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At the annual conference of the Association for Radiation Research held in Oxford 26-28 June 2017, one session focused on the potential of targeting stem cells in radiation oncology. Cancer stem cells (CSCs) are highly tumorigenic cells capable to self-renew and to give rise to all other tumour cells. Emerging clinical evidence links CSCs to the risk of tumour relapse and suggests that therapeutic targeting of CSC populations in combination with radiotherapy might be a promising approach to improve local tumour control. This editorial outlines the concept of cancer stem cells in radiation biology and the main avenues for tumour radiosensitisation by anti-CSC therapies.

Introduction

Treatment resistant and high metastatic potential cells have been recognised in cancer therapy for many years. More than twenty years ago, Dick and coauthors used the specific cell surface proteins to isolate a population of tumorigenic cells which they called leukaemia initiating cells of leukaemia stem cells [1, 2]. Soon after, cells which have tumour initiating properties and stem cell characteristics were found in many tumour types and therefore renamed cancer stem cells (CSCs) [3]. Similar to normal stem cells, these cells are able to self-renew and generate all cell types within a tumour [4]. Due to their self-renewal properties and genomic instability, CSCs play a key role in tumour initiation, metastasis, recurrences and therapy resistance [5]. In contrast to non-CSCs, CSC are thought to be quiescent but reenter the cell cycle depending on the microenvironmental factors, for example after anti-cancer therapy, enabling them to reestablish the tumour and/or produce metastasis [6, 7]. The hierarchical organisation of tumours however was recently revisited as CSCs were shown to display plasticity enabling transit between non-CSCs and CSCs [6-9]. CSCs are thought to reside in a specific niche, the tumour microenvironment [10], which crucially determines the CSC fate and tumour malignant potential [11]. Many signals from the environment are thought to modulate the number of CSCs and metastastic risk, such as signalling pathways involving transforming growth factor beta (TGFβ), WNT, Notch and Hedgehog which play a critical role in stemness, metastatic potential and treatment response [12, 13].

The use of specific cell surface proteins (markers), to select/enrich for CSCs using flow cytometry tremendously contributed to current knowledge but is disputed due to the lack of universal markers owing to tumour heterogeneity [10, 14, 15]. Probably multiple markers are

needed to distinguish CSC from the bulk tumour. Emerging evidence suggests a role for CSCs in radiotherapy failure. To permanently cure cancer by radiotherapy as well as by other types of curative anti-cancer therapy, all CSCs and non-CSCs that are able to re-acquire CSC characteristics must be eliminated.

The concept of cancer stem cells in radiation biology

The curability rate of radiation therapy depends on the eradication of the cells with tumorigenic potential, i.e., the CSCs. After high dose radiotherapy, tumours may regrow from only one of a few surviving CSCs [16, 17]. To achieve cure, i.e. permanent local tumor control, all CSCs must be reproductively inactivated. The first method to evaluate the reproductive potential of single tumour cells *in vitro* was developed in 1956 by Puck and Marcus who analysed the colony forming potential of single HeLa cells presented as surviving fractions following X-ray irradiation [18]. Since then colony formation assays are considered to be a standard for in vitro analysis of the intrinsic tumour cell radiosensitivity. However, *in vitro* plating efficacy does not systematically always correlate with the fraction of CSCs *in vivo*, and usability of *in vitro* clonogenic assay to evaluate CSC inactivation remains questionable [19-21]. Recent developments in 3D culture techniques allowing growth of patient CSC-containing tumour organoids [22] and subsequent organoid-based radiation survival studies [23] may become instrumental in the investigation of CSC dynamics.

To directly analyse a relationship between tumour, take rate and tumour cure by *in vivo* irradiation, Hewitt and Wilson used a serial dilution assay of the leukaemia cells in CBA mice and demonstrated a linear dependence of the log survival rate of the leukemic cells from the dose. This study showed that eradication of a higher number of CSC requires increasing radiation dose [24]. The dilution assay was later employed by Hill and Milas for 25 different murine tumours of spontaneous origin to correlate the fraction of tumorigenic cells, or CSCs defined as TD_{50} (the number of tumour cells required for a 50% tumour take rate), and tumour radiocurability defined as tumour control dose 50%, TCD_{50} (the irradiation dose required for tumour control in 50% of the animals) [21]. This study demonstrated a significant inverse correlation between the fraction of CSCs in the experimental tumours (TD_{50} values) and tumour radiocurability (TCD_{50} values). Another study by Baumann et al. used 10 human squamous cell carcinoma xenografts in mice irradiated with single doses under clamp hypoxia or with fractionated irradiation. The results of this study showed a significant correlation of TCD50 values for these irradiation protocols and demonstrated that the number of CSCs and their intrinsic radiosensitivity might be the most critical determinants of tumour radiocurability [25].

