a more active daughter cell that produces a progeny of more specialized cells undergoing terminal differentiation.

Recent advances in cancer biology support the critical role of an uncommon cellular population with stem-like features in tumor generation and propagation (3-6). This population, commonly referred to as cancer stem cells (CSCs), displays three characteristics: i) expression of a repertoire of markers common to stem and progenitor cells, ii) unlimited growth in vitro using media optimized for normal stem cell cultures and iii) ability to reproduce the parental tumor upon injection in immunocompromised mice.

The concept that a transformed stem cell is the progenitor of an entire tumor population implies that cancers are organized in a stringent hierarchy with a CSC at the apex of the pyramid (“hierarchical model”), in a distortion of the functional architecture of a normal tissue. Consistent with this hypothesis, increasing evidence suggests that CSCs aberrantly exploit molecules and pathways governing the self-renewal program. This is confirmed by the marked asymmetry in the distribution of self-renewal components between CSCs and their differentiated progeny (7,8), and by the anti-tumor activity displayed by inhibitors of the self-renewal pathway in many preclinical models (9,10).

It also appears that CSCs successfully secure appropriate microenvironmental stimuli by displacing normal stem cells from their niches. The interaction between CSCs and the microenvironment is
bidirectional, as indicated by the trans-differentiation process employed by CSCs to generate vascular precursors (11,12). Like their normal counterpart, CSCs exhibit multifaceted defensive machinery that protects them from the effects of antiblastic compounds (13). In addition, the CSC fraction is probably enriched after chemotherapy as demonstrated by the increased expression of “stemness” markers in early breast cancer patients receiving primary systemic therapy (14).

The mechanisms underlying chemoresistance can be schematically subdivided in CSC-intrinsic and CSC-extrinsic (or indirect). The first group includes proficient DNA repair machinery, high expression of ATP-binding cassette (ABC) drug transporters and altered cell cycle kinetics. The latter group includes microenvironmental influences that indirectly contribute to chemoresistance (Figure 1).

**CSC-intrinsic mechanisms of chemoresistance**

Preservation of the genetic code from exogenous or endogenous injuries is critical to maintain normal cellular function. After cells sense DNA damage, they begin repair activities that restore the original sequence of the genome. Alternatively, severe lesions lead to the elimination of irreversibly damaged cells by triggering programmed cell death. Several, partly overlapping, repair signals are involved in the maintenance of genome integrity (15). Each pathway corrects a specific form of genetic lesion that, in turn, reflects the type of damage induced by the causal agents. Considerable evidence indicates that embryonic (16) and adult (17) stem cells have greater ability to repair their genetic code than their offspring. However, aged stem cells tend to accumulate genetic/epigenetic mutations as a consequence of a declined ability in correcting genetic lesions (18), which may account for the increased cancer incidence with aging. While DNA damage repair is crucial to prevent malignant transformation, transformed cancer cells take advantage of improperly activated repair pathways exploiting them to overcome chemotherapy-induced cell death.
Glioblastoma stem-like cells (GBM-SCs) are resistant to chemotherapy, independently of their ability to extrude cytotoxic drugs (19). After exposure to ionizing radiation, GBM-SCs activate ATM and Chk1 undergoing cell cycle arrest and repairing their DNA more readily than non-CSCs (20). Likewise, lung cancer stem cells (LCSCs) exposed to genotoxic stress activate Chk1 and Chk2, while differentiated lung cancer cells are vulnerable to chemotherapy (unpublished data). When chemotherapy is combined with Chk1 inhibitors, LCSCs undergo cell death through mitotic catastrophe. Hyperactivation of the ATR/Chk1 pathway also protects colon cancer stem cells (CCSCs) from platinum derivatives, whereas this chemoresistant phenotype is reverted by the inhibition of ATR/Chk1 signaling (21).

