Clinical Cancer Research

Molecular Pathways: Targeting Cancer Stem Cells Awakened by Chemotherapy to Abrogate Tumor Repopulation

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Abstract

Cytotoxic chemotherapy remains the first-line therapy for many advanced solid tumors; hence, understanding the underlying mechanisms to overcome chemoresistance remains a top research priority. In the clinic, chemotherapy is administered in multiple cycles that are spaced out to allow the recovery or repopulation of normal tissues and tissue stem cells between treatment cycles. However, residual surviving cancer cells and cancer stem cells can also repopulate tumors during the gap periods between chemotherapy cycles. Tumor repopulation is a phenomenon that has not been well studied; it is often overlooked due to current customized experimental study strategies. Recent findings reveal an alarming role for dying cells targeted by chemotherapy in releasing mitogens to stimulate active repopulation of quiescent cancer stem cells. Therefore, new therapeutic options to abrogate tumor repopulation will provide new avenues to improve chemotherapeutic response and clinical outcome. *Clin Cancer Res; 22(4); 802–6. ©2015 AACR.*

Background

Cytotoxic chemotherapy remains the first-line therapy for many advanced solid cancer types. There are many types of cytotoxic chemotherapy (1). Cell-cycle–dependent chemotherapeutic drugs that target dividing cancer cells would concurrently target normal cells that divide rapidly during normal tissue homeostasis. These drugs cause undesirable side effects within normal tissues with a high turnover rate, including myelosuppression (e.g., neutropenia) in bone marrow, mucositis (inflammation) in the intestinal tract and alopecia (or hair loss) in hair follicles. Therefore, to alleviate some of these side effects that can lead to severe complications or potentially life-threatening toxicities, chemotherapeutics are administered in multiple cycles of fractionated doses that are spaced out to allow normal cells and tissue stem cells to recover or repopulate between treatment cycles (reviewed in refs. 2, 3). However, residual surviving cancer cells can also repopulate tumors during the gap periods between chemotherapy cycles, which is a major cause of treatment failure that is often overlooked. In the past decade, experimental data from preclinical models have demonstrated that repopulation of tumor cells occurs between and during chemotherapy cycles in many solid tumors (reviewed in refs. 2, 3). The term "repopulation" is defined as "proliferation of surviving tumor cells during or after cytotoxic chemotherapy." However, most laboratory studies overlook the biologic phenomenon of tumor repopulation by exposing cancer cells to long-term continuous chemotherapy to select for chemoresistant clones *in vitro*, followed by high-throughput molecular analyses to compare molecular changes that occur between these chemoresistant clones and their parental cells. Similarly, most *in vivo* studies administer one single dose or continuous administration of chemotherapy drugs followed by downstream molecular, phenotypic, and functional analyses. Such study designs do not take into account the concept of tumor repopulation, the identity of repopulating tumor cells, or the consequential of these repopulating clones following multiple chemotherapy treatment cycles (like those administered in the clinic). This will be the main focus of the summary and discussion here.

Awakening of dormant or quiescent cancer stem cells to repopulate tumor

An elegant recent study by Kreso and colleagues specifically labeled single cancer cells derived from colon cancer patients by lentiviral lineage tracking and examined their repopulation dynamics in response to the chemotherapeutic drug oxaliplatin (4). Under steady-state conditions without chemotherapy, they observed several different types of clones within these colon cancer patient-derived xenografts: tumor-propagating clones that persisted throughout multiple serial passages, clones that persisted transiently but became undetectable later on, and dormant/quiescent clones that became reactivated to expand following multiple serial transplantation passages (4). Following oxaliplatin chemotherapy, they observed a marked heterogeneity in the response of individual colon cancer clones to chemotherapy. Particularly, there was a significant enrichment of dormant/quiescent clones that became reactivated, verifying a selective response of these dormant/quiescent clones to chemotherapy. On the other hand, while those presumably quickly proliferating tumor-propagating clones were sensitive to chemotherapy killing, some tumor-propagating clones did persist through selective

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pressure of chemotherapy, although their growth kinetics became slower (4). Further DNA copy-number variation profiling and targeted deep sequencing confirmed that oxaliplatin chemotherapy did not necessarily induce evolution of new genetically distinct subclones as most studies would presume; on the contrary, chemotherapy altered the proportion of preexisting clonality. Such interesting findings challenged the conventional strategies to identify "chemoresistance mechanisms or predictive signatures of therapeutic response" by comparing molecular differences at the genomic level, which may not necessarily yield a straightforward answer to understanding chemoresistance. These findings also added another level of intratumoral cellular complexity to understanding chemotherapeutic response, pointing to the existence of dormant or quiescent subpopulations within tumors that could become "awakened" and expanded in response to injury and cell death induced by chemotherapy.

