Evaluation of premature senescence and senescence biomarkers in carcinoma cells and xenograft mice exposed to single or fractionated irradiation

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Abstract. The purpose of the present study was to elucidate whether premature senescence contributes to the outcome of radiotherapy (RT) and to validate senescence biomarkers in vitro and in vivo. Cultured human cancer cell lines and xenografted mice were exposed to single (SR; 2, 6 or 12 Gy) or fractionated radiation (FR; 3 x 2 Gy or 6 x 2 Gy), and premature senescence was assessed using senescence-associated β-galactosidase (SA-β-Gal) activity, hypophosphorylation of pRb and p21 accumulation. A variety of senescence-associated biomarkers including cathepsin D (CD), the eukaryotic translation elongation factors eEF1A1, eEF1B2, decoy receptor 2 and Dec1 were further validated in vivo or in vitro. We demonstrated the beneficial tumor suppressive role of ionizing radiation (IR)-induced premature senescence in vitro and in vivo. FR inhibited tumor growth via induction of premature senescence as effectively as an equivalent SR dose (≥6 Gy). In addition, CD and eEF1 were valuable biomarkers of cellular senescence in either SR- or RF-exposed carcinoma cells or xenograft mice. Our results suggest that 2 Gy of a conventional RT regime could achieve a better clinical outcome if premature senescence could be increased through an improved understanding of its molecular action mechanism.

Introduction

Ionizing radiation (IR)-induced apoptosis has been known to play a primary role in the therapeutic effect of IR. Many studies have addressed the mechanism of IR-induced apoptosis and the most effective strategies to induce it in radiotherapy (RT)-targeted cancer cells (1-4). However, it has been reported that apoptosis may not be the exclusive or even the primary mechanism underlying tumor regression due to cancer therapy; many studies have indicated that premature senescence also plays a crucial role in tumor regression as well as cancer prevention (5-7). Cellular senescence is induced by a variety of signals, such as telomere shortening, oncogene activation, ROS and DNA damage (8,9). Senescent cells undergo functional and morphological changes including senescence-associated β-galactosidase (SA-β-Gal) activity and large and flat morphology (10,11). Previous studies showed that cancer cells may become prematurely senescent rather than apoptotic in response to DNA damage depending on cellular context, although the mechanism of this alternate response is unclear. Senescent cells are found in pre-malignant lesions in mice and humans, and induction of senescence prevents malignant progression in certain mouse tumor models (12-14). Premature senescence may be induced in cancer cells using lower doses of a drug than required to induce apoptosis (15,16), indicating that cancer therapies targeting cellular senescence may reduce damage of normal tissues. However, despite the interest in the clinical application of premature senescence to increase RT efficacy, its importance in IR-exposed cells or tissues remains to be further validated.

RT is an essential therapeutic modality for a wide range of malignant tumors. Although 40-60% of cancer patients receive RT, its clinical usage is hindered by the significant morbidity of radiation-induced injury to surrounding normal tissue. A major challenge in radiation oncology is minimizing...
the detrimental effects of RT on normal tissue while maximizing its tumoricidal effects (17,18). Fractionated radiation (FR) treatment reduces the damage to the surrounding normal cells while maintaining the probability of tumor control (19). In the clinical setting, the total IR dose is carefully determined and is often fractionated to reduce the injury to normal tissues. Due to differences in the repair of sub-lethal damage in normal vs. tumor cells, 2 Gy fractional doses are typically used in conventional FR (20,21). It is not well known whether FR or any IR modality effectively induces premature senescence in vitro, mainly due to the shortage of reliable biomarkers of senescence as well as the technical difficulty of validating permanent cell cycle arrest. Although several novel markers of senescence have been identified (15,22), their reliability, diagnostic and prognostic values require further characterization.

In the present study, we assessed IR-induced cancer cell senescence and senescence biomarkers in vitro and in vivo, and compared the relative effectiveness of single radiation (SR) vs. FR.

**Materials and methods**

**Cell culture and animals.** H460 and MCF7 cells were cultured as described by Byun et al (15). Female BALB/c athymic nude mice (Orient Co., Seongnam, Korea) were maintained and studied as described by Byun et al (15).

