Cancer Stem Cells: Targets and Potential Biomarkers for Radiotherapy

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Abstract
Cancer stem cells (CSC) have the unique ability to cause tumor recurrences if they survive treatment. Radiotherapy has curative potential because it has been functionally shown to sufficiently inactivate CSCs. It is well known that CSCs mediate the radiation resistance of tumors by tumor-specific factors, such as the pretreatment number of CSCs and repopulation or reoxygenation during fractionated radiotherapy. CSCs appear to have a higher intrinsic radioresistance than non-CSCs, a factor that is especially important for the development of predictive biomarkers that, if this finding holds true, can only be successfully established if they are stem-cell specific. Recent clinical data imply that stem-cell–related surface markers may be directly used as predictors for the radiocurability of tumors with comparable risk factors, such as histology and size. Future studies need to address the question of which additional markers need to be considered if more heterogeneous patient collectives are investigated. With the goal of developing a direct targeting approach, investigators are currently evaluating several drugs that are intended to target CSCs by inhibiting stem-cell–related signal transduction pathways. We need to preclinically test such drugs as combined-modality therapies in combination with radiotherapy to evaluate their curative potential, and optimize them by increasing their specificity to CSCs over normal tissue stem cells to avoid increased radiation toxicity.

Background
Radiotherapy has a curative potential in many solid human tumors. Locally advanced, unresectable head and neck carcinoma or non–small cell lung cancer can be cured by radiotherapy alone or in combination with chemotherapy in 10% to 50% of patients (1, 2). In early tumor stages of some cancers [e.g., anal cancer (3) and early non–small cell lung cancer (4)], primary radiotherapy alone or in combination with chemotherapy can achieve local control rates similar to those obtained with surgery. The current stem cell hypothesis implies that permanent local tumor control or recurrence after treatment depends on the inactivation or survival of CSCs after treatment (5, 6).

Currently, a CSC is defined as a cell that has the ability to self-renew and to differentiate into all subpopulations of cells that compose a tumor (7). In the case of clinical radiotherapy, this definition translates into the fact that all CSCs need to be inactivated before a permanent tumor cure can be achieved and that a CSC that survives after treatment will cause a tumor recurrence (5). Thus, an evaluation of permanent local tumor control functionally measures the survival of CSCs (5). With the use of modern markers and cell-sorting techniques, it is now possible to sort tumor cells into subpopulations that are enriched for CSCs and those that contain fewer CSCs. The "stemness" of the sorted cells can be functionally validated by quantitative transplantation assays and evaluation of the TD_{50} (i.e., the cell number that is necessary to reach a tumor growth in 50% of the host animals, referred to as the take dose). The TD_{50} is known to correlate linearly with the TCD_{50} (i.e., the irradiation dose that is necessary to locally control 50% of the tumors) after single-dose irradiation under homogeneous hypoxia (6). The biologic assay of applying a single dose of irradiation under homogeneous hypoxia (i.e., clamped blood flow) is used to exclude the impact of different oxygenation levels and factors of resistance specific to fractionated irradiation (see below) that can additionally affect local tumor control. The observation of a correlation of tumorigenicity with radiation tumor control probability highlights the importance of the pretreatment number of CSCs for local tumor control after radiotherapy. The use of both traditional functional radiobiologic assays (e.g., TCD_{50}) and modern marker-based assays (e.g., sorting of cells expressing markers that are enriched in CSC) allows us to investigate the functional effects as well as the biologic behavior of CSCs. With the increasing importance of combined radiotherapy and systemic agents (e.g., molecular-targeted agents), CSC-relevant endpoints are crucial for preclinical evaluations. Especially in modern combined-treatment approaches, in...
many cases, a dissociation of the endpoints can occur, and sole evaluations of tumor regression or tumor growth delay in preclinical radiation studies can largely overestimate the efficacy of new treatments (8). A combined treatment that inactivates a high proportion of the non-CSC mass of tumor cells can lead to major effects on tumor regression and tumor growth delay without improving local tumor control, whereas combined treatments that increase the inactivation of CSCs can improve local tumor control (9).

**Radioresistance of Tumors Is Mediated by CSCs**

CSCs mediate the radioresistance of tumors by a number of mechanisms, such as their intrinsic cellular radioresistance and absolute number before treatment. Another group of mechanisms is specific for resistance against fractionated irradiation (i.e., the clinically relevant application of radiotherapy in small daily doses). Examples of the latter mechanisms include the capacity to recover and repair sublethal damage between irradiation fractions, the repopulation capacity between fractions, tumor hypoxia, and likely also other microenvironmental factors [e.g., lactate (ref. 5; Fig. 1)].