Indeed, other studies in animal models of glioblastoma (5) and medullobastoma (6), and patient-derived xenografts from bladder urothelial carcinomas (7), supported the existence of a quiescent tumor subpopulation or cancer stem cells. Chen and colleagues used *in vivo* lineage tracing to identify a quiescent tumor subpopulation that coexpressed Sox2 within a mouse model of glioblastoma (*hGFAP-Cre*; $Nf1^{fl/+}$; $P53^{fl/fl}$; $Pten^{fl/+}$ mice; ref. 5). The DNA alkylating chemotherapeutic drug temozolomide was able to target and diminish the proportion of proliferating glioblastoma progenitor cells, while repopulation of tumor was driven by residual quiescent cancer stem cells that were recruited to divide after temozolomide treatment was discontinued. Similarly, Vanner and colleagues demonstrated a rare and quiescent $Sox2^+$ tumor subpopulation within a mouse model of medulloblastoma ($PTCH1^{+/-}$ mouse model of SHH subtype; ref. 6). These $Sox2^+$ cells contain cancer stem cell properties with both self-renewal and differentiation potential *in vivo*. The S-phase–specific chemotherapeutic drug cytarabine was used and shown to effectively target proliferating differentiated cells, while $Sox2^+$ cancer stem cells did not respond to drug treatment, and instead their frequency was significantly increased following drug treatment (6). Independently, our group used immortalized cancer cell–derived and patient-derived xenografts from bladder urothelial carcinomas to demonstrate the existence of a quiescent tumor subpopulation that could be enriched by cytokeratin (CK) 14 expression (7). These $CK14^+$ urothelial carcinoma cells exhibited functional cancer stem cell properties as sphere-forming stem cells *in vitro* and as tumorigenic cells *in vivo*. Combination chemotherapeutic treatment of the cytidine nucleoside analogue gemcitabine and the DNA alkylating agent cisplatin was effective in diminishing proliferating urothelial carcinomas cells, while quiescent $CK14^+$ cancer stem cells were spared and recruited into cell division by chemotherapy, leading to an increased frequency following chemotherapy (7). In addition to the previously proposed intrinsic resistance of cancer stem cells to chemotherapeutic drugs (refs. 8–14; reviewed in refs. 15, 16), the above intriguing observations collectively confirmed an active response and "reawakening" of quiescent cancer stem cells into cell division, contributing to repopulation of residual tumors following cytotoxic chemotherapy (Fig. 1A).

Mitogens released by dying cancer cells "reawaken" quiescent cancer stem cells for tumor repopulation

The next important question to be addressed would be the following: What are the underlying mechanisms contributing to the reawakening of quiescent cancer stem cells into active cell division and tumor repopulation?

During normal tissue homeostasis, quiescent tissue stem cells generally serve as a reserve until being challenged by emergency circumstances such as tissue injury or stress. For instance, quiescent stem cells within the hair follicle bulge region of skin epidermis do not contribute to daily epidermal homeostasis until being challenged by a physical full-thickness wound or incision wound (17, 18). Similarly, quiescent stem cells (lineage-negative epithelial stem/progenitor cells) within normal distal lung were recently demonstrated to respond by cellular proliferation to repair injury induced by influenza or bleomycin treatment (19). A dormant or quiescent neural stem cell within the subventricular zone of the brain was also demonstrated to be primed and could be recruited in response to brain ischemia (20, 21). Further, two functional distinct phases of quiescent satellite muscle stem cells and long-term hematopoietic stem cells were reported, namely G_0 and G_{Alert} phases, that could reversibly transit between these two phases in response to injury-induced signals (22, 23). Genes involved in mitochrondrial metabolism seemed to correlate with the ability of stem cells in the G_0 state to transit into the G_{Alert} state, priming these G_{Alert} quiescent stem cells into accelerated entry to the cell cycle for sustaining proliferation and differentiation necessary to repair tissue injury (23). Because the cancer types discussed above contain functionally distinct quiescent cancer stem cells, they likely retain safeguard mechanisms similar to those in tissue stem cells for responding to wounding or tissue injuries (24, 25). Therefore, it seems rational for us in drawing a parallel perspective to hypothesize that the molecular mechanisms involved during the process of wound response within an organ may be shared during chemotherapy-induced damages within tumors.