Since the discovery of CSC markers, CSC specific phenotypes have been identified for the most human tumours enabling development of the CSC-based predictive tests for radiation oncology [26, 27]. The findings obtained in the retrospective clinical studies show that analysis of CSC-specific signatures as surrogate markers of CSC density in the pre-treatment tumour biopsies might be used for the prediction of radiotherapy outcome and treatment selection [19, 26-29]. Isolation of the putative CSC populations from established cell lines and primary tumour specimens and characterisation of their relative radiosensitivity and associated molecular characteristics demonstrated multiple intrinsic mechanisms that may shield CSCs from the radiation-induced damage including enhanced DNA repair, protection against oxidative stress and activation of the pro-survival signalling pathways [28]. In addition to the inherent mechanisms, radiosensitivity of CSCs within their niche is also regulated by the different microenvironmental cues such as oxygen tension, metabolites, interaction with extracellular matrix, and by multiple growth factors and inflammatory cytokines secreted by the cancerous and non-cancerous cells [30, 31]. Taken together, the experimental and clinical findings suggest that both density of CSCs prior to radiotherapy and their intrinsic radioresistance are important determinants of the patients' outcome after radiotherapy.

CSC as predictive and prognostic biomarkers

The discovery of membrane markers to select/enrich for stem cell populations pioneered by Dick et al. has been instrumental in the isolation and characterisation of CSCs [1, 2]. The first solid tumour where CSCs were identified was breast cancer. Clarke and colleagues used $CD44^{+}/CD24^{-/low}$ markers to isolate breast cancer cell population significantly enriched for tumour initiating cells [32]. Since then, CSC specific markers were identified and characterised for many other tumour entities, and studies on their prognostic and predictive values are important [14, 33, 34]. Using such markers, gene expression profiles specific for stem cells were found. As such, developmental genes such as the Yamanaka reprogramming factors, Sox2, Oct4, Klf4 and c-Myc [35] and many others could be related to cancer stemness, EMT and metastatic potential. Importantly, the tumour expression of CD133, CD44, and $CD44^{+}/CD24^{-}$ were associated with the response to (chemo-)radiotherapy, for e.g. non-small cell lung cancer [36], larynx [37], oesophageal cancer [29], head and heck squamous cell carcinoma [38] and others [26, 34]. Although patient populations tested so far are too small to use CSC markers as a robust predictor of response, CSC markers alone or in combination with e.g. environmental factors such as hypoxia and established markers could in the future be used for patient stratification into the groups with different resistance to radiotherapy and for selection of the specific targets for personalised medicine in combination with radiation.

CSC heterogeneity and plasticity of CSC state

For a long time it has been assumed that stem cell hierarchy is rather stringent, however, recently it became apparent that cells within a tumour can develop plasticity, and non-CSCs can be converted to the CSC state. The tumour microenvironment seems influences this process. The group of Piccolo showed that at least in some tumours the plasticity may be caused by hippo-, mechano- and Wnt signalling transcriptional activators YAP and TAS. YAP/TAZ can re-programme cancer cells into CSCs leading to tumour initiation, progression and metastasis [39]. Also, micro-environmental factors such as hypoxia [40] or those induced by irradiation may enhance epithelial to mesenchymal transition (EMT) and invasion [41]. However, increasing evidence suggests that CSC populations in solid malignancies are heterogeneous [42] and stemness is not always coupled to EMT but can be also associated with other factors such as genetic background [43, 44], metabolic reprogramming [45] and radiation-induced polyploidy [46]. Plasticity creates the problem that both bulk tumour and CSCs should be obliterated during cancer treatment [7].

Development of anti-CSC therapies for tumour radiosensitisation

Taking into account the relevance of CSC in tumour curability, a large number of studies have been conducted in the last decade to develop the experimental approaches of CSC targeting for tumour radiosensitisation. These strategies for example include inhibition of the developmental pathways implicated in CSC maintenance (e.g. Wnt, Hedgehog, Notch) [47-49], DNA damage signalling (e.g. ATM, Chk1, Chk2) [50-52], epigenetic mechanisms (e.g. HDAC, EZH2) [53, 54], metabolic program [55], ROS scavenging system [56] as well as promoting apoptotic signalling and cell death [57]. A number of the ongoing clinical studies for advanced malignancies are aiming to assess the therapeutic potential of CSC-targeted therapies including clinical trials for Wnt, Notch, Hedgehog pathway inhibition and development of the anti-CSC vaccines [13, 58]. The evaluation of these clinical studies and further research are needed to assess whether there is a benefit in combinating these treatments with radiotherapy.

Conclusion and outlook

Emerging evidence linking CSCs to tumour growth and therapy failure suggests that therapeutics targeting CSC population might be a promising approach to increase local tumour control in combination with radiotherapy. A number of clinical trials are currently underway to investigate the therapeutic potential of CSC therapies, although the number of clinical studies for

their combination with radiotherapy is still limited. The relevance of CSCs for radiotherapy resistance is supported by the fact that CSC-related signatures correlate with patient outcome and can be used in combination with established biomarkers for treatment individualisation. Recent advances in the field are enabling not only the identification of CSCs in human tumours and tracing their progeny in mice models, but also the high-throughput sequencing of individual tumour cells and building a clonal lineage tree that describes the evolution of CSCs and their genetic heterogeneity. Nevertheless, many questions remain about the contribution of the microenvironment and epigenetics to the regulation of tumour radiocurability, and particularity to the processes of CSC maintenance and tumour cell reprogramming during radiotherapy. Gaining insight into these processes should identify new potential therapeutic targets for tumour radiosensitisation.

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