The phosphatidylinositol-3 kinase (PI3K)/Akt pathway is often deregulated in high-grade primary brain tumors and mediates different pro-tumorigenic activities (22). Moreover, an intricate connection links PI3K/Akt and DNA repair machinery (23). Indeed, in glioblastoma cells inhibition of PI3K or Akt prolongs ionizing radiation-induced DNA damage as demonstrated by the delayed clearance of gamma-H2A.X foci (24). Accordingly, Akt inhibitors efficiently target GBM-SCs, determining a reduction of viable cells and abrogating neurosphere formation (25).

DNA sensor and repair pathways act in concert with apoptotic signaling to decide cell fate. Thus, the imbalance of the apoptotic machinery towards an anti-apoptotic state favors cancer cell survival (26). Interleukin-4 (IL-4) is known to amplify the expression of anti-apoptotic mediators in different epithelial cancers (27). The chemotherapy-resistant phenotype of CSCs seems to be sustained, at least in part, by the release of IL-4 in a process that is abrogated by either a neutralizing antibody or a mutant form of IL-4 (28). Since IL-4 can be produced in a number of tumors (29,30), it is likely that other CSC types can exploit IL-4 to counteract the cytotoxic activity of chemotherapeutic drugs.
The multidrug resistance (MDR) phenotype is a further critical hurdle for chemotherapy efficacy. ABC drug transporters are the main players in this phenomenon, as they actively extrude from cancer cells a variety of structurally and functionally unrelated drugs of natural origin (31). Both normal stem cells and their malignant counterpart express high levels of ABC pumps (32). In fact, the ability of CSCs to actively exclude the HOECHST 33342 dye has been exploited to facilitate their isolation and purification. These CSCs, defined as side population (SP) by the above indicated assay, have been studied in different malignancies including acute myeloid leukemia (AML) and neuroblastoma. The AML SP is characterized by a greater proficiency in extruding daunorubicin and mitoxantrone compared with the non-SP (33), and a similar pattern has been documented for neuroblastoma stem-like cells (34). Moreover, the doxorubicin-selected breast cancer cell line MCF-7/ADR acquires stem-like properties and a molecular portrait dominated by epithelial-to-mesenchymal transition (EMT)-related and self-renewal-related genes (35). The gain of this stem-like state is coupled with the overexpression of both MDR-linked genes and the cyclophosphamide-metabolizing enzyme aldehyde dehydrogenase 1 (ALDH1).

A further mechanism involved in CSCs resistance to chemotherapy is cell quiescence. In normal stem cells, prolonged exit from the cell cycle ensures longevity of adult tissues by avoiding that stem cells exhaust their proliferative potential (36). Quiescent stem cells efficiently repair DNA damage and re-enter the cell cycle to reconstitute the damaged tissue after exposure to cytotoxic injury (37). In a malignant context, quiescent CSCs are mostly spared by chemotherapy-induced cytotoxicity and are therefore capable of reconstituting the original tumor. Initial evidence connecting quiescence to CSC chemoresistance comes from label-retaining approaches indicating that pancreatic adenocarcinoma label-retaining cells (LRCs) encompass the operative criteria of CSCs and survive 5-Fluorouracil treatment, unlike their non-LRCs counterpart (38). Similarly, putative ovarian CSCs display lower proliferative activity, enhanced tumorigenicity in xenograft models and increased resistance to cisplatin compared with the non-CSC fraction (39).
**Indirect mechanisms of chemoresistance**

The interplay between CSCs and the microenvironment is a dynamic process leading to the continuous remodeling of both compartments. Experimental evidence confirms the critical role of the EMT in the development of cancer metastases and chemoresistance. Recent findings have demonstrated that EMT is induced by the activation of a transcriptional complex influenced by different paracrine-acting signals, including the self-renewal-associated pathways Hedgehog (40), Notch (41) and Wnt (42). This complex leads to radical cytoskeletal rearrangements culminating in a switch toward a mesenchymal-like phenotype. Cells undergoing these morpho-functional changes are typically located at the tumor-stroma interface, where they gain pro-metastatic traits coupled with increased clonogenicity and enrichment in stem cell-associated markers (43).