During tissue wound repair, Li and colleagues proposed a role for cells undergoing programmed cell death in releasing mitogens and promoting healing of excision wounds in skin and other organs (26). While the release of cytokines and factors from dying cells was previously thought to elicit an immune response for cell clearance (27), these authors demonstrated that the release of prostaglandin E_2 (PGE_2) acted downstream of the executor caspases 3 and 7, which acted as a mitogen to promote wound healing by stimulating proliferation in a paracrine manner (26). Particularly, caspase 3 or 7 cleavages led to enhanced activity of calcium-independent phospholipase A_2 ($iPLA_2$), which in turn increased synthesis and release of arachidonic acid, which is the upstream phospholipid to be metabolized into PGH_2 and finally PGE_2 (26). Independently, caspase-3–activated $iPLA_2$ had been implicated to mediate chemotactic activity by recruiting monocytes, likely contributing to recruitment of immune cells to wound sites for clearance of dead cells by phagocytic engulfment (28). Unfortunately, cancer cells undesirably adapted this wound-response mechanism following radiation- (29) and chemotherapy-induced cell death (7). Results from our group demonstrated that many pro-inflammatory factors, including PGE_2, were released from urothelial carcinoma cells during chemotherapy-induced cell death (Fig. 1B), which drove these chemotherapy-treated tumors into a wound-response state as demonstrated by global RNA-sequencing analyses followed by gene-set enrichment analyses confirming an enrichment of a wound-response gene signature, when compared with vehicle-treated xenograft tumors (7). The pro-inflammatory phospholipid PGE_2 contributed to enhanced ability to generate sphere-forming stem cells *in vitro* and

