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Award Article

Acquired Radioresistance of Cancer and the AKT/GSK3β/cyclin D1 Overexpression Cycle

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Radioresistance/Fractionated radiation/AKT/Cyclin D1 overexpression.

Fractionated radiotherapy (RT) is widely used in cancer therapy for its advantages in the preservation of normal tissues. However, repopulation of surviving tumor cells during fractionated RT limits the efficacy of RT. In fact, repopulating tumors often acquire radioresistance and this is the major cause of failure of RT. We have recently demonstrated that human tumor cells acquire radioresistance when exposed to fractionated radiation (FR) of X-rays every 12 hours for 1 month. The acquired radioresistance was associated with overexpression of cyclin D1, a result of a series of molecular changes; constitutive activation of DNA-PK and AKT with concomitant down-regulation of glycogen synthase kinase-3β (GSK3β) which results in suppression of cyclin D1 proteolysis. Aberrant cyclin D1 overexpression in S-phase induced DNA double strand breaks which activated DNA-PK and established the vicious cycle of cycling D1 overexpression. This overexpression of cyclin D1 is responsible for the radioresistance phenotype of long-term FR cells, since this phenotype was completely abrogated by treatment of FR cells by the API-2, an AKT inhibitor or by a Cdk4 inhibitor. Thus, targeting the AKT/GSK3β/cyclin D1/Cdk4 pathway can be an efficient modality to suppress acquired radioresistance of tumor cells. In this article, I overview the newly discovered molecular mechanisms underlying acquired radioresistance of tumor cells induced by FR, and propose a strategy for eradication of tumors using fractionated RT by overcoming tumor radioresistance.

INTRODUCTION

Radiotherapy (RT) is one of the major modalities of cancer treatment with excellent tumor control, preservation of normal tissues and less systemic influences. Originally, therapeutic radiations were delivered as a single fraction of a large dose and this approach often resulted in serious side effects such as acute reactions and later skin damage.[1] Treatment schedule was invented in which radiation was delivered in small multiple fractions (FR) which improved preservation of normal tissue functions. Differential repair capacities of slowly proliferating normal tissue cells and rapidly proliferating tumor cells underlie the mechanism of sparing sever damage to the former in the fractionated RT.[2,3] General protocol of fractionated RT consists of daily exposures to a fraction dose of around 2 Gy for 5 to 7 weeks. Although tumors receive a large total dose by multiple FR, they sometimes recur with radioresistance which eventually leads to treatment failures of RT.[4] Repopulation of tumor cells is frequently observed during fractionated RT and the rate of proliferation accelerates with increasing the overall treatment time in clinical situation. This accelerated repopulation of tumor cells are associated with the acquisition of radioresistance. The phenomenon of acquired radioresistance by repeated exposure of low-dose X-ray has been reported around 1950.[5–7] In the past, mechanisms of acquired radioresistance include; selection of intrinsic radioresistant cells in a heterogeneous population, induction of mutations toward radioresistance following initial irradiation and the change in the tumor microenvironment to protect tumor cells (tumor bed changes).[8] However, the precise mechanism behind acquired radioresistance remains elusive and elucidation of the molecular mechanisms of acquired radioresistance is essential for the development of a more effective fractionated RT.

The acquisition of radioresistance is also reported in the name of radioadaptive response which has been defined by the induction of radioresistance to subsequent high dose of radiation by a single exposure to a low priming dose of 0.01–0.2 Gy.[9] Adaptive response persists up to 20 to 66 hour after exposure to priming dose of irradiation in mouse fibroblasts and in human lymphocytes, respectively and the resistance subsides thereafter, showing the transient nature of the phenotype.[10,11] In contrast, acquired radioresistance report-

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ed previously by Shimura *et al*. manifests after exposure of FR with 0.5 Gy for 1 month and the resistance persisted for another 1 month after cessation of FR.[12] Thus, the acquired radioresistant phenotype is long lasting and possibly irreversible.

