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Soy Isoflavones Protect Normal Tissues While Enhancing Radiation Responses

Gilda G. Hillman, PhD

Soy isoflavones have demonstrated chemopreventive and anticancer properties in epidemiology and biological studies, in addition to their function as antioxidants in prevention of cardiovascular disease. We have explored the potential of soy isoflavones, as a safe biological approach, to enhance the efficacy of radiotherapy for local tumor control and limit normal tissue damage in solid tumors. This review presents studies investigating the interaction between soy isoflavones and radiation in different malignancies, including prostate cancer, renal cell carcinoma, and nonsmall cell lung cancer. Soy isoflavones were found to be potent sensitizers of cancer cells to radiation causing increased cell killing in vitro in human tumor cell lines and greater tumor inhibition in vivo in preclinical orthotopic murine tumor models. In the course of these studies, radioprotection of normal tissues and organs in the field of radiation was observed both in a clinical trial for prostate cancer and in preclinical models. The mechanisms of radiosensitization and radioprotection mediated by soy isoflavones are discussed and emphasize the role of soy isoflavones in increasing radiation effect on tumor and mitigating inflammatory responses induced by radiation in normal tissues. Soy isoflavones could be used as a safe, nontoxic complementary strategy that simultaneously increases radiation effectiveness on the malignancy while reducing damage in normal tissues in the field of radiation.

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Introduction

Recent advances in conventional radiotherapy (RT) for cancer have led to improved local control by targeting of tumors with high precision under image guidance, yet, tumor recurrence still occurs. Local tumor control and cure could be achieved by increasing RT dose delivered to the malignancy; however, this is countered by greater doses to surrounding normal tissue resulting in greater toxicity. A complementary strategy, which simultaneously increases radiation effectiveness on the malignancy while reducing toxicity in normal tissues, is required. New approaches to enhance the efficacy of RT for local tumor control and limit normal tissue damage are constantly under investigation. We have explored soy isoflavones (SIF) as a new and safe biological approach to favorably modify clinical responses to radiotherapy. For the past 18 years, our studies have demonstrated the radiosensitizing effects of SIF on tumors in different preclinical tumor models including prostate, kidney, and lung.[1-5] These studies also revealed that SIF caused radioprotection of normal tissues leading to a decrease in inflammatory responses and reduced tissue damage in irradiated organs.[4,5]

Isoflavones, extracted from soy beans, consist of genistein, daidzein, and glycitein, which are phytochemicals or phytoestrogens, ie, plant estrogens.[6] SIF are similar in their chemical structure to estrogens, but have weak estrogenic activity.[6,7] Genistein, the most biologically active compound, has a higher binding affinity to β estrogen receptor and a lower affinity to α estrogen receptor compared to estradiol.[8] Genistein can affect estrogen metabolism and exert a favorable role in the prevention of hormone-related cancers.[7,8]

The interest in SIF compounds stems from their chemoprevention properties documented in epidemiology studies which compared the frequency of cancer in different countries and have linked diet to cancer incidence.[7,9] A high consumption of

Department of Oncology, Radiation Oncology Division, and Department of Biochemistry, Microbiology and Immunology, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI

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Address reprint requests to Gilda G. Hillman, PhD, Department of Oncology, Radiation Oncology Division, and Department of Biochemistry, Microbiology and Immunology, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Scott Hall, Room 8255, 540 E. Canfield, Detroit, MI 48201. E-mail: hillmang@karmanos.org

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soy in Asian populations in China and Japan was shown to be associated with a lower incidence of prostate cancer and breast cancer compared to Western countries.[6,9-11] High levels of SIF have been found in the serum, urine, and prostatic fluid of Asian men consuming a soy rich diet, which could contribute to decreased incidence of prostate cancer.[12]

Initial work on cancer cells indicated that soy chemopreventive properties may be due to their anticancer properties.[13,14] Inhibition of human cancer cell growth by SIF involved the modulation of genes related to the control of the cell cycle and apoptosis.[7] Controlled clinical trials showed that SIF are nontoxic and safe for human use in contrast to chemotherapeutic drugs, but SIF have limited therapeutic activity for established solid cancers.[15,16] In contrast to toxic chemotherapy agents often used in combination with RT for solid tumors, testing a nontoxic natural compound as a complementary approach to RT was a novel concept to investigate.

In experimental studies, SIF were found to be potent sensitizers of cancer cells to RT and chemotherapy, as demonstrated in human cancer cell lines in vitro and in preclinical tumor models in vivo by us and others.[6,7,14] The mechanism of SIF radiosensitization in tumors could involve targeting signaling pathways promoting tumor growth, invasion, and metastasis. These pathways, which are constitutively activated in cancer cells, are further upregulated by radiation as a survival response to oxidative damage and are implicated in cancer resistance to RT and chemotherapy.[2,17,18] Besides their potential use in cancer, SIF have antioxidant properties and have shown benefits for prevention of cardiovascular disease and bone disease, protecting normal tissues from treatment-induced toxicity.[19] SIF could have a differential benefit for cancer and normal tissue when used in conjunction with RT for cancer. The goals of this review are to summarize our studies on SIF radiosensitization and radioprotection effects in metastatic murine tumor models of three major malignancies including prostate cancer (PCa), renal cell carcinoma (RCC) and nonsmall cell lung cancer (NSCLC). These studies demonstrated that SIF consistently showed a dual capability to protect normal tissue from radiation injury and simultaneously enhance radiation damage in the malignancy.

SIF Radiosensitization: Increased Tumor Response by SIF and RT

Prostate Cancer

In Vitro Potentiation of Cell Killing by SIF and RT in Human PCa Cell Lines

The treatment of metastatic PCa remains a clinical challenge. RT is a conventional treatment for PCa, however, a high percentage of patients have intermediate to high-risk localized PCa and are at risk of recurrence and progression to metastasis after RT.[20] At this stage, treatment options and efficacy are limited despite the advances in sophisticated new androgen-depleting agents.[21] Given the chemopreventive and anticancer properties of genistein demonstrated in numerous studies,[7,13,14] we have explored the potential of pure genistein or a mixture of SIF to improve the local control of PCa by RT.

In vitro studies performed on human PCa cancer cell lines showed that SIF inhibit tumor cell growth by modulation of genes related to the control of the cell cycle and apoptosis.[7] Genistein was found to potentiate the effect of radiation, in vitro, on human PCa cell lines PC-3 and C4-2B derived from castration-resistant advanced PCa.[18,22-24] A synergistic increase in cancer cell killing was observed with low physiological doses of 15 μM of genistein combined with low doses of photon radiation (2-3 Gy) akin to those used in conventional fractionated RT.[18,22-24] The combined treatment was more effective by pretreatment of the cells with genistein prior to radiation than