

Cancer Stem Cells and Radiotherapy: New Insights Into Tumor Radioresistance

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Solid tumors are histologically heterogeneous and include tumor cells, stroma, inflammatory infiltrates, and vascular structures. Furthermore, it has long been recognized that subpopulations of cancer cells exist within the tumors that resemble the developmental hierarchy of the normal tissue from which the tumor arose. In recent years, the cancer stem cell model of tumorigenesis has received increasing attention. This model posits that tumors are driven and maintained by a minority subpopulation of cells that have the capacity to self-renew (i.e., give rise to progeny with similar properties as themselves) and to generate the more differentiated progeny which make up the bulk of a tumor (1). The former population has been termed cancer stem cells (CSCs), tumorigenic cancer cells, or tumor-initiating cells, by various investigators, to indicate that only they can give rise to new tumors when transplanted into immunodeficient animals.

Evidence for the existence of CSCs initially came from studies of acute myelogenous leukemia (AML), among which a subset of leukemic cells was identified that gave rise to AML in immunodeficient mice and that displayed a similar cell surface immunophenotype as normal hematopoietic stem cells (2). Subsequently, CSCs were isolated from solid tumors, including glioblastoma, medulloblastoma (3), breast cancer (4), melanoma (5), and prostate cancer (6). The existence of CSCs has profound implications for cancer biology and therapy because it is likely that eradication of CSCs is the critical determinant in achieving cure. It has been proposed that CSCs may be particularly resistant to chemotherapy and radiation therapy, although good evidence supporting this notion has been lacking. In this issue of the Journal, Phillips et al. (7) report evidence that cells from breast cancer cell lines that resemble breast cancer-initiating cells are radioresistant compared with the remainder of breast cancer cells. Similarly, a recently published report by Bao et al. (8) suggests that glioblastoma stem cells are radioresistant and may therefore contribute to treatment failures.

Phillips et al. chose established breast cancer cell lines as their model system. Specifically, they applied conditions that were previously used for culturing mammospheres from primary breast cancer specimens (9) to culture nonadherent cells that they isolated from two adherent breast cancer cell lines. These nonadherent subpopulations could be grown as spheroids in mammosphere media and contained a larger fraction of cells than the adherent subpopulation with the CD24^{-low}/CD44⁺ phenotype, which was previously shown to identify breast tumor-initiating

cells in primary patient specimens (4). When the spheroids or adherent cultures were irradiated in vitro, the cells arising from spheroids were more radioresistant, with an absolute difference in mean surviving fraction at 2 Gy of approximately 20%. Importantly, both cell populations were irradiated as single-cell suspensions, thus removing the complicating factor of low oxygen tension at the center of the spheroids. The authors also examined several molecular assays relevant to radiosensitivity, including levels of reactive oxygen species and phosphorylation of histone H2AX, both of which were decreased in spheroid cultures. Furthermore, fractionated radiation appeared to increase the percentage of nonadherent CD24^{-low}/CD44⁺ cells in monolayer cultures, suggesting that the relative radioresistance of this subset may lead to their expansion during a course of radiotherapy. Thus, the authors present evidence that CSC-like cells from breast cancer cell lines appear to be radioresistant.

The findings of Phillips et al. are clearly provocative. However, close inspection of the study reveals several important issues that should be considered when interpreting CSC-related studies. First, as the authors point out, attempting to apply stem cell concepts initially described in primary tumors to cell lines is fraught with pitfalls. Because established cell lines have been passaged in artificial conditions for many generations, it is possible that selection for growth in tissue culture has blurred the distinction between tumorigenic and nontumorigenic cells. Indeed, a recent study on CSC-like subpopulations in murine ovarian cancer cell lines found that both the CSC-like and non-CSC-like subpopulations gave rise to tumors in nude mice but that the non-CSC-like cells formed tumors somewhat more slowly than the CSC-like population (10).

Second, culture conditions could contribute to functional differences between CSCs and non-CSCs found in vitro. The nonadherent spheroids in the study by Phillips et al. were grown in

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DOI: 10.1093/jnci/djj505

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the presence of epidermal growth factor and basic fibroblast growth factor but the adherent cells were not, and both growth factors have been shown previously to decrease radiosensitivity (11,12). Accordingly, when these growth factors were added to the adherent cultures, their radioresistance was similar to that of the nonadherent spheroids. Although the authors document a modest increase in the percentage of CD24^{-low}/CD44⁺ cells in the monolayer cultures after addition of the growth factors that may at least partially explain the increased radioresistance, the differential culture conditions complicate interpretation of their findings.

