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. By its curative potential, radiotherapy has functionally proven to sufficiently inactivate CSC. It is well known that CSC mediate radiation resistance of tumors by tumor-specific factors like pre-treatment CSC number or repopulation and reoxygenation during fractionated radiotherapy. The intrinsic radioresistance of CSC appears to be higher compared to non-CSC, a factor that is especially important for the development of predictive biomarkers that, if this finding holds true, could be successfully established only if they are stem-cell specific. Recent clinical data imply that stem-cell-related surface markers may be directly used as predictors for radiocurability of tumors with comparable risk factors like histology and size. Future studies need to address the question which additional markers need to be considered if more heterogeneous patient collectives are investigated. On the way to a direct targeting-approach, several drugs are currently being evaluated aiming to target CSC via inhibition of stem-cell related signal transduction pathways. Such drugs need to be preclinically tested as combined modality therapies in combination with radiotherapy to evaluate their curative potential, and need to be optimized by increasing the specificity to CSC over normal tissue stem cells to avoid increased radiation toxicity.
Background

Radiotherapy has a curative potential in many solid human tumors. Locally advanced, irresectable head and neck carcinoma or non-small cell lung cancer can be cured by radiotherapy alone or in combination with chemotherapy in 10-50% of the patients (1, 2). In earlier tumor stages of some entities, e.g. anal cancer (3) or early NSCLC (4) primary radiotherapy alone or in combination with chemotherapy even reaches similar local control rates as surgery. Based on the cancer stem cell (CSC) hypothesis, a permanent local tumor control or recurrence after treatment depends on the inactivation or survival of cancer stem cells (CSC) by the treatment (5, 6).

The current understanding of CSC defines a cancer stem cell as a cell that has the ability to self-renew and to differentiate into all subpopulations of cells that comprise a tumor (7). In the case of clinical radiotherapy this definition translates into the fact that all CSC need to be inactivated to reach a permanent tumor cure and that a surviving CSC after treatment will cause a tumor recurrence (5). Thus, evaluating permanent local tumor control functionally measures survival of CSC (5). Using modern markers and cell sorting techniques, it is today possible to sort tumor cells into subpopulations that are enriched for CSC and subpopulations that contain less CSC. The stemness of the sorted cells is functionally validated by quantitative transplantation assays and evaluation e.g. of the TD$_{50}$, i.e. the cell number necessary to reach a tumor growth in 50% of the host animals (take dose 50%). The TD$_{50}$ is known to correlate linearly with the TCD$_{50}$, i.e. the irradiation dose necessary to locally control 50% of the tumors, after single dose irradiation under homogeneous hypoxia (6). The biological assay of applying a single dose irradiation under homogeneous hypoxia, i.e. clamped blood flow, is used to exclude the impact of different oxygenation levels and of specific factors of resistance specific to fractionated irradiation (see below) that would additionally impact local tumor control. These data underline the importance of the pre-treatment number of CSC for local tumor control after radiotherapy. Using both, the “traditional” functional radiobiological assays like TCD$_{50}$ and the modern marker-based assays, i.e. sorting of cells expressing markers that are enriched in CSC, allows us to investigate the functional effects as well as biological behavior of CSC. With increasing importance of combined radiotherapy and systemic, e.g. molecular targeted agents, CSC-relevant endpoints are of utmost importance for their preclinical evaluation. Especially in such modern combined treatment approaches, there is in many cases a dissociation of the endpoints, and sole evaluation of tumor regression or tumor growth delay in pre-clinical radiation research 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, while only combined treatments that increase the inactivation of CSC do improve local tumor control (9).

Radioresistance of tumors is mediated by cancer stem cells

Cancer stem cells mediate radioresistance of tumors by a number of mechanisms, e.g. their intrinsic cellular radioresistance or their absolute number before treatment. The other group are the mechanisms specific for resistance against fractionated irradiation, i.e. the clinically relevant application of radiotherapy in small daily doses. Examples for the latter mechanisms are the capacity to recover and repair sublethal damages between irradiation fractions, the
repopulation capacity between fractions, tumor hypoxia and likely also other microenvironmental factors like lactate (5).

There is increasing evidence for a higher intrinsic radioresistance of CSC compared to non-CSC tumor cells, a fact that is especially important in light of the development of predictive biomarkers that usually measure all tumor cells, i.e. mainly the bulk of non-CSC. In extensive experiments, an increase of the fraction of cells positive for the putative CSC marker CD133 (CD133+ cells) could be shown by ex-vivo sorting after in vivo irradiation of subcutaneous or orthotopic glioma xenografts. Stemness of the CD133+ cells was confirmed by transplantation assays. The increase was more pronounced after fractionated irradiation (5x3 Gy) as compared to single dose irradiation (1x9 Gy), a fact that 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 (10). Another mechanism of a higher radioresistance of CSC versus non-CSC may be an increased potential of defense against reactive oxygen species (ROS) in cancer stem cells. For classical photon radiotherapy, ROS is a critical mediator of radiation damage in tumor or normal tissue cells, as major parts of the radiation-induced DNA damage are indirect effects by ROS accumulation. Recent data suggest that genes involved in ROS scavenging are highly overexpressed in the human breast cancer CSC-enriched subpopulation of CD44+ CD24\(^{low}\) Lin\(^-\) cells compared to non-tumorigenic cells and that biochemical levels of ROS are lower in normal mammary stem cells and in CD44+ CD24\(^{low}\) Lin\(^-\) breast cancer cells compared to non-mammary repopulating units or non-tumorigenic cells. CD44+ CD24\(^{low}\) Lin\(^-\) cells showed a higher clonogenic cell survival and less DNA damage after in-vitro irradiation compared to their marker-negative counterpart and pharmacological modulation of the ROS levels could outweight this effect (11). These data show an interesting mechanism of CSC-dependent radioresistance that warrants further in-vivo evaluation.