**Antibodies.** Rabbit anti-eEF1A1 and anti-eEF1B2 antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-phospho-pRb and mouse anti-p53 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and Novocastra Inc. (Newcastle, UK), respectively. Rabbit anti-p21, goat anti-cathepsin D, rabbit anti-DecR2, mouse anti-DEC1, goat anti-β-actin, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Irradiation.** Cells were irradiated to γ-ray with 137Cs γ-ray source (Atomic Energy of Canada Ltd., Mississauga, Canada) at a dose rate of 3.2 Gy/min. Irradiation of cells was based on three single irradiation doses (2, 6 and 12 Gy), while fractionated irradiation of cells was conducted daily to 2 Gy three times with 4 h time intervals for single day or two consecutive days (3 x 2 Gy or 6 x 2 Gy). Local regional irradiation of xenografted tumor was performed under anesthesia using a 60Co source irradiator (Theratron 780; Atomic Energy of Canada Ltd.) operating at 1.3 Gy/min. When xenografted tumor volume reached 200-250 mm³, xenografted regions of mice were exposed to SR or FR with the same procedures.

**Relative cell number, clonogenicity, SA-β-Gal staining and western blotting.** Analyses of relative cell number, clonogenicity, SA-β-Gal activity and western blotting were performed as described by Byun et al (15).

**Xenograft mouse model.** H460 lung cancer cells (5x10⁶ cells) were injected subcutaneously to the right side proximal hind legs of 6 week-old nude mice. Xenograft tumor growth was monitored every day by measuring tumor width and length using a digital caliper. Tumor volume was calculated by the following equation: Volume = 0.5 x Width² x Length.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay.** Apoptosis in tumor tissue was assayed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (ApopTag® Peroxidase In Situ Apoptosis detection kit; Millipore, Temecula, CA, USA) following the manufacturer’s protocol. Sections were counterstained with hematoxylin. Negative control sections were incubated with distilled water in the absence of TdT.

**Immunohistochemistry.** Tumor tissues from xenografted mice were fixed and embedded in paraffin. Immunohistochemistry was performed with the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturer’s protocol. Immunoreactive sites were visualized by 3,3'-DAB. Subsequently, the slices were counterstained by hematoxylin.

**Results**

**Effect of SR and FR on premature senescence in carcinoma cell lines.** To investigate whether equivalent total doses of SR or FR induced premature senescence, we exposed MCF7 and H460 carcinoma cells to a range of doses of SR (2, 6 or 12 Gy) or FR (3 x 2 Gy or 6 x 2 Gy). In both cell lines, 6 or 12 Gy SR dose-dependently decreased clonogenicity and relative cell number, increased SA-β-Gal activity and induced large and flat cell morphology (Figs. 1A-C and 2A-C). These results indicate that either 6 or 12 Gy SR effectively induced cellular senescence rather than cell death. Since RT is conventionally delivered in fractions of 2 Gy, we compared the effects of 6 or 12 Gy FR on MCF7 and H460 cells (Figs. 1 and 2). The same total dose of FR resulted in similar effects as SR on clonogenicity, relative cell number and cell death in both cell lines. Thus, our data demonstrate that premature senescence is the major response of carcinoma cells to 6 or 12 Gy of IR, regardless of SR or FR.