We have established long-term FR cells of HepG2 and HeLa by exposure to 0.5 Gy of X-rays every 12 hours for 1 month and analyzed DNA damage response (DDR) of those cells. We have found that acquired radioresistance is a result of the constitutive activation of AKT/glycogen synthase kinase-3β (GSK3β)/cyclin D1/Cdk4 pathway which is induced by a positive feedback loop mediated through the cyclin D1 overexpression cycle triggered by FR.[12] Inhibition of the AKT/GSK3β/cyclin D1/Cdk4 pathway using an AKT inhibitor of AKT/PKB signaling inhibitor-2 (API-2) or a Cdk4 inhibitor of 2-Bromo-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione can suppress acquired radioresistance of tumor cells.

This paper reviews DDR of long-term FR cells and molecular mechanisms of acquired radioresistance induced by FR. The AKT/GSK3β-mediated cyclin D1 overexpression cycle is important in acquired radioresistance and is the potent molecular target to enhance the efficacy of fractionated RT.

Disruption of G1/S Checkpoint by Cyclin D1 Overexpression Following Long-term FR

In order to elucidate the molecular mechanisms underlying acquired radioresistance of tumor cells induced by FR, we have studied DDR of long-term FR in human tumor cells of HepG2 and HeLa. Molecular mechanisms of DDR are well studied using a single radiation (SR) exposure regime, and multiple pathways of cell cycle checkpoint, DNA repair and cell death are involved.[13–15] Cell cycle checkpoint is activated to block the progression of cells to allow the time for proper repair of DNA damage.[16] The G1/S checkpoint operates through inactivation of cyclin-dependent kinases (CDKs) via degradation of positive regulators of CDKs such as cyclin D1 and Cdc25A, and by activation of a negative regulator such as p21 through the p53 pathway. Inactivation of CDKs leads to dephosphorylation of Rb which then sequesters E2F to prevent progression through the G1/S phase of the cell cycle.[17–21] In contrast to these DDR for SR, DDR for long-term FR remains to be elucidated.

The G1/S checkpoint mediated by cyclin D1 degradation was observed after SR in parent cells before acquisition of radioresistance, whereas long-term FR cells entered S-phase after the same SR with subsequently arrested in late S-phase or G2-phase. Disruption of the G1/S checkpoint in long-term FR cells is a result of resistance of radiation-induced cyclin D1 degradation, which is necessary for activation of early G1/S checkpoint.[22] GSK3β kinase is the protein kinase that phosphorylates cyclin D1 on threonine286 (Thr286) to facilitate its degradation on cell cycling. This phosphorylation also regulates cyclin D1 degradation in response to DNA damage.[23–25] The AKT-mediated phosphorylation of GSK3β on serine 9 decreases its kinase activity on Thr286 of cyclin D1, which inhibits the nuclear export and the cytoplasmic proteasomal degradation of cyclin D1.[26,27] Thus, AKT positively regulates G1/S cell cycle progression through inactivation of GSK3β, leading to nuclear accumulation of cyclin D1. Upon SR, activation of AKT and inactivation of GSK3β were observed transiently in parent cells as reported by Tan *et al*.[28, 29] In contrast, long-term FR constitutively activates DNA-PK and AKT, which in turn inactivate GSK3β-mediated cyclin D1 degradation.[12] Consequently, the G1/S checkpoint was disrupted in long-term FR cells by the down-regulation of cyclin D1 degradation through the constitutive active AKT/GSK3β pathway.

ATM signaling has been shown to play a critical role in the regulation of cyclin D1 degradation in response to DNA damage via F-box protein FBXO31.[30,31] However, this pathway is thought to be inactivated by the defect of cyclin D1 phosphorylation through the AKT/GSK3β pathway in long-term FR cells.

Acquired Radioresistance by Constitutive Active DNA-PK/AKT/GSK3β/cyclin D1 Pathway

