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At the Crossroads of Cancer Stem Cells, Radiation Biology and Radiation Oncology

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Abstract

Reports that a small subset of tumor cells initiate and sustain tumor growth, are resistant to radiation and drugs, and bear specific markers, have led to an explosion of cancer stem cell research. These reports imply that the evaluation of therapeutic response by changes in tumor volume is misleading, as volume changes reflect the response of the sensitive rather than the resistant tumorigenic cell population. The reports further suggest that the marker based selection of the tumor cell population will facilitate the development of radiation treatment schedules, sensitizers and drugs which specifically target the resistant tumorigenic cells that give rise to treatment failure. This review presents evidence that contests the observations that cancer stem cell markers reliably identify the subset of tumor cells which sustain tumor growth, and that the marker identified population is radioresistant relative to the marker-negative cells. Experimental studies show that cells and tumors that survive large radiation doses are not more radioresistant than unirradiated cells and tumors, and also show that the intrinsic radiosensitivity of unsorted colony forming tumor cells, in combination with the fraction of unsorted tumor cells that are tumor initiating, predicts tumor radiocurability.

Keywords

Cancer stem cells; Cancer stem cell markers; radiation resistance; radiocurability

Introduction

Reports that a small percentage of tumor cells are tumorigenic, bear specific markers and are treatment resistant, have stimulated and sustained cancer stem cell research for the past dozen years. The reports suggest that the assessment of treatment efficacy by changes in tumor volume is misleading, as volume changes reflect the response of the predominant sensitive non-stem tumor cell population rather than the resistant tumor initiating and sustaining population. The reports also form the basis for designing treatments that specifically target the marker bearing tumor sub-population. This article presents evidence that contests the notion that cancer stem cell markers identify all tumorigenic cells and that

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the marker identified population is resistant to radiation. Specifically, studies indicate that: (1), recurrent tumors are equally or more radiocurable than parental non-irradiated tumors, (2), cells from tumors surviving large sub-curative doses of radiation are not more radioresistant than cells from un-irradiated tumors and (3), the slopes of radiation dose vs. percent tumor cure curves, do not differ from the slopes of radiation dose vs. percent survival of non-selected in vitro colony forming tumor cells. Furthermore, studies show that the number of tumor-initiating cells per tumor, in combination with the in vitro measured radiation sensitivity of non-selected colony forming tumor cells from the same tumor, predicts the radiation control dose of isografted murine and xenografted human tumors.

Not all tumor cells are tumorigenic, and the fraction which is, is dynamic

In 1973, Hewitt et al reported that the number of injected cells from 5 spontaneous murine tumors that was needed to achieve a 50% successful transplantation take rate (TD₅₀), in recipient syngeneic mice, ranged from 21 cells to 24,000 cells [1]. That is, the fraction of injected cells that was tumor-initiating ranged from approximately 1 in 21 to 1 in 24,000. Additionally, and similar to previously reported in vitro studies [2], the study demonstrated that the expression of a cell's tumorigenic potential was influenced by its microenvironment. Specifically, when unirradiated tumor cells were mixed with lethally irradiated tumor cells immediately prior to injection, the number of unirradiated cells needed to initiate tumors decreased in 4 of the 5 tumor types. For example, the TD₅₀ decreased from 190 cells to 14 cells, and for another, from 6,900 cells to 4.4 cells. Similar to the impact of lethally irradiated cells, Matrigel, a matrix-like protein substance containing various growth factors also reduces the number of injected tumor cells needed to initiate tumors in immunodeficient mice [3,4]. Thus, while only a fraction of tumor cells appear to be capable of initiating and sustaining tumor growth, the expression of the tumorigenic potential is dependent on microenvironmental factors.

The size of the tumor-initiating cell fraction impacts radiocurability

Hill and Milas evaluated the relationship between the fraction of tumor cells that were tumor initiating, and the tumor's radiocurability [5]. A significant correlation was observed between the tumorigenic fraction of 25 spontaneous murine tumors and the radiation dose required to achieve permanent local tumor control ($P = 0.01$). Additionally, the relationship between the fraction of injected tumor cells capable of initiating tumors and the fraction of the same cells which formed colonies in vitro was examined in a subset of 12 spontaneous mammary carcinomas. Although the fraction of cells which formed colonies was larger than the fraction that initiated tumors, the two values significantly correlated ($P = 0.01$). The study thus demonstrated a significant relationship between the fraction of tumor cells capable of forming tumors in vivo, the fraction that formed colonies in vitro, and the tumor control dose. These data generally support the cancer stem cell hypothesis.