**Evaluation of senescence markers in SR- or FR-exposed carcinoma cell lines.** We reported CD, eEF1A1 and eEF1B2 and others have reported DecR2 and Dec1 as senescence markers (15,22); however, the reliability of these markers requires further validation. We examined expression changes of these markers during premature senescence in SR- or FR-exposed cells (Fig. 3). In either cell line, expression of CD was increased and eEF1A1 and eEF1B2 were decreased by 6 or 12 Gy of SR or FR. Notably, p53 and p21 accumulated not only in 6 or 12 Gy of SR- or FR-exposed cells, but also in 2 Gy of SR-exposed cells which did not undergo either apoptosis or cellular senescence. The changes in expression of CD, eEF1A1 and eEF1B2 closely correlated with hypophosphorylation of pRb, a reliable indication of cell cycle arrest. In contrast to the report of increased Dec1 and DecR2 expression in oncogene-induced prematurely senescent tumor cells (13), after IR exposure in our experiments, DecR2 expression decreased in both cell lines...
Figure 1. The cellular response in SR- or FR-exposed MCF7 cells. (A) Clonogenicity and (B) relative cell number were decreased by 6 or 12 Gy of SR or FR. (C) Phase contrast images of SA-β-Gal-stained cells (blue). Note the large and flat morphology of SA-β-Gal-positive cells. (D) Propidium iodide (PI) positivity increased in IR-exposed cell cultures. Clonogenicity was assessed at 7 days, and relative cell number and SA-β-Gal positivity were examined 4 days after exposure to various doses of SR (2, 6 or 12 Gy) or FR (3 x 2 Gy or 6 x 2 Gy). C, unexposed control cells; SR, single radiation; FR, fractionated radiation.

Figure 2. The cellular response in SR- or FR-exposed H460 cells. (A) Clonogenicity and (B) relative cell number were decreased by 6 or 12 Gy of SR or FR. (C) Phase contrast images of SA-β-Gal-stained cells (blue). Note the large and flat morphology of SA-β-Gal-positive cells. (D) Propidium iodide (PI) positivity increased in IR-exposed cell cultures. Clonogenicity, relative cell number, and SA-β-Gal positivity were analyzed as described in Fig. 1. C, unexposed control cells; SR, single radiation; FR, fractionated radiation.
and Dec1 was evidently increased only in H460 cells. These data indicate that IR exposure induced senescence effectively in either SR (6 or 12 Gy)- or FR (3 x 2 Gy or 6 x 2 Gy)-exposed H460 and MCF7 cells, and we further validated CD, eEF1A1 and eEF1B2 as valuable markers of cellular senescence.

**Effect of SR and FR on apoptosis, cell proliferation and premature senescence in tumor tissue of xenograft mice.** We next examined whether IR-induced premature senescence was involved in inhibition of tumor growth in vivo. We developed a mouse xenograft model by subcutaneously transplanting H460 cells in athymic nude mice and exposed mice to either SR or FR (Fig. 4A). The xenograft tumor volume was markedly reduced in a dose-dependent manner following FR and SR exposure (Fig. 4B).

Discussion

RT is based on eliminating the disease by depriving the proliferative potential of the tumor cells. Frequently, the desired effect of tumor treatment is achieved through the promotion...
of apoptosis, particularly in solid tumors (23-25). Although failure to undergo apoptosis in response to radiation may be a mechanism of radiation resistance, it is gradually being recognized that the loss of reproductive capacity in solid tumors can occur through alternative pathways including mitotic catastrophe, autophagic cell death and cellular senescence (26,27). Haugstetter et al (28) showed that senescence index could predict the treatment outcome in 30 metastatic colorectal

![Figure 5](image1)

**Figure 5.** Evaluation of premature senescence in tumor tissues of xenografted mice exposed to SR or FR. (A) H&E staining and TUNEL assay (brown precipitate). (B) Ki-67 immunostaining (brown) and SA-β-Gal activity (blue) in tumor sections derived from xenografted mice exposed to either SR or FR. (C) Quantification of Ki-67 immunostaining and SA-β-Gal positivity. C, unexposed control cells; SR, single radiation; FR, fractionated radiation.

![Figure 6](image2)