There is increasing evidence that CSCs have a higher intrinsic radioresistance than non-CSC tumor cells, a fact that is especially important in light of the development of predictive biomarkers that usually measure all tumor cells, that is, mainly the bulk of non-CSCs. In extensive experiments, Bao and colleagues (10) showed an increase in the fraction of cells positive for the putative CSC marker CD133 (CD133⁺ cells) by ex vivo sorting after in vivo irradiation of subcutaneous or orthotopic glioma xenografts. The stemness of the CD133⁺ cells was confirmed by transplantation assays. The increase was more pronounced after fractionated irradiation (5 × 3 Gy) compared with single-dose irradiation (1 × 9 Gy), which may be explained by repopulation (see below) or repair of sublethal damage. The authors found a preferential activation of DNA-damage checkpoints in marker-positive versus marker-negative cells. Another mechanism of a higher radioresistance of CSCs versus non-CSCs may be an increased potential of resistance against fractionated irradiation in vivo (i.e., the colony-forming ability of cells in vitro) does not essentially translate into stemness in vivo (9, 16). These results are in line with data regarding the putative CSC marker CD44, which has been shown to be functionally relevant for the radioresistance of colorectal cancer cells (17). CD44 expression was shown to correlate with in vivo plating efficiency in laryngeal cancer cell lines and in patients with early laryngeal cancer with local tumor control after radiotherapy (18). Of interest, in this dataset, no correlation was observed between the in vitro intrinsic radiosensitivity of the cell lines and CD44 expression, suggesting a possibly higher impact of the number of CSCs or clonogens compared with their intrinsic radioresistance for local tumor control. This notion is supported by the fact that the known major differences in vivo radiosensitivity between tumors of different histologies can only be explained in part by their different intrinsic radiosensitivities (19–21), whereas other factors, such as tumor microenvironmental parameters, substantially affect their radiocurability in vivo.

Another well-investigated mechanism is the increase in the number of CSCs between irradiation fractions by tumor cell repopulation. This was shown in extensive experiments that revealed a steeper increase of the TCD₅₀ with time after tumor cell inoculation or a priming dose in preirradiated tumors compared with previously untreated tumors, whereas a comparison of the TCD₅₀ for tumors of similar size yielded no difference. The steep increase of the TCD₅₀ in the preirradiated tumors with a dose loss of 2.1 Gy per day versus 1.3 Gy per day in control tumors is explained by an induction of accelerated repopulation of tumorigenic cells.
by irradiation (22). Repopulation of CSCs affects the number of CSCs and is now known to be one of the most important determinants of local tumor control after fractionated irradiation (23–26). By applying modern real-time imaging techniques and tracking CSCs by using the absence of 26S proteasome activity as a marker, Vlashi and colleagues (27) were able to show an increase in the percentage of CSCs 72 hours after irradiation with a dose of 5 Gy in a human glioma model. At the same time, the percentage of proliferating cells measured by Ki-67 staining increased to a higher extent in marker-positive than in marker-negative cells, which can be interpreted as an effect of CSC repopulation.

An important factor that may lead to differences in the in vitro and in vivo radioresistance of clonogenic tumor cells or CSCs is the tumor micromilieu. Hypoxia increases the radioresistance of tumor cells, including stem cells, thereby reducing the tumor control probability (28, 29). Furthermore, independently of hypoxia, the tumor lactate content (30) inversely correlates with local tumor control, suggesting a higher radioresistance of CSCs in high-lactate tumors (31, 32). Tumor hypoxia was shown to increase

the fraction of cells expressing the putative stem-cell marker CD133 in a medulloblastoma cell line in vitro (33, 34) and to promote maintenance of the embryonic stem cell pluripotent potential (35). Hypoxia-inducible factor (HIF)-2α and HIF-regulated genes appear to be preferentially expressed in glioma stem cells compared with nonstem tumor cells and normal neural stem cells (36). Extended exposure to hypoxia may, via HIF-2α, even promote a phenotypic shift of non-CSCs to stem-like cells with self-renewal capabilities (37).

Overall, CSCs mediate the radioresistance of tumors by tumor-specific factors, such as the CSC number, repopulation, and/or reoxygenation. CSCs appear to have a higher intrinsic radioresistance than non-CSCs, a factor that is especially important for the development of predictive biomarkers that, if this finding holds true, can be successfully established only if they are stem-cell specific.