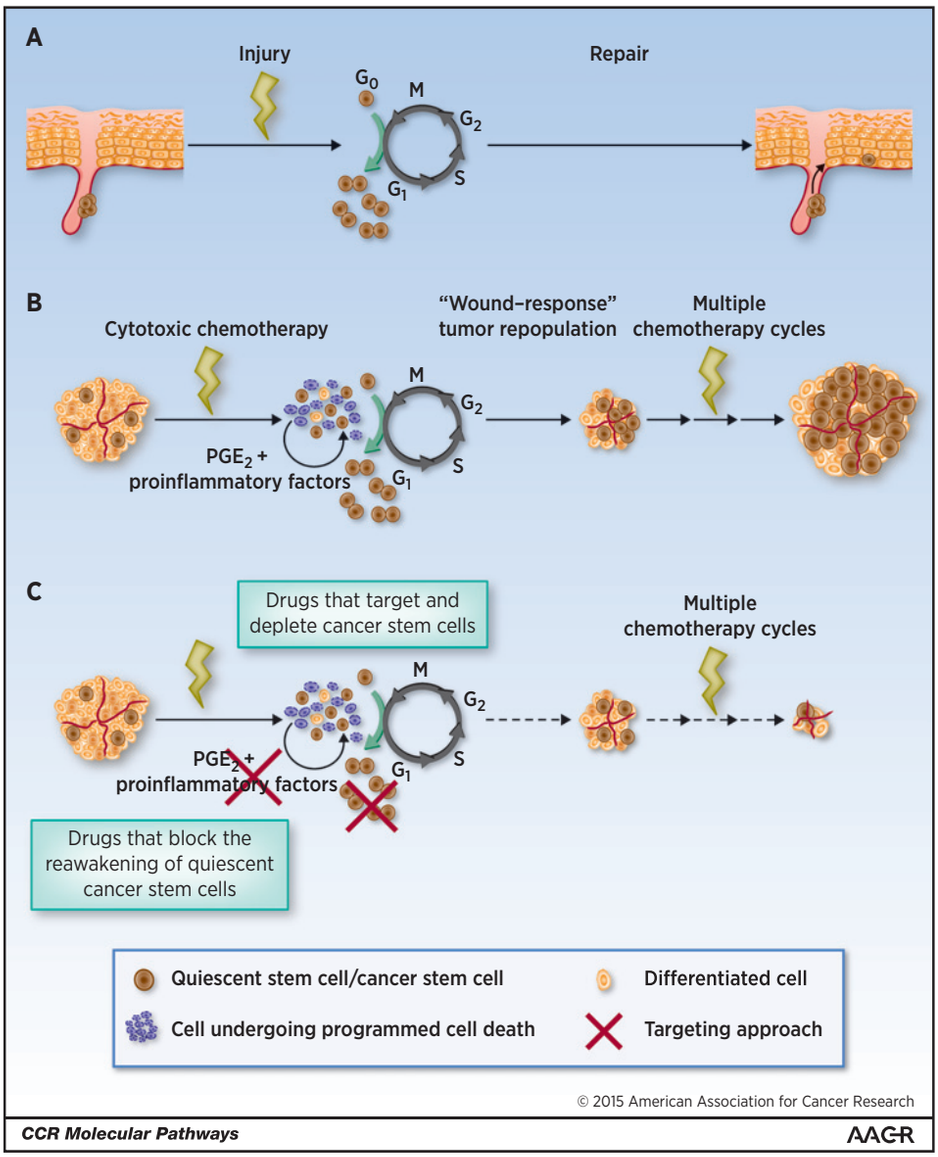

Figure 1.
Schematic diagram illustrating a "wound-response"–like tumor repopulation driven by cancer stem cells (7). A, tissue injuries induce reactivation of quiescent stem cells to repopulate and repair wound sites. B, cytotoxic chemotherapy effectively induces cell death in differentiated cancer cells, while dying cells release pro-inflammatory factors (including PGE_2) that stimulate reawakening of quiescent cancer stem cells into cell divisions and tumor repopulation. Recurrent wound-response–like tumor repopulation following multiple chemotherapy cycles ultimately leads to chemoresistance. C, two targeting approaches were proposed to abrogate tumor repopulation and chemoresistance: (i) drugs that effectively block the reawakening of quiescent cancer stem cells; (ii) and drugs that directly target and deplete cancer stem cells.

induced recruitment of quiescent cancer stem cells into cell division for tumor repopulation (Fig. 1B; ref. 7). Because chemotherapy was administered in multiple cycles as clinical regimen, successive rounds of wound-induced tumor repopulation driven by PGE_2 and other pro-inflammatory factors ultimately led to marked expansion of cancer stem cells and treatment failure following multiple chemotherapy cycles (Fig. 1B; ref. 7).

Clinical–Translational Advances

Current adjuvant therapies developed to enhance chemotherapeutic response focus on targeting the molecular basis adapted by cancer cells to evade drug resistance. These include, but are not limited to, reduced drug uptake by altering expression of nucleoside transporters and copper transporters, enhanced drug export by increased expression of ATP binding cassette protein transporters, enhanced drug metabolism via cytochrome P450 superfamily enzymes, enhanced survival by promoting prosurvival pathways or overexpression of antiapoptotic proteins, and alterations in the components of DNA repair pathways.

However, mounting evidence demonstrating the existence of quiescent cancer stem cells in multiple tumor types (presented earlier), that they can either evade cell-cycle–dependent chemotherapy by hibernating in their dormant state, or they can be "reawakened" by chemotherapy-induced damages to actively participate in a wound repair type of tumor repopulation, adds another level of complexity to the existing multifactorial problem of chemoresistance. Many recent clinical trials were initiated to investigate whether modifying the schedule of chemotherapy to reduce the gap periods between treatment cycles may alter therapeutic response and clinical outcome. These does-dense chemotherapy trials were designed to achieve maximum tumor killing by shortening the intervals between chemotherapy treatments without altering the dosage. Such a condensed treatment schedule

became feasible due to the co-administration of colony-stimulating factors, e.g., GM-CSF/G-CSF/CSFs, to induce mobilization and differentiation of hematopoietic stem and progenitor cells for enhancing leukocyte recovery (30, 31). While in theory the hypothesis for dose-dense chemotherapy was appealing, clinical trials in early breast cancers demonstrated some impact on disease-free survival with no significant benefit on overall survival (32–34). Therefore, new approaches to directly target tumor repopulation may be more efficacious. Here, we propose at least two approaches to target tumor repopulation fueled by quiescent cancer stem cells as adjuvant therapies to enhance chemotherapeutic response:

1. drugs that effectively block the reawakening of quiescent cancer stem cells (Fig. 1C); and
2. drugs that directly target and deplete cancer stem cells (Fig. 1C).