Cyclin D1 is an important regulator of cell cycle progression at G1/S transition. Cyclin D1 accumulates during progression of the G1-phase and it is degraded in the S-phase.[32] Deregulation of cyclin D1 in the S-phase perturbs DNA replication and triggers DNA damage checkpoint.[33–35] Similarly, cyclin A overexpression induces DNA double strand breaks (DSBs) in human and mouse fibroblasts.[36] These results indicate that deregulation of cell cycle regulators, cyclin D1 and cyclin A is associated with induction of DSBs. We have shown DSBs in the S-phase of long-term FR cells by γ-H2AX foci, a hallmark of DSBs[37,38] and by neutral comet assay.[12] Formation of DSBs in long-term FR cells can be suppressed when cyclin D1 was down-regulated by cyclin D1 siRNA. This indicates that DSBs are mediated by cyclin D1 overexpression. Interestingly, cyclin D1 overexpression prevented DNA replication by suppressing the progression of replication forks in long-term FR cells (Shimura and Ochiai unpublished observation). These results suggest that cyclin D1-mediated DSBs are thought to be generated by erroneous DNA replication. Indeed, DSBs are made by cooperation of BLM helicase with Mus81 nuclease during processing of stalled replication forks that induced by treatment with a low-dose aphidicolin, inhibitor of DNA polymerase α.[39,40] However, further investigation is necessary to clarify the underlying mechanism of cyclin D1-mediated DSBs.

Cyclin D1-mediated DSBs trigger DDR in which DNA damage checkpoint and homologous recombination repair are involved. Furthermore, radiation-induced DNA damage

was rapidly repaired in long-term FR cells compared to parent cells.[12] Thus, long-term FR cells promote radioresistance by efficient DNA repair via constitutive activation of DDR mediated through cyclin D1 overexpression. Figure 1 is the schematic representation of the feedback loop in long-term FR cells, leading to cyclin D1 overexpression and acquisition of radioresistance. Long-term FR activates DNA-PK and AKT which in turn inactivate GSK3β to prevent nuclear export and cytoplasmic degradation of cyclin D1. Nuclear accumulation of cyclin D1 in the S-phase perturbs DNA replication and induces DSBs which then activate DNA-PK, triggering activation of AKT and suppression of GSK3β-mediated cyclin D1 degradation. This completes positive feedback loop of cyclin D1 overexpression. Thus, cyclin D1 overexpression cycle is continuously activated in long-term FR cells even after cessation of FR. DNA-PK is reported to activate AKT in response to various genotoxic stresses including low doses of irradiation to promote cell survival against lethal radiation damage.[41,42] Our analysis revealed that DNA-PK/AKT/GSK3β/cyclin D1 pathway is associated with acquired radioresistance of tumor cells triggered by FR.

Aberrant overexpression of cyclin D1 by down-regulation of proteolysis is frequently noted in various tumor cells and is linked to the development of cancer.[43,44] Overexpression of cyclin D1 is strongly correlated with the poor prognosis of oral carcinoma and head and neck squamous cell carcinoma after RT or chemo-RT.[45,46] Although our observations of acquired radioresistance by cyclin D1 overexpression have to be verified in other tumor cells and in clinical cases of human tumors undergoing RT, the level of cyclin D1 is thought to be worth monitoring along the course of the treatment for assessing the efficacy of fractionated RT.

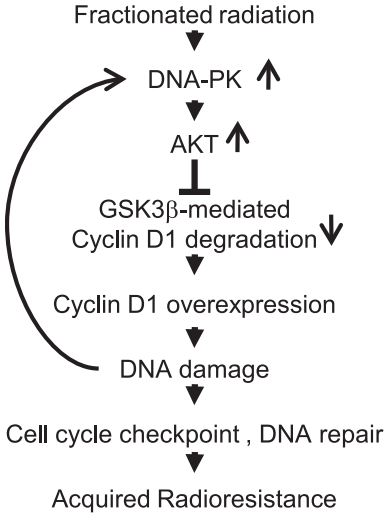

Fig. 1. Acquired radioresistance by cyclin D1 overexpression cycle. A positive feedback loop depicting the roles of DNA-PK, AKT, GSK3β and cyclin D1 in cyclin D1 overexpression and the resulting acquisition of radioresistance are represented. DNA-PK/AKT/GSK3β-mediated cyclin D1 overexpression resulted in acquired radioresistance of tumor cells by constitutive activation of DDR in long-term FR cells.