Do cancer stem cell markers identify all and only cancer stem cells?

Commonly cited cancer stem cell markers include cell surface proteins (eg. CD24, CD44, and CD133), cells exhibiting an enhanced capacity for exclusion of dyes such as Hoechst 33342, and cells exhibiting an elevated activity of the enzyme aldehyde dehydrogenase

(ALDH). A mechanistic relationship between the various markers and cancer stem cell characteristics such as indefinite self-renewal and radiation sensitivity remains under investigation but is not well established [6-8].

In seminal studies, Al-Hajj et al reported that greater than 1000 non marker-selected cells from xenografts were required for the initiation of orthotopic tumors in non-obese diabetic SCID (NOD/SCID) mice, whereas, as few as 100 CD44⁺CD24^{-/low} cells were capable of initiating tumors in the same mice. Ponti et al [10] reported that culturing breast tumor cells under conditions used to grow normal breast stem cells, lead to the growth of non-adherent spheres. Ninety-five to 98 percent of mammosphere cells expressed the CD44⁺CD24^{-/low} profile. Tumors could be initiated by the injection of 3000 mammosphere cells vs. greater than 100,000 unselected cells from the MCF7 mammary tumor cell line. In contrast to these results, in estrogen receptor negative human breast cancers, Meyer et al [11] reported that both CD44⁺CD24^{-/low} and CD44⁺CD24⁺ cells were equally tumorigenic in NOD/SCID mice, while cells selected for a three marker combination, CD44⁺CD49^{hi}CD133/2^{hi}, exhibited even greater tumorigenicity. Additionally, cells from different xenografts, all bearing the same marker, exhibited notable differences in tumorigenicity. Ginestier et al reported that 50,000 CD44⁺CD24^{-/low}Lineage⁻ALDH⁻ breast cancer cells were not tumorigenic when injected in humanized NOD/SCID mice, whereas in the subset of CD44⁺CD24^{-/low}Lineage⁻ cells exhibiting high ALDH1 activity, as few as 20 tumor cells were capable of initiating tumors [12].

Concurrent with these breast tumor studies, it was reported that as pertained to normal brain, a small fraction of cells from brain tumors cultured in low-attachment culture dishes without serum but supplemented with growth factors, gave rise to the growth of cell spheres [13-16]. Cells expressing the stem cell marker CD133⁺ were reported to be capable of forming neurospheres, but not CD133⁻ cells [14]. Singh et al reported that as few as 100 CD133⁺ cells were capable of initiating brain tumors in NOD/SCID mice whereas no tumors arose from 5-10 × 10⁴ CD133⁻ cells [17]. Similarly, Bao et al reported that 300 and 1000 CD133⁺ cells from xenografts and primary human glioblastomas were able to initiate intracranial tumors in NOD/SCID mice, whereas 2 × 10⁶ CD133⁻ cells were not tumor initiating [18]. Subsequent studies, however, have reported that both CD133⁻ and CD133⁺ brain tumor cells are tumorigenic [19].

To summarize, the percentage of tumorigenic cells in a marker selected population is generally substantially higher than in a non-selected or marker negative population. Nevertheless, the majority of marker selected cells are not tumorigenic, and marker negative cells may also exhibit tumorigenicity. Additionally, cells from different tumors of the same tumor type, although bearing the same marker or marker combination, are not equally tumorigenic. References [20,6,7] further examine the distribution of proposed cancer stem cell markers in tumors and normal tissues as well as marker specificity and the tumorigenicity.

Are tumors and tumor cells surviving large doses of radiation radioresistant?

