Radiation induces the generation of cancer stem cells: A novel mechanism for cancer radioresistance (Review)

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Abstract. Radioresistance remains a major obstacle for the radiotherapy treatment of cancer. Previous studies have demonstrated that the radioresistance of cancer is due to the existence of intrinsic cancer stem cells (CSCs), which represent a small, but radioresistant cell subpopulation that exist in heterogeneous tumors. By contrast, non-stem cancer cells are considered to be radiosensitive and thus, easy to kill. However, recent studies have revealed that under conditions of radiation-induced stress, theoretically radiosensitive non-stem cancer cells may undergo dedifferentiation subsequently obtaining the phenotypes and functions of CSCs, including high resistance to radiotherapy, which indicates that radiation may directly result in the generation of novel CSCs from non-stem cancer cells. These findings suggest that in addition to intrinsic CSCs, non-stem cancer cells may also contribute to the relapse and metastasis of cancer following transformation into CSCs. This review aims to investigate the radiation-induced generation of CSCs, its association with epithelial-mesenchymal transition and its significance with regard to the radioresistance of cancer.

Contents

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1. Introduction

As one of the main treatments for cancer, radiotherapy has been widely used in the clinic for >100 years (1). With the development of advanced radiotherapy techniques, radiotherapy has become an extremely efficacious treatment for cancer. However, radioresistance and subsequent relapse and metastasis of cancer occurs in numerous patients that have received advanced radiotherapy (2). Previous studies have reported that intrinsic cancer stem cells (CSCs), which represent a small subpopulation of cancer cells that exist within heterogeneous tumors, are responsible for radioresistance in various types of cancer (3-6). By contrast, non-stem cancer cells, which are the differentiated progeny of CSCs that account for a substantial part of the tumor, are hypothesized to be radiosensitive and thus easy to kill using radiotherapy, resulting in the short-term regression of tumors.

In 1994, Lapidot first reported the existence of a particular subpopulation of cells in leukemia, which were finally termed CSCs or cancer initiating cells (7,8). CSCs are defined as a small cancer cell population within a tumor that has the capacity to self-renew and differentiate into the heterogeneous lineages of cancer cells that comprise the tumor (9). At present, it is postulated that tumor development is driven by the self-renewal and multi-lineage differentiation of CSCs, while their differentiated offspring do not possess the ability to self-renew and extensively proliferate, therefore losing tumorigenic potential (10). Tumors have been demonstrated to be heterogenic with hierarchical organization (11-13) and CSCs are considered to lie at the peak of the tumor hierarchy (8). Despite accounting for only a small proportion of the tumor mass, CSCs have been identified as the main reason for the development of therapeutic resistance, recurrence and metastasis (14-19), which indicates that the elimination of CSCs, rather than non-stem cancer cells, is important for the treatment of cancer. Therefore, recent studies have focused on developing novel treatment strategies that target CSCs (20-23).

To study these cells, CSCs must be identified and isolated from the tumor bulk or cancer cell lines. The most widely used method for identifying/isolating CSCs is based on the expression of specific cell surface marker or sets of markers (24). A number of specific cell surface markers of CSCs have been identified in a number of diverse human cancers, such as cluster of differentiation (CD)34$^+$CD38$^-$ for CSCs of acute myelomonocytic leukemia (25) and CD133$^+$ for CSCs of central nervous system tumors (26) and colon cancer (27). Recently, Schatton *et al* (24) extensively reviewed the specific cell surface markers of CSCs of diverse human cancers. It has been reported that the activity or expression of certain enzymes and membrane transporters in CSCs are different from that in non-stem cancer cells. For example, the activity of aldehyde dehydrogenase 1 (ALDH1) in CSCs is increased in various cancer types, including breast (28), lung (29) and pancreatic cancer (30). Furthermore, the cell membrane adenosine triphosphate-binding cassette (ABC) transporter is overexpressed in the CSCs of ovarian cancer (31), nasopharyngeal carcinoma (32), glioma (33) and lung cancer (34). Notably an isolation method for CSCs, which is based on the enzymatic activity of ALDH1, has been developed and is now widely accepted (28,35-38). Furthermore, side population assays, a well-known and extensively used technique for isolation of CSCs, are based on the fact that the overexpression of ABC transporter in CSCs effectively effuse the Hoechst dye (39). In addition to surface markers and functional markers, CSCs exhibit unique characteristics, including upregulation of anti-apoptotic proteins, increased efficiency of DNA repair and dormancy/slow cell cycle kinetics (40). These characteristics, together with functional markers, are reported to contribute to the resistance of CSCs to therapy (41-44).