**Figure 6.** Expression of the indicated proteins in tumor tissue of xenografted mice exposed to either SR or FR. Western blot analyses were performed with specific antibody for each protein in tissue lysates obtained from three mice per group at 1 or 7 days post-irradiation. C, unexposed control cells; SR, single radiation; FR, fractionated radiation.
cancer patients. Our data showed that premature senescence rather than apoptosis is the predominant response in vitro and in vivo to either 6 or 12 Gy of SR or FR (Figs 1, 2 and 5). Apoptotic cells were rare in vivo 1 or 7 days after SR or FR exposure (Fig. 5A). As the in vivo system may be more clinically relevant than in vitro culture, this suggests that apoptosis may not be the principal mechanism of IR-mediated tumor regression in the clinical situation. Chang et al (29) demonstrated that induction of senescence-like phenotype seems to be common response to IR under the conditions of minimal cytotoxicity, providing the evidence that senescence may be an important determinant of cancer treatment outcome. Of note, IR exposure is effective at reducing tumor cell proliferation through multiple mechanisms, depending on the complexity of the cellular context (25). We and others found that loss of PTEN activity in either glioma cells or a mouse model of prostate cancer results in widespread premature senescence rather than apoptosis (12,25). Ruth and Roninson (30) reported that expression of P-glycoprotein (Pgt) inhibits IR-induced apoptosis. However, this effect of Pgt had no effect on radiation resistance since inhibition of apoptosis is associated with a concurrent increase in mitotic catastrophe and senescence in SR-damaged cells. Adjunctive therapy of tyrosine kinase inhibitor or poly(ADP-ribose) polymerase inhibitor with SR potentiates terminal growth arrest associated with senescence in vitro and in vivo, suggesting that tumor cell senescence is a mechanism for tumor targeting therapy in combination with IR (31,32). DeMasters et al (33) demonstrated that vitamin D3 analog delayed FR (5 x 2 Gy)-induced senescence arrest in breast cancer cells. Based on these results, not only the genetic background of the cancer patients but also the drug selection for combinational treatment should be carefully considered when predicting the primary mechanism underlying tumor regression after RT. Further studies are needed to elucidate how widespread this phenomenon may be and whether premature senescence practically contributes to the treatment outcome in animal models and cancer patients.

Molecular changes associated with cell cycle arrest, such as changes in phospho-Rb, p53 and p21 in IR-exposed cells occurred in similar patterns in SR- and FR-treated cells. However, since these molecules change under the conditions of not only permanent cell cycle arrest but also transient cell cycle arrest, they are indicators of cell cycle arrest (15). Expression of CD, eEF1A1 and eEF1B2 was evidently altered in cells exposed to the same total doses of SR or FR (6 or 12 Gy; Figs 3 and 6) and were not altered in transient cell cycle arrest (15). CD, eEF1A1 and eEF1B2 expression correlated more closely with the SA-β-gal labeling index than the accumulation of p53 and p21 did (Figs. 3 and 6), providing further support of our previous report that these molecules are promising markers for the detection of senescence (15). Expression of CD, eEF1A1 and eEF1B2 in vivo was more sensitive to FR than SR (Fig. 6), indicating that conventional FR could be the preferred regimen for inducing premature senescence. However, since we did not detect any change in DeR2 in IR-induced senescent cells, neither in vitro nor in vivo, DeR2 expression may depend upon the specific cellular context and may not be a general marker for cellular senescence. Similarly, although Decl expression increased following SR or FR exposure in vitro, Decl expression did not correlate with cellular senescence in vivo (Figs. 3 and 6). Although several recently identified biomarkers, such as HP1α, PML, p16, p5 and 12C-FDG may indicate cellular senescence, these need to be further evaluated before their clinical application in vivo (13,24,27,28,34). SA-β-Gal activity assay is the gold standard for senescence detection but is not suitable for clinical use since it is an enzymatic assay that requires fresh, frozen tissue sections (24). Our results strongly suggest that analysis of CD, eEF1A1 and eEF1B2 expression in combination with p21, pRB and proliferation markers such as Ki-67 or poly(ADP-ribose) polymerase, is a promising method to assess senescence in vitro and in vivo.

In summary, we have shown that premature senescence was the predominant pathway for IR-mediated tumor regression in vitro and in vivo, in response to 6 and 12 Gy of either SR or FR. Reliable assays for senescence may provide prognostic value on IR-mediated tumor regression in clinically relevant situations, and might be a more accurate prognostic factor than the apoptotic index in the treatment of solid tumors. Moreover, our data further support that CD, eEF1A1 and eEF1B2 are promising biomarkers for the detection of IR-induced premature senescence in vivo. Our data may contribute to the clinical application of premature senescence for the prediction of RT outcome.

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References