Like the EMT, hypoxia is also emerging as a critical regulator of the CSC pool. Hypoxia derives from different cooperating factors such as the chaotic and dysfunctional vasculature supplying malignant tumors and poor oxygen diffusion within rapidly expanding neoplasms. Low oxygen tension activates the family of hypoxia-inducible factors (HIFs), which trigger adaptive changes at multiple levels including the generation of new blood vessels in the attempt to ensure sufficient oxygen and nutrients (44). However, the abnormal architecture of newly formed vessels limits drugs perfusion, determining a sub-optimal concentration of chemotherapeutic agents within the tumor. Besides this mechanistic hypoxia-mediated drug resistance, direct evidence connects HIF factors and CSCs. Indeed, cancer cells cultured under low oxygen conditions or low pH express higher levels of stemness markers, acquire stem-like phenotype and over-express stemness-related genes (45-47). Furthermore, based on functional similarities between adult stem cells and their malignant counterpart, it has been proposed that hypoxic areas within a tumor act as niches for CSCs (48).
Clinical-Translational Advances

It is possible to speculate that different mechanisms of chemoresistance are preferentially, if not exclusively, responsible of distinct phases of cancer relapse and progression. The temporal pattern of disease recurrence characteristic of many solid tumors suggests that the slow replication kinetics of CSCs could account for the limited efficacy of adjuvant chemotherapy in eradicating microscopic residual disease. Conversely, altered mechanisms of DNA repair, overexpression of ABC transporters and improper activation of anti-apoptotic signaling may be predominant during metastatic progression, when differentiated tumor cells are killed by chemotherapy and resistant CSCs are forced to re-enter the cell cycle to numerically restore the tumor population.

When considering chemotherapy-enhancing therapeutic approaches, attention often turns to agents interfering with DNA repair. Poly-ADP ribose polymerase (PARP) inhibitors are the prototype drugs of this class. The logic behind the development of PARP inhibitors relies on the concept of synthetic lethality, defined as the co-occurrence of two genetic events, which lead to cell death. To exploit this concept, cancer cells defective for a specific DNA repair pathway are exposed to compounds that inhibit a different signaling avenue partially overlapping the defective one. The combined (genetic and pharmacological) abrogation of two redundant DNA repair pathways results in the increased sensitivity of cancer cells to specific DNA-damaging agents. Different PARP inhibitors have demonstrated encouraging activity against tumors with inherent defects in DNA repair such as breast (49) and ovarian (50) carcinomas harboring BRCA1 or BRCA2 germline mutations. Chk1 inhibitors have also recently entered clinical development in combination with gemcitabine, irinotecan and cytarabine. When referred to Chk1 inhibitors, the principle of synthetic lethality involves the p53 tumor suppressor. p53-defective cells are unable to undergo G1 arrest and, as a result, depend on Chk1 to activate cell cycle checkpoints in response to DNA-damaging agents (51). Thus, in p53-defective CSCs, a synthetic lethality-driven regimen should include a
Chk1 inhibitor and a DNA-damaging agent, even though the ability of Chk1 inhibitors to preferentially kill p53-deficient cells is still debated (52). Given the close relationship between DNA repair and apoptosis, compounds targeting anti-apoptotic proteins, such as Bcl-2 family member inhibitors, might be useful in p53 wild-type tumors. With this approach, sequential oncogenic activities can be selectively blocked by taking into account the temporal and functional connections between different therapeutic targets.

If the inhibition of various components of the DNA repair pathway is currently exploited to potentiate the activity of alkylating agents, inhibitors/modulators of ABC drug transporters have been developed as chemosensitizers in order to increase the intracellular levels of ABC pumps substrates such as taxanes, anthracyclines and vinca alkaloids. After the failure of 1st and 2nd generation ABC inhibitors, more potent and specific 3rd generation antagonists have been synthesized and are currently undergoing clinical development (32). Even though direct proof of the anti-tumor activity of such compounds is still missing, ABC inhibitors offer the possibility to block pumps distributed in different body sites, such as the blood-brain barrier, thus improving drug bio-distribution within sanctuary sites.