A recent study revealed that like induced stem cells, non-stem cancer cells can dedifferentiate into CSCs via epithelial-mesenchymal transition (EMT) (45). In addition, it has been reported that radiotherapy induces cancer cells to undergo EMT, which results in the development of cancer cell radioresistance (46). Recent studies have confirmed that radiation can induce non-stem cancer cells to obtain the phenotype and functions of CSCs, including high resistance to radiotherapy (47,48). These results indicate that radiation can directly result in the generation of new CSCs from non-stem cancer cells and that these transformed non-stem cancer cells therefore become radioresistant and thus survive radiotherapy treatment (47,48). These findings indicate that non-stem cancer cells, in addition to intrinsic CSCs, contribute to relapse and metastasis of cancer following transformation into CSCs. This review will investigate the radiation-induced generation of CSCs, its association with EMT and its significance in cancer radioresistance.

2. CSCs exhibit a critical function in cancer cell radioresistance

Radiotherapy is one of the common approaches for cancer therapy. It may be used alone or in combination with chemotherapy and/or surgery. Radiotherapy has demonstrated therapeutic effects for the majority of cancer types and exhibits curative potential in a number of solid human tumors (49), including head and neck carcinoma (50) and non-small cell lung cancer (51). However, despite continuous advances in radiotherapy technology, a high proportion of patients succumb due to tumor recurrence and metastasis as a result of radioresistant cancer cells (2). Increasing evidence has revealed that CSCs are the main contributor to cancer radioresistance in the majority of tumor types, such as glioblastoma (3), head and neck cancer (4), breast cancer (5) and pancreatic cancer (6). Furthermore, Baumann *et al* (52) reported that the radioresistance of a tumor depends on the number of CSCs present within the tumor itself. Therefore, it was hypothesized that CSCs are responsible for the failure of radiotherapy (53).

Although the mechanism that confers radioresistance to CSCs remains unclear, significant advances in this area of study have been made. A number of potential factors are hypothesized to be involved in the radioresistance of CSCs. Desai *et al* (54) demonstrated that altered regulation of DNA repair genes, which contributes to enhanced double-strand break resolution, resulted in the radioresistance of human lung CSCs. Furthermore, compared with adherent prostate cancer cells (prostate cancer non-stem cells), cells in prostatospheres (prostate CSCs) exhibited higher expression levels of DNA repair proteins following exposure to ionizing radiation, which efficiently repair radiation-induced DNA injury (55) and therefore confer a survival advantage to CSCs. Bao *et al* (16) reported that CD133$^+$ glioma stem cells conferred glioma radioresistance via preferential activation of the DNA damage checkpoint response, as well as increased DNA repair capacity. Recently, Diehn *et al* (17) reported that, compared with non-tumorigenic cells, breast CSCs possessed increased free radical-scavenging ability due to the increased expression of free radical scavenging systems, which may reduce reactive oxygen species-mediated DNA damage and cell death after radiation. The Notch (56), c-Jun N-terminal kinase (57) and protein kinase Cδ signaling (58) pathways are also hypothesized to contribute to CSC radioresistance.

The tumor microenvironment also contributes to the radioresistance of CSCs. Jamal *et al* (59) reported that CD133$^+$ glioblastoma cells grown as intracranial xenografts repaired DNA damage more efficiently than those grown *in vitro*, as demonstrated by a more rapid decrease in level of radiation-induced γH2AX and tumor suppressor p53-binding protein 1 foci, the indicators of DNA damage, in the CD133$^+$ glioma cells grown *in vivo*. In a study using explant model and neurospheres culture models derived from surgical glioblastoma multiforme specimens, radiation was found to significantly reduce neurosphere formation in the neurospheres cultures model, but not in the explant model (60), which confirmed the involvement of the tumor microenvironment in CSC radioresistance.

3. Origins of CSCs

Although the function of CSCs in therapy resistance of cancer has been confirmed, the origin of CSCs remains controversial. Several hypotheses regarding the origin of CSCs have been suggested to date, including cell fusion between adult stem cells and transformed or normal somatic cells, horizontal gene transfer from apoptotic cells into normal stem/progenitor cells, chromosome derangements and gene mutations in stem/progenitor and differentiated cells and inflammatory

microenvironment stimulation, all of which have been reviewed by Bu and Cao (61). However, the present review focused on EMT as a potential mechanism by which CSCs are generated.

EMT is a unique dedifferentiation process that is involved in embryonic development, whereby cells lose epithelial features and gain mesenchymal properties (62). EMT has also been identified in cancers derived from numerous tissue types, including esophageal (63), breast (64), colon (65), ovarian (66) and thyroid gland tissues (67). The cells undergoing oncogenic EMT observed in cancer exhibit similar characteristics to those undergoing developmental EMT, such as spindle-shaped morphology, loss of cellular polarity, disintegration of tight junctions and adherens junctions, downregulation of E-cadherin (epithelial cell marker) and upregulation of N-cadherin and vimentin (mesenchymal markers) and an increase in migratory and invasive ability. The EMT process transforms the epithelial phenotype exhibited by cancer cells into a mesenchymal phenotype, resulting in cells that are more invasive, metastatic and resistant to therapy (68). Therefore, EMT is hypothesized to promote progression and aggressiveness of tumors (62) and notably, increased expression of EMT markers in tumors is associated with distant metastasis and poor prognosis (69). Therefore, these results indicate an association between EMT and CSCs.

It has been reported that EMT-derived cells exhibit potential for multi-lineage differentiation that is similar to mesenchymal stem cells (70). Furthermore, the induction of the EMT process in immortalized human mammary cells results in the expression of stem cell markers and an increased ability to form mammospheres, which are similar to those of stem cell-like cells isolated from cultured human mammary epithelial cells (45). These findings indicate that EMT generates mammary cells with stem cell properties from normal mammary epithelial cells. Notably, the study also indicated that after undergoing EMT, experimentally immortalized human mammary epithelial cells dedifferentiated into CSCs, as demonstrated by the increased formation of colonies in soft agar suspension culture and tumor spheres, which indicate the *in vitro* tumorigenicity and stemness of cells, respectively (45). In addition, the *in vivo* tumorigenic capacity assay also demonstrated that the immortalized human mammary epithelial cells that had undergone EMT formed tumors more efficiently than those that were undergoing the EMT process upon subcutaneously injecting them into athymic nude mice (45). These findings indicate that EMT promotes the generation of CSCs from more differentiated neoplastic cells. Similarly, Morel *et al* (71) confirmed that breast CSCs possessing stem and tumorigenic traits may be generated from non-tumorigenic mammary epithelial cells through EMT. Another similar study using a breast cancer model also demonstrated that EMT *in vivo* generates breast CSCs, even if the process of EMT is incomplete or aberrant (72). Furthermore, a clinical study using thyroidectomy specimens obtained from patients with anaplastic thyroid carcinoma (ATC) and contiguous differentiated thyroid carcinoma (DTC) revealed that nestin, a marker for stem cell phenotype, was overexpressed in ATC, while no expression of E-cadherin was observed in ATC. By contrast, contiguous DTC specimens were negative for nestin and positive for E-cadherin expression (67). This study confirmed that EMT is associated with the acquisition of a stem cell phenotype in ATC, however, the significance of the study is limited by the small case series: The authors suggested that a further study based on a larger series of cases is required.

4. Radiation induces EMT in cancer

The association between radiation and EMT has gained increasing attention recently. A number of studies have confirmed that radiation can induce EMT or phenotypic changes similar to EMT (73-75). For example, in KYSE-150R cells, a radioresistant esophageal cancer cell line, phenotypic changes similar to EMT are induced by radiation, including decreased E-cadherin and increased Snail and Twist expressions (76), which are also observed in nasopharyngeal carcinoma (77) and colorectal cancer (78). Furthermore, a number of pathways have been reported to contribute to radiation-induced EMT of cancer cells. In lung cancer cells, radiation increases EMT by regulating epithelial and mesenchymal cell markers via the Janus kinase 2/ p21-activated kinase 1/Snail signaling pathway (79). Furthermore, Yuan *et al* (80) reported that B lymphoma Mo-MLV insertion region 1 exhibits a central function in the regulation of radiation-induced EMT via activation of phosphoinositide 3-kinase/protein kinase B signaling in breast cancer cells. In addition, in a study using cervical cancer cells, low-dose radiation was demonstrated to activate the nuclear factor-κB (NF-κB) pathway, which subsequently resulted in EMT of cervical cancer cells (81).

In contrast with phenotypic changes, the characteristic changes in the behavior of cancer cells that have undergone EMT post-radiation are more attractive to investigators. The finding that cancer cells that have obtained mesenchymal phenotypes by EMT are more resistant to therapy implies that radiation-induced EMT may have conferred radioresistance to these cancer cells, which contribute to the relapse of cancer following radiotherapy. This hypothesis has been confirmed by numerous studies involving various types of cancer. Chang *et al* (46) revealed that prostate cancer cells exhibiting EMT after radiation therapy become more resistant to radiation. Similar results have also been reported in other types of cancer, including pancreatic cancer (82), colorectal cancer (83), breast cancer (84), lung cancer (79), nasopharyngeal carcinoma (77), hepatocellular carcinoma cells (85) and gastric cancer (86).

5. Radiation induces the generation of CSCs

The observation that radiation induces EMT of cancer cells, which drives the dedifferentiation of adult cancer cells into CSCs, indicates that radiation may result in the generation of CSCs from differentiated cancer cells. It has been demonstrated that CSCs can be enriched both *in vitro* and *in vivo* by radiation, which indicates the possibility of radiation-induced generation of CSCs. Wang *et al* (87) demonstrated that the proportion of prostate cancer stem-like cells in a human prostate cancer cell culture increased significantly following exposure to radiation. The authors postulated that radiation eliminated the radiosensitive adult cancer cells in the culture by inducing apoptosis, which resulted in the enrichment of radioresistant CSCs. Al-Assar *et al* (88) reported that breast

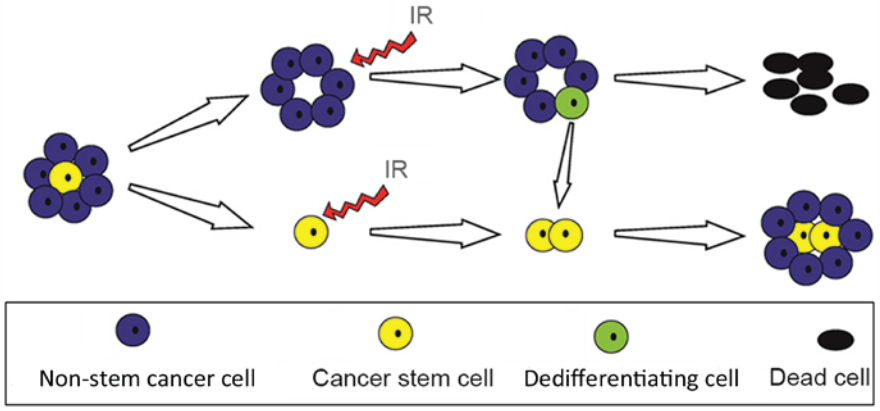

Figure 1. Radiation-induced generation of CSCs contributes to the relapse and metastasis of cancer. CSCs are a small, but radioresistant cell subpopulation that exist within heterogeneous cancer masses. Under conditions of radiation-induced stress, CSCs survive following IR; however, the majority of non-stem cancer cells are killed via various mechanisms such as induction of cell apoptosis or mitotic death. However, a small number of non-stem cancer cells undergo dedifferentiation and transform into CSCs via unknown mechanisms. The newly generated CSCs, together with the intrinsic CSCs, subsequently contribute to relapse and metastasis of cancer. CSCs, cancer stem cells; IR, irradiation.

CSCs in xenografts exposed to radiation were enriched, as demonstrated by an increased number of $CD24^-$/epithelial-specific $antigen^+$ cancer cells, a marker of breast CSCs, in xenografts. The enrichment of breast CSCs in xenografts exposed to radiation was also considered as the result of different radioresistance between CSCs and adult cancer cells, which was consistent with the aforementioned speculation of Wang *et al* (87). This explanation was undoubtedly reasonable, but may be not complete. Thus, there may be another reasonable source causing an increase in the absolute number of CSCs and subsequently resulting in CSCs enrichment upon radiation: Radiation-induced generation of CSCs. This source cannot be ignored, since the possibility that cancer cells without stemness markers could obtain stemness markers upon exposure to irradiation was not excluded in the aforementioned studies of Wang *et al* (87) and Al-Assar *et al* (88).

In 2012, Lagadec *et al* (48) revealed for the first time that the enrichment of breast CSCs following radiation was involved in the induction of stem cell-like properties in non-stem cancer cells. In this study, the non-stem breast cancer cells ($ALDH1^-$ cells) in single cell suspensions obtained from fresh human breast specimens or established cells lines, were isolated using fluorescence-activated cell sorting after ALDH1 staining (48). These non-stem breast cancer cells were subsequently exposed to various dose of radiation. Following 5 days of irradiation, the number of $ALDH1^+$ cells in the irradiated non-stem breast cancer cell population increased significantly in a dose-dependent manner, which indicated that radiation promoted the non-stem breast cancer cells to exhibit a CSC phenotype. Furthermore, the generated breast CSCs induced by radiation exhibited increased mammosphere formation, increased tumorigenicity and expressed the same stemness-related genes as breast CSCs obtained from non-irradiated samples. Furthermore, these induced breast CSCs exhibited resistance to radiation. This study confirmed that radiation induced the generation of CSCs, which was also reported by Wang *et al* (89). Additionally, Ghisolfi *et al* (47) demonstrated that radiation induced stem cell-like properties in non-stem hepatocarcinoma cells, as demonstrated by the findings that non-side population (CSC-depleted population) cells from HepG2 and Huh7 cells exhibited increased sphere formation and stemness gene expression after exposure to radiation.

To date, no studies have investigated the involvement of EMT in the radiation-induced generation of CSCs. However, studies investigating the mechanism underlying radiation-induced generation of CSCs have indicated the potential association between EMT and the generation of radiation-induced CSCs (90,91). Lagadec *et al* (48) reported that inhibition of Notch receptor expression reduced the ability of the cells to form mammospheres, and therefore concluded that the ionizing radiation-induced translation of non-stem breast cancer cells was Notch-dependent. Previous studies have revealed that Notch signaling mediates EMT via direct or indirect regulation of Snail expression (92-94), a key transcription factor regulating EMT, or via epigenetic mechanisms involving miRNA (95). In another study by Wang *et al* (89), the expression of NF-κB in breast cancer cells was elevated after radiation exposure, which contributed to the expression of stemness genes. Inhibition of NF-κB blocked radiation-induced stemness *in vitro* and *in vivo*, which indicated that the NF-κB pathway was involved in the radiation-induced generation of breast CSCs. Similar to the Notch pathway, the NF-κB pathway was also reported to contribute to EMT via transcriptional regulation of genes involved in EMT, including Snail (96), zinc-finger E-box-binding (ZEB)1 and ZEB2 (97) and Twist (98). These findings suggest that EMT is involved in the radiation-induced generation of CSCs.

The observation that radiation induces the generation of CSCs from differentiated cancer cells highlights a novel interaction between radiation and cancer, which may be key to understanding cancer radioresistance. The killing effect of radiation on cancer cells has been well established and is widely used in the clinic as the main approach for cancer therapy. Previous studies have recognized that radiotherapy can effectively kill the majority of differentiated cancer cells in the hierarchical cancer tissue during treatment, however, the intrinsic radioresistant CSCs in the cancer tissue survive

radiotherapy and therefore this results in the relapse and metastasis of cancer (2,99). The findings that radiation can induce the generation of fresh CSCs from non-stem cancer cells and that the novel CSCs exhibit radioresistant traits similar to the intrinsic CSCs indicates that the newly generated CSCs induced by radiation may be partly responsible for the radioresistance of cancer (Fig. 1).

6. Conclusion

Previous studies have established that the relapse and metastasis of cancer is due to the existence of intrinsic, radioresistant CSCs in hierarchical cancer tissue (100-104). Recent evidence indicates that radiation converts non-stem cancer cells into CSCs, which exhibit similar radioresistance to intrinsic CSCs (90). These results provide novel insights with regard to the mechanism of cancer radioresistance, through which the differentiated and radiosensitive non-stem cancer cells that should be killed by radiotherapy are able to survive radiotherapy. After radiotherapy-induced stresses disappear, these newly generated CSCs, together with the intrinsic CSCs, contribute to the relapse and metastasis of cancer. Future studies investigating the underlying pathways driving this transformation may lead to the development of treatment approaches that block the generation of induced CSCs and subsequently enhance the efficacy of radiation treatment.

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