The small percentage of marker bearing tumorigenic cells within a much larger population of marker-bearing non-tumorigenic cells confounds an assessment of the radiation sensitivity of the tumorigenic cells based on marker expression. This pertains regardless of the response metric. While an increase in the fraction of cells exhibiting a marker following irradiation may be indicative of greater resistance of the marker bearing cells, the assay requires the passage of several hours to days between radiation and marker quantification, to clear the radiation sterilized cells from the analyzed population. The findings are also contingent on the absence of radiation-induced reprogramming of marker-negative tumor cells into marker-positive cells as has been reported [21,22]. In short, increased marker expression following irradiation is not equivalent to an evaluation of the radiation sensitivity of demonstrably tumorigenic cells that survive irradiation.

Suit [23] exposed isotransplants of a spontaneous murine mammary tumor to various dose levels. In tumors receiving a TCD₉₅ dose, (the dose which on average achieved permanent local control of 95% of treated tumors) a recurrent tumor was excised and isotransplanted into syngeneic mice. The TCD₅₀ of the recurrent transplanted tumor was lower than pertained to unirradiated tumors, i.e., 51.3 vs. 59.9 Gy. Ando et al [24] reported the TCD₅₀ of a recurrent isotransplanted spontaneous fibrosarcoma was substantially lower than the parental tumor, i.e., 58.0 vs. 78.9 Gy. The tumors' stem cell fractions, resolved by the TD₅₀ transplantation assay, were similar in the non-irradiated control and recurrent tumors. Majima et al [25] also found that the radiosensitivity of tumor clones, isolated from recurrent murine tumors following large subcurative doses of radiation, were not more radioresistant than clones from unirradiated tumors. The enhanced radiocurability of heavily irradiated recurrent tumors is consistent with earlier in vitro studies first reported by Sinclair [26]. An increasing fraction of cells surviving increasingly large single radiation doses, exhibited increasing radiosensitivity, i.e., "heritable non-lethal damage".

Studies reporting a similar or greater sensitivity of tumors and cells surviving large single-fraction irradiation do not preclude the possibility that multiple smaller doses of radiation administered over days to weeks, may select for a pre-existing relatively resistant tumorigenic sub-population. This possibility warrants investigation, however it is informative to note that Yaromina et al reported a highly significant correlation between the single-dose TCD₅₀ of 8 human squamous cell carcinomas and their 30 fraction TCD₅₀ values, ($R = 0.91$, $P = 0.002$) [27].

In vitro colony forming tumor cells and tumorigenic cells in situ are equally sensitive to radiation

In 2006, Bao et al reported that CD133⁺ brain tumor-initiating cells were radioresistant compared to CD133⁻ cells [18]. Several subsequent studies reported greater resistance of marker positive vs. marker negative cells from brain, breast and other tumor types [28-35], with various mechanisms being proposed as the cause of radioresistance [29]. However,

other investigators have not found CD133⁺ or CD44⁺CD24⁻ tumor cells to be more resistant than their marker negative counterparts [36-41]. For example, in early passaged xenografts, Zielske et al reported the enrichment of the CD44⁺CD24⁻Lin⁻ALDH1⁺ breast tumor stem cell population two weeks following irradiation, suggesting resistance and survival of the marker identified population [36]. In contrast, radiation lead to the depletion of CD44⁺CD24⁻Lin⁻ALDH1⁺ cells in xenografts initiated from a different patient.

The colony formation assay has long been considered the gold-standard in vitro assay for assessing the intrinsic radiosensitivity of tumor cells. Few if any of the thousands of published in vitro radiation survival curves exhibit a decrease in slope indicative of a small resistant sub-population of cells at high radiation doses. In addition to in vitro colony formation studies, the TCD₅₀ assay provides a powerful quantitative tool for directly assessing the sensitivity of tumorigenic cells in their in situ environment. Rofstad et al exposed human melanoma xenografts to a range of doses and measured the slope of a curve fit to percent tumor cure vs dose. The slopes of the curves did not differ from the slopes of curves fit to percent surviving fraction of cells from the same tumors that were exposed to various doses of radiation in vivo, and then plated for in vitro colony formation [42]. Baumann et al examined the in situ cell sensitivity of the human squamous cell carcinoma FaDu by evaluation of the TCD₅₀ of tumors of increasing size and number of colony forming cells [43]. After adjusting for differences in the sensitivity of cells irradiated under oxygenated conditions in vitro, and hypoxic conditions in vivo, their study showed that the slope of a curve fit to the dose to achieve 50% tumor control vs. clonogens per tumor, did not differ from the survival curve slope of FaDu cells irradiated in vitro. Interrogation and validation of the equivalent sensitivity of colony forming tumor cells and the in situ sensitivity of tumorigenic cells is illustrated in Figure 1. Studies by both Rofstad et al and Baumann et al demonstrated that the radiation sensitivity of colony forming tumor cells and cells which lead to treatment failure if not sterilized, did not differ.