A further strategy aimed at eliminating CSCs entails the use of differentiation-inducing agents that enhance chemosensitivity while determining a depletion of the CSC pool. The pro-differentiation activity of the bone morphogenetic protein 4 (BMP4) on GBM-SCs may be exploited for the treatment of high-grade gliomas (53). Likewise, BMP4 has been recently shown to promote apoptosis, differentiation and chemosensitization of colon CSCs through the inhibition of PI3K/AKT (54). Of note, the combined use of BMP4, 5-fluorouracil and oxaliplatin can induce tumor eradication in CSC-based model of colon cancer. Since the sequential use of differentiating agents and chemotherapy has shown considerable efficacy in acute promyelocytic leukemia (55), it is likely that the increasing research on cancer stem cells will promote the use of similar strategies.
in solid cancers. In this context, it is conceivable that protective signaling coming from the microenvironment could counterbalance the activity of differentiation-inducing agents. Thus, we envision that co-targeting intrinsic and extrinsic mechanisms associated with CSC maintenance by combining differentiation-inducing agents with anti-angiogenic compounds or inhibitors of EMT/hypoxia-associated effectors may lead to a deeper depletion of the CSC pool.

The ability to easily expand in vitro CSCs from several solid tumors has radically modified the preclinical models of human cancer based on cancer cell delivery in immunocompromised mice. Many standard cancer cell lines generate tumors whose phenotype appears extremely different from human tumors. It is extremely likely that the orthotopic transplantation of CSCs in the appropriate murine background will allow more reliable testing of anti-cancer agents by taking into account the specific molecular settings of the tumor-initiating cells of each human malignancy (56,57). Such models are very flexible and may allow to test potential treatments of both adjuvant and metastatic therapies.

In this regard, the discovery of CSCs has also questioned the general approach presently employed to validate novel pharmacological compounds. Currently, anti-cancer drugs are initially tested in metastatic patients and, if found effective, are then moved to the adjuvant setting. However, all evidence concerning CSCs suggests that this approach might be conceptually wrong, and that employing parameters of rapid tumor response - evaluated in metastatic disease - might underestimate the benefit of multiple drugs. It is likely that inhibitors of paracrine-acting pathways could offer greater opportunities as adjuvant therapies by targeting minimal residual disease, than compounds producing rapid tumor shrinkage, which may be more suitable for the metastatic or neoadjuvant setting. The lack of benefit from bevacizumab (58) and cetuximab (59) as adjuvant therapy in colorectal cancer patients, despite their pivotal role in the management of metastatic disease, corroborates this hypothesis. Likewise, a phase II study comparing FOLFOX or FOLFIRI
plus bevacizumab with or without the Smoothened inhibitor GDC-0449 in metastatic colorectal cancer patients failed to reach its primary endpoint (60), despite the encouraging activity of GDC-0449 in tumors with activating mutations of the Hedgehog pathway (61). Similar unsatisfactory results were observed in a randomized placebo-controlled phase II trial with GDC-0449 as maintenance therapy in advanced ovarian cancer (62).

Finally, it is worth considering that the development of chemotherapy-enhancing agents aimed at eliminating CSCs must take into account safety issues. To this end, research efforts should be oriented towards a deeper characterization of adult stem cells in order to avoid, or at least minimize, the inhibition or crucial mechanisms for normal stem cell maintenance. This aspect is even more relevant when anti-CSC drugs are considered for the treatment of pediatric patients and young adults.

**Figure 1.**

CSC-intrinsic mechanisms of chemoresistance include A) High expression of MDR pumps that actively extrude chemotherapeutic agents of natural origin, B) A proficient DNA repair machinery that removes DNA adducts formed by alkylating agents, C) Chemotherapy kills rapidly proliferating cells while slow-cycling/quiescent CSCs are spared from chemotherapy-induced cell death. Microenvironmental stimuli (D) associated with EMT and hypoxia indirectly contribute to chemoresistance through the generation of cancer stem-like cells. Abbreviations: CSCs: cancer stem cells, EMT: epithelial-to-mesenchymal transition.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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