Given the effects of selection for growth in tissue culture, CSC-like subpopulations within established cell lines might not necessarily display the same cell surface immunophenotypes as CSCs within tumors. In general, the surface proteins that are used for identifying CSCs have not been shown to be necessary or sufficient for conferring stem cell-like properties and thus must currently be viewed as purification markers without functional implications. Therefore, rather than simply ascribing CSC-like properties to cells of a given surface immunophenotype, it is crucial to show that this population has the functional characteristics of CSCs. To accurately compare results from different studies, it is imperative to show, in each experimental system, that purported CSC-like cells can self-renew and give rise to more differentiated progeny and that non-CSCs lack these properties. Currently, this difference can be most convincingly demonstrated by serial transplantation of CSC-like and non-CSC-like cells in animal models (1).

Another important point relevant to all CSC-related studies concerns the purity of CSC populations. Given the number of CSCs needed to initiate tumors in rodent models (generally in the range of hundreds to thousands), it is possible that only a subset of cells within these populations will turn out to be true CSCs. In the system used by Phillips et al., the spheroid cultures contained approximately 40% CD24^{-low}/CD44⁺ cells and thus may have contained an even smaller fraction of true CSC-like cells. The presence of non-CSCs within the CSC population could cloud functional analyses because results might reflect the properties of the contaminating cells (possibly early progenitors) rather than those of CSCs. It will therefore be important to reevaluate functional characteristics, such as sensitivity to therapeutics, once purer populations of CSCs can be isolated.

Phillips et al. provide evidence that CSC-like cells may be intrinsically radioresistant, a finding that could clearly have important clinical implications. However, it is possible that *in vivo* microenvironmental effects either positively or negatively modulate this property. For example, if it turns out that CSCs tend to be located in hypoxic regions of tumors, this would likely affect their sensitivity to radiotherapy via the oxygen enhancement ratio. Alternatively, if the cell cycle distribution of CSCs and non-CSCs *in vivo* differs (and one might well expect this to be true), cell cycle effects might contribute to *in vivo* radiosensitivity. To address such questions, effects of ionizing radiation on CSCs also should be examined in the context of whole tumors, ideally both in xenograft models and in human patients.

The contemporaneous study by Bao et al. both confirms and extends the findings of Phillips et al. These authors studied xenografts from primary glioblastoma multiforme specimens, thus addressing many of the aforementioned concerns regarding established cell lines. Prior work identified CD133⁺ cells as the tumorigenic population in primary glioblastoma multiforme

specimens (3), and Bao et al. found that these cells were radioresistant compared with CD133⁻ tumor cells. Importantly, they showed that CD133⁺ cells accumulated after irradiation both *in vitro* (i.e., neurosphere cultures) and *in vivo* (i.e., xenografts). Furthermore, they demonstrated that the modest enrichment of CD133⁺ cells after irradiation has biologic relevance by showing that a slight increase in the percentage of CD133⁺ cells in suspensions used to initiate tumors dramatically increased their growth rate. Akin to the work by Phillips et al., Bao et al. also examined multiple molecular markers of radiation damage, including apoptosis, histone H2AX phosphorylation, and phosphorylation of genes involved in DNA damage repair, and found that by each of these measures, CD133⁺ cells were more radioresistant. They concluded that CD133⁺ cells can activate DNA damage checkpoint responses to a greater degree than CD133⁻ cells and thus repair DNA damage more efficiently. Intriguingly, by using an inhibitor of the checkpoint kinases Chk1 and Chk2, they were able to radiosensitize the CD133⁺ cells.

Taken together, the two studies suggest that radioresistance may be a general property of CSCs and that this may be due to their ability to more efficiently repair DNA than non-CSCs. As with most provocative studies, they raise more questions than they answer: Are CSCs of all tumor types radioresistant? Can the accumulation of CSCs after radiotherapy seen *in vitro* and in xenografts be documented in patients? Does the percentage of CSCs within human tumors predict radiosensitivity? Will CSCs be similarly resistant to chemotherapeutic agents? How about the newer targeted therapy agents? Will it be possible to develop radio- or chemosensitizers that preferentially sensitize CSCs compared with normal tissues?

The findings by Phillips et al. and Bao et al. have important clinical implications. In light of these studies, it is even clearer that identifying and characterizing CSCs for every tumor possible is of paramount importance and will likely lead to new therapeutic avenues. In the case of radiotherapy, it seems possible that response assays that capture the effects of radiation on CSCs rather than the bulk of a tumor could prove to be more sensitive predictors of treatment outcome than traditional measures of treatment response. Also, work on radiosensitizers should begin to focus on preferentially affecting CSCs compared with normal tissues and normal tissue stem cells. With these studies, the often quoted notion that solid tumor CSCs may contribute to treatment failure has begun to be substantiated.

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