The number of tumorigenic cells per tumor and their in vitro measured radiation sensitivity, determines the tumor control dose

Treatment failure occurs secondary to the survival and growth of one or more tumor initiating cells. If the radiation sensitivity of unsorted in vitro colony forming tumor cells does not differ from the sensitivity of the tumorigenic cells in situ, with knowledge of tumor volume, approximate number of tumor cells per unit tumor volume, and the fraction of tumor cells which are tumorigenic, an equation based on standard radiobiologic principles should predict a tumor's 50% tumor control dose [44]. To assess this possibility, the tumorigenic fraction of four spontaneous murine tumors in syngeneic mice and two human tumors in Nude mice was evaluated by the TD₅₀ assay. These data were combined with the in vitro radiation sensitivity of the unsorted tumor cells from the tumors, to predict the tumors' 50% tumor control doses. The number of tumor initiating cells per tumor and their intrinsic radiosensitivity, predicted the measured 50% tumor control doses with a rank-order correlation coefficient of 1.0, $P = <0.01$. Adjusting for the oxygen dependent difference in radiosensitivity between uniformly oxygenated cells in vitro, and uniformly hypoxic tumors, the average difference between the predicted and measured TCD₅₀ values was

approximately 3 Gy [44]. These results provide evidence that elimination of the tumorigenic cell fraction is required for the successful radiotherapy of cancer, and these tumorigenic cells are not more resistant than the bulk colony forming tumor cells.

Summary

- The size of the tumorigenic cell fraction is tumor specific and is significantly influenced by the tumor cells' microenvironment.
- Only a small subset of marker-positive cells initiate tumors upon injection into immunodeficient mice.
- A pan tumor-marker or marker combination identifying all and only tumorigenic cells in individual tumors of the same type or across tumor types has not been identified.
- A positive correlation exists between the fraction of unselected tumor cells capable of initiating tumors, the fraction of the same cells which form colonies in vitro, and the radiation dose required to sterilize tumors.
- Recurrent tumors surviving large single-fraction doses of radiation are equally or more curable by radiation than their parental non-irradiated tumor.
- The slope of % tumor cure vs. dose curves measured in vivo, does not differ from the surviving fraction vs. dose curves of unselected colony forming cells measured in vitro.
- The number of tumor-initiating cells per tumor and their radiation sensitivity, predicts the radiation dose required to achieve permanent local tumor control.

These results indicate that colony forming tumor cells constitute an appropriate cell population for targeting, enhancing, and quantifying the radiation sensitivity of tumor cells which give rise to treatment failure if not sterilized. They caution against over-reliance on "markers" for the identification of the tumorigenic cell population and its radiosensitivity.

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Figure 1. Key features of the relationship between radiation dose and the loss of cell reproductive capacity in vitro and in vivo

A, in vitro, a linear increase in dose results in an exponential decrease in the surviving fraction of colony forming tumor cells. The presence of a small radiation resistant subpopulation of cancer stem cells in the unsorted tumor cell population would result in a decrease in the slope of the survival curve at high doses. This is not observed. **B**, the same non-selected tumor cells are used to initiate tumors and the dose required for permanent local tumor control in 50% of treated mice (TCD₅₀) is determined. The tumor control dose increases with increasing tumor size and number of colony forming cells per tumor. For similarly oxygenated tumors and cells in vitro, the slope of the change in dose needed to compensate for an increase in the number of clonogens per tumor (Y/X in panel **B**), does not differ from the change in dose needed to sterilize the same fraction of tumor cells in vitro (Y/X in panel **A**). Thus, the radiation sensitivity of unsorted colony forming tumor cells in vitro is same as that of cells which give rise to treatment failure if not sterilized in vivo.