In fact, preclinical studies convincingly demonstrated efficacious results for these approaches.

There are examples in preclinical models for testing drugs that block the reawakening of quiescent cancer stem cells for tumor repopulation. In xenografts from urothelial carcinomas, blocking PGE_2 release by a neutralizing antibody or using the FDA-approved drug celecoxib was effective in diminishing paracrine effects of neighboring dying cells to promote expansion of cancer stem cells induced by chemotherapy (7). More importantly, celecoxib treatment was sufficient to block chemotherapy-treated xenografts from entering into a wound-response state, thereby preventing subsequent wound-induced tumor repopulation and eventual chemoresistance (Fig. 1C), as measurable by significantly reduced volume of xenograft tumors and significant reduction in the frequency and size of distal metastatic foci in the lung (7). The fact that celecoxib is efficacious in improving chemotherapeutic response in a primary xenograft model derived from an original patient who failed chemotherapy, this provided convincing proof-of-concept evidence for testing such adjuvant therapeutic approaches with comparable concepts in other preclinical models and subsequent human clinical trials. Indeed, other approaches that block tumor repopulation (although not specifically targeting cancer stem cells) have been proven effective to enhance chemotherapeutic response in xenograft models of ovarian cancer (35–37), glioma (38), breast cancer (39, 40), prostate cancer (40), and mesothelioma (41).

On the other side of this equation, there have been proposals based on the conceptual assumption that driving dormant stem cells into the cell cycle could sensitize them to cell-cycle–dependent chemotherapy. In fact, experimental evidence based on this concept existed primarily in the context of leukemic stem cells. In a patient-derived xenograft model of human acute myeloid leukemia, dormant cancer stem cells residing in the endosteal bone marrow niche were induced into entering the cell cycle by G-CSF and were demonstrated to become sensitized to the chemotherapeutic drug cytarabine *in vivo* (42). While this strategy was effective in diminishing the frequency of leukemic stem cells, residual stem cells clearly persisted (presumably dormant ones) and were able to re-initiate leukemia upon retransplantation. However, caution is necessary before initiating such an approach, because the molecular mechanisms regulating the balance between driving stem cell exhaustion and uncontrolled expansion are not understood currently.

In certain clinical settings, blocking tumor repopulation alone may be sufficient to improve clinical outcome because the bulk tumor would be surgically removed following neoadjuvant chemotherapy. However, in certain cancer types such as medulloblastomas and gliomas that are highly infiltrating to the normal brain regions, the surgical margins are difficult to determine, and sometimes it is presumably not feasible to remove all bulk tumor cells by surgery. Therapeutics that enable direct targeting and effective killing of cancer stem cells may be more desirable in such scenarios. Preclinical studies using the herpes simplex virus-thymidine kinase suicidal gene approach to ablate glioma cancer stem cells plus temozolomide chemotherapy demonstrated prolonged survival when those results were compared with results in the treatment group receiving temozolomide alone (5). Another preclinical study identified two aureolic acids, dactinomycin and mithramycin, in targeting $Sox2^+$ medulloblastoma quiescent stem cells. These cancer stem cell-targeting drugs were effective in killing neural sphere forming cells *in vitro* and prevented xenograft growth following engraftment of $Sox2^+$ cancer stem cells *in vivo* (6). It would be predicted that combination of such a cancer stem cell–targeted approach, together with tumor debulking drugs, would yield optimal efficacy. In fact, these targeted approaches in combination with tumor debulking chemotherapy also demonstrated success in preclinical models of breast cancer (43–45) and head and neck cancer (46).

While most of these concepts that block quiescent cancer stem cells from tumor repopulation or direct their specific targeting are still in the preclinical phase, they are yielding promising efficacy. It is hoped that the next era of human clinical trials will unravel their effectiveness in modulating therapeutic response and patient survival. Nonetheless, accumulating basic knowledge in understanding the regulation of stem cell quiescence versus emergency-induced proliferation will undoubtedly provide an important foundation for designing more specific anticancer strategies to target this "good/evil" switch.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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