Beside these potential differences in the intrinsic radioresistance between CSC and non-CSC, cancer stem cells impact local tumor control after radiotherapy in patients by other important (and today very well investigated) mechanisms. The impact of the absolute number of CSC on local tumor control after radiotherapy has been shown already in the 80s by proving a significant correlation between transplantability (TD\(_{50}\)) and tumor cure rate (TCD\(_{50}\)) after single dose irradiation under homogeneous hypoxia in different xenograft models (6). These data are in line with the observation in preclinical and clinical studies that tumor volume inversely correlates with curability by irradiation (12, 13). In other experiments, tumor curability after single dose irradiation did not correlate with single factors, but with a combination of transplantability and intrinsic radiosensitivity in vitro (14). Consistently, even curability after clinical relevant fractionated irradiation, which is expected to be influenced by several other factors, strongly correlates with the number and intrinsic radiosensitivity of CSC, the latter indirectly measured by TCD\(_{50}\) after single dose irradiation under homogeneous hypoxia (9, 15). In these experiments, also the plating efficiency in vitro correlates with local tumor control after fractionated irradiation in vivo (not published). This is despite the well known fact that clonogenicity, i.e. the colony forming ability of the cells in vitro is not essentially translating into stemness in vivo (9, 16). These results are in line with data on the putative CSC marker CD44 that has been shown to be of functional relevance the tumorigenicity of colorectal cancer cells (17). CD44 expression correlates with the in vitro plating efficiency in laryngeal cancer cell lines and in patients with early laryngeal cancer with
local tumor control after radiotherapy (18). Interestingly, in this dataset no correlation was observed between in vitro intrinsic radiosensitivity of the cell lines and CD44 expression, suggesting a possibly higher impact of the number of CSC or clonogens compared to their intrinsic radioresistance for local tumor control. The latter is supported by the fact that the known major differences of in vivo radiosensitivity between tumors of different histologies can only in part be explained by their different intrinsic radiosensitivity (19-21), while other factors like tumor micromilieu parameters importantly impact their radiocurability in vivo.

Another well investigated mechanism is the increase of the number of CSC between irradiation fractions by tumor cell repopulation. This has been shown in extensive experiments, where a steeper increase of the TCD50 with time after tumor cell inoculation or after a priming dose was shown after pre-irradiation compared to previously untreated tumors while a comparison of the TCD50 for tumors of similar size yielded no difference. The steep increase of the TCD50 in the pre-irradiated 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 CSC impacts the number of CSC and is today known to be one of the most important determinants of local tumor control after fractionated irradiation (23-26). By application of modern real-time imaging techniques and tracking of CSC by using the absence of 26S proteasome activity as a marker, recent data could show an increase of the percentage of CSC 72h after irradiation with 5x3 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 versus marker negative cells (27), which can be interpreted as an effect of repopulation of CSC.

An important factor that may lead to differences between in-vitro and in-vivo radioresistance of clonogenic tumor cells or CSC is the tumor micromilieu. Hypoxia increases radioresistance of tumor cells including stem cells, thereby reducing tumor control probability (28, 29). Also tumor lactate content independently from hypoxia (30) inversely correlates with local tumor control, suggesting a higher radioresistance of CSC in high-lactate tumors (31, 32). Tumor hypoxia has been 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 HIF2α and HIF-regulated genes appear to be preferentially expressed in glioma stem cells compared to non-stem tumor cells and to normal neural stem cells (36). Extended exposure to hypoxia may, via HIF2α, even promote a phenotypic shift of non-CSC to stem-like cells with self-renewal capabilities (37).

Overall, CSC mediate radioresistance of tumors by tumor-specific factors like CSC number, repopulation and/or reoxygenation. The intrinsic radioresistance of CSC appears to be higher compared to non-CSC, a factor that is especially important for the development of predictive biomarkers that, if this finding holds true, could be successfully established only if they are stem-cell specific.

**Clinical-Translational Advances**
Overall, CSC determine local tumor control after radiotherapy by various mechanisms. Modern marker-based techniques allow us today to visualize cell populations enriched for CSC and provide a rational basis for the development of biomarkers predicting local tumor control after radiotherapy and of CSC targeting strategies that in combination with radiotherapy may lead to considerable advances in tumor curability in the clinics.