Clinical–Translational Advances

CSCs determine local tumor control after radiotherapy by various mechanisms. Today, modern marker-based
techniques are allowing us to visualize cell populations that are enriched for CSCs. This approach provides a rational basis for the development of biomarkers that are predictive for local tumor control after radiotherapy and for CSC-targeting strategies that in combination with radiotherapy may lead to considerable advances in tumor curability in the clinical setting. Recently, for the first time, the applicability of a CSC-related surface marker as a biomarker bearing predictive potential for local tumor control after radiotherapy was shown in patients with laryngeal cancer (18, 38). Consistent with preclinical experiments showing the importance of the intertumoral heterogeneity of CSC density for local tumor control after radiotherapy, a comparison of different gene signatures revealed a significant correlation with local tumor control only for the CD44 mRNA and CD44 immunohistochemical score. This hypothesis-driven approach was confirmed by a data-driven approach that also defined nohistochemical score. This hypothesis-driven approach was the most significant marker for discriminating control after radiotherapy, a comparison of different gene

parameters for predicting tumor cure by radiation (5, 6, 9, 15). Overall, the data support CD44 as a promising candidate biomarker that needs to be further validated in terms of its predictive potential for individualized patient treatments (38). However, for tumors other than early laryngeal cancer, the implementation of such a biomarker will be more difficult due to the greater heterogeneity in the size and micromilieu of such tumors, which may necessitate a combination of biomarkers for CSCs with information about the tumor micromilieu and size (38).

Another aspect to consider is the existence of splicing variants of CD44 (CD44v). The multiple isoforms of CD44 are involved in cellular functions such as motility and proliferation (39–42). Although the value of CD44v as CSC-dependent cell surface markers has not sufficiently been investigated so far, the targeting of such variants appears to be a promising strategy for combined radio-oncologic treatment approaches. As shown in preclinical in vivo experiments, anti-CD44v6–directed antibodies that were conjugated with a cytotoxic chemotherapeutic agent significantly improved local tumor control in combination with fractionated irradiation in a head and neck squamous cell carcinoma model (43). The next step after these results is to validate the stemness of CD44v6-positive tumor cells. However, the functional data suggest an effective inactivation of CSCs, raising a question as to whether this is specific for CSCs or also applies to non-CSC tumor cells. This distinction is relevant in terms of biomarker development, rather than the potential clinical advantage for tumor cure. Another issue is the expression of the target in epithelial normal tissues, which limits the clinical applicability of the approach.

Currently, a number of drugs and genetic approaches are being developed that specifically target signaling pathways that are required for stem cell self-renewal and normal cell development, such as Hedgehog, Notch, and Wnt (44, 45). For example, targeting of Delta-like ligand 4 (DLL4), an important component of the Notch signaling pathway, has been shown to reduce the stem-cell content of colorectal and breast cancer models (46). Most of these drugs are in preclinical testing, and some have been introduced in early clinical trials (47–49). A major difficulty that must be overcome for a successful introduction into the clinics will be the specificity of these drugs. Targeting pathways that are not specific for CSCs but are also relevant in normal tissue stem cells may lead to considerable side effects. This is especially relevant when such drugs are combined with radiotherapy, because acute radiation toxicity is known to be caused by a radiogenic depletion of normal tissue stem cells (50, 51). Attempts to target the CSC niche present even more difficulties because such niches are likely dependent on tumor and host factors and can hardly be defined anatomically (52). Current data suggest that hypoxia may be critical for maintaining a CSC niche (refs. 53 and 54; see above). Recently, inhibition of self-renewal and tumorigenicity was shown after targeting of HIFs via knockdown in glioma (36). The diversely expressed HIF-1α and the more selectively expressed HIF-2α are both factors that are stabilized by hypoxia, dimerize with the HIF-β subunit, and then bind to hypoxia-regulated genes, thereby modulating processes such as angiogenesis, cell survival, and motility. As outlined above, it has been known for a long time that the extent of hypoxia correlates inversely with radiocurability (29, 55, 56). Furthermore, clinical trials have shown that hypoxic cell sensitizers or modulation of hypoxia can improve local tumor control in head and neck cancer (57). Today, tumor hypoxia can be visualized and quantified with the use of modern MRI- (58) or positron emission tomography–based imaging techniques, and this information can be implemented into radiotherapy treatment planning (59). Established approaches that involve targeting of hypoxia by hypoxic cell sensitizers (57), methods to modulate hypoxia [currently in clinical trials (60)], and strategies that integrate information about hypoxia in dose-painting approaches [i.e., a local integrated boost to hypoxic regions (61)] can help to improve local tumor control, although they are not CSC-specific. At least in glioma versus normal neural stem cells, the hypoxia-dependent factor HIF-2α seems to be specific to CSCs (36, 37). Inhibition of such specific targets may be a promising strategy to further enhance the potential of hypoxia-targeted combined treatments.

The first step toward a potential clinical application of stem-cell–related surface markers as predictors for tumor radiocurability has been made. Several drugs that aim to target CSCs via inhibition of stem-cell–related signal transduction pathways are currently being evaluated. We need to preclinically test these drugs in combination with radiotherapy to evaluate their curative potential and optimize
them by increasing their specificity to CSCs over normal tissue stem cells to avoid increased acute radiation toxicity.

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No potential conflicts of interest were disclosed.

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