Cyclin D1 expression is regulated both at the transcriptional and the post-translational levels. The amounts of mRNAs for *CCND1* (the *cyclin D1 gene*) were not dramatically different among parent cells and long-term FR cell. Therefore, cyclin D1 overexpression was not due to the mutation of cyclin D1 gene, such as gene amplification, but due to the decreased degradation of the protein mediated by the AKT/GSK3β pathway. Immunostaining analysis of cyclin D1 revealed that cells with overexpressing cyclin D1 were not existed in parent cells before FR, but it appeared by exposure of long-term FR (Shimura unpublished observation). Thus, cyclin D1 overexpression is acquired trail as a result of epigenetic change mediated through the DNA-PK/AKT/GSK3β pathway. Consequently, we propose that acquired radioresistance is attributed to alterations of DDR induced by long-term FR rather than selection of radioresistant cells or the change in the tumor microenvironment.

Targeting the AKT/GSK3β/cyclin D1/Cdk4 Survival Signaling Pathway for Suppression of Tumor Radioresistance

Accumulating evidence suggests that the phosphatidylinositol-3-OH kinase (PI3K)/AKT signaling pathway regulates cellular processes of proliferation and survival that contribute to tumor progression.[26,27,47] In response to growth signals and DNA damage signals via a variety of growth factor tyrosine kinases, PI3K generates phosphatidylinositol-3,4,5-triphosphate, which is a lipid messenger essential for the translocation of AKT to the plasma membrane, where it is phosphorylated and activated by 3-phosphoinositide-dependent kinase 1 (PDK1).[47] The PI3K/AKT pathway is also implicated both in radioresistance and chemoresistance of human tumor cells.[48–53] AKT is known to block apoptotic pathways by regulating several target molecules including proapoptotic and antiapoptotic proteins.[47,54,55] Active AKT, a common mediator of cell survival signals induced by radiation through multiple intracellular signaling pathways,[56,57] modulates apoptosis and increases the apoptotic threshold. Thus, the AKT activity of tumor cells determines a cell's fate after irradiation. Inhibition of the AKT pathway can modulate apoptosis sensitivity of tumor cells to increase radiation-induced cell death and improve clinical outcome. Although AKT is involved in regulation of cell survival and proliferation, downstream targets of the AKT signaling pathway responsible for tumor radioresistance are still elusive. We have shown that the treatment with an AKT inhibitor of API-2[58] or siRNA targeted cyclin D1 successfully suppressed acquired radioresistance of tumor cells. Furthermore, treatment with either API-2 or Cdk4 inhibitor was also

effective in suppressing resistance to anti-tumor drug of cisplatinum (II)-diammine-dichloride (CDDP) in the cells with acquired radioresistance. These treatments rendered acquired radioresistant cells susceptible to radiation and CDDP with increased apoptosis. Cyclin D1 overexpression using with cyclin D1 expressing vector invalidated radio- and chemo-sensitization of API-2 in acquired radioresistant cells, whereas the Cdk4 inhibitor can suppress the radio- and chemo-resistance in those cells with cyclin D1 overexpression. Thus, cyclin D1/Cdk4 is downstream of the AKT pathway and is the most important AKT target regulating radio- and chemo-sensitivity of tumor cells. These results suggest that manipulation of cyclin D1 and Cdk4 may be useful for enhancement of radio- and chemo-sensitivity in acquired radioresistant cells induced by FR. In addition to the regulation of cell survival, AKT is associated with cell growth, hypoxia and cell metabolism of tumor cells. These cellular functions are thought to be associated with acquired radioresistance. Further characterization of the AKT on acquired radioresistance is currently under investigation.

Due to the expansion of the understanding of the molecular mechanisms underlying radioresistance, the combination of RT with molecular-targeted agents that sensitize tumors to radiation is now being applied for cancer treatment.[59–61] A number of therapeutic agents have been shown to induce cyclin D1 degradation.[62] We have shown that treatment with API-2 alone has no effect on induction of cell death in HeLa cells and have no detectable side effects on body weight or activity in mice.[58,63] Thus, combination of radiation with API-2 could suppress acquired radioresistance of tumor cells without harmful effects. However, careful evaluation is required to confirm the feasibility of API-2 in fractionated RT.

In this review, we have described the molecular mechanisms of acquired radioresistance induced by cyclin D1 overexpression. We would propose the importance of cyclin D1 on radiosensitivity and chemosensitivity of tumors, and that the AKT/GSK3β/cyclin D1/Cdk4 pathway may serve as a new target to enhance efficacy of RT.

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