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 has been demonstrated in laryngeal cancer patients (18, 38). Consistent with preclinical experiments showing the importance of 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 CD44 mRNA and CD44 immunohistochemical score. This hypothesis-driven approach has been confirmed by a data-driven approach that also defined CD44 as the most significant marker discriminating between cures and recurrences. In laryngeal cancer cell lines, CD44 expression correlated with in vitro plating efficiency but not with the intrinsic radiosensitivity in vitro (18). Although clonogenic cells in vitro do not necessarily reflect CSCs in vivo (5, 9, 16), these data support that CD44 expression correlates with the number and not with the intrinsic radiosensitivity of CSCs and that the number or density of CSC is an important parameter to predict radiation tumor cure (5, 6, 9, 15). Overall the data support CD44 as a promising candidate biomarker that needs to be further validated for its predictive potential in 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 larger heterogeneity of tumor sizes and tumor micromilieu, which may necessitate a combination of biomarkers on CSC with informations on the tumor micromilieu and tumor size (38).

Other aspects are the existence of splicing variants of CD44 (CD44v). The multiple isoforms of CD44 are involved in cellular functions like motility or proliferation (39-42). Although the value of CD44 splicing variants as CSC-dependent cell surface markers has not sufficiently been investigated so far, their targeting appears to be a promising strategy for combined radio-oncological treatment approaches. As demonstrated in preclinical in-vivo experiments, anti-CD44v6 directed antibodies 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 consequent next step after these results is to validate the stemness of CD44v6 positive tumor cells, however the functional data implicate an effective inactivation of CSC and the remaining question is whether this is specific for CSC or also applies for non-CSC tumor cells, a discrimination that is not relevant for the potential clinical advantage in terms of tumor cure but for biomarker development. Another issue is the expression of the target also in epithelial normal tissues which limits the clinical applicability of the approach.

Currently, a number of drugs and also genetic approaches are developed that specifically target signaling pathways like Hedgehog, Notch and Wnt that are required for stem cell self renewal and normal cell development (44, 45). For example, targeting of Delta-like-4-ligand, an important component of the Notch signaling pathway, has been demonstrated to reduce the stem cell content of colorectal and breast cancer models (46). Most of these drugs are in preclinical testing, some have been introduced in early clinical trials (47-49). A major difficulty for a successful introduction into the clinics will be the specificity of these drugs. Targeting
pathways that are not specific for CSC 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, where acute radiation toxicity is known to be caused by a radiogenic depletion of normal tissue stem cells (50, 51). Even more difficult is the approach to target the CSC niche, as 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 cancer stem cell niche (53, 54), see above). Recently, inhibition of self renewal and tumorigenicity has been shown after targeting of Hypoxia-inducible factors (HIFs) via knockdown in glioma (36). The diversely expressed HIF1α and the more selectively expressed HIF2α are both factors that are stabilized by hypoxia, dimerize with the HIFβ subunit and then bind to hypoxia-regulated genes, thereby modulating processes like angiogenesis, cell survival and motility. As outlined above, it is known for a long time that the extent of hypoxia inversely correlates with radiocurability (29, 55, 56) and clinical trials have shown that hypoxic cell sensitizers or modulation of hypoxia can improve local tumor control in head and neck cancer (57). Tumor hypoxia can today be visualized and quantified using modern MRI- (58) or PET based imaging techniques and this information can be implemented into radiotherapy treatment planning (59). Established approaches of targeting hypoxia by hypoxic cell sensitizers (57) and ongoing clinical trials that modulate hypoxia (60) or strategies to integrate information on hypoxia in dose painting approaches, i.e. 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, hypoxia-dependent factor Hypoxia-inducible factor 2α (HIF2α) seems to be specific to CSC (36, 37). Inhibition of such specific targets may be a promising strategy to further enhance the potential of hypoxia-targeted combined treatments.

Overall, the first step for a potential clinical application of stem-cell-related surface markers as predictors for tumor radiocurability has been done. Several drugs are being evaluated aiming to target CSC via inhibition of stem-cell related signal transduction pathways. Such drugs need to be preclinically tested in combination with radiotherapy to evaluate their curative potential, and need to be optimized by increasing the specificity to CSC over normal tissue stem cells to avoid increased acute radiation toxicity.
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Figure legends

Figure 1: Radiotherapy (RTx) has a curative potential in solid human tumors. Whether a tumor has a good chance to be permanently locally controlled or has a high risk to recur after treatment is importantly affected by CSC. CSC-related parameters, like their pre-treatment total number, their repopulation capacity during fractionated radiotherapy, their intrinsic radioresistance as well as micromilieu conditions provide a good basis for the development of biomarkers predictive for local tumor control and can also be targets for combined treatment approaches in radiation oncology, aiming to improve tumor control probability.
Radiotherapy (RTx)

Permanent local tumor control

Follow-up

High risk of recurrence

- Pretreatment CSC number: Low to High
- CSC increase during fractionated RTx (repopulation capacity): Low to High
- Intrinsic radioresistance of CSC: Low to High
- Tumor micromilieu: Oxic to Hypoxic

Development of biomarkers
Targeting for combined treatment approaches with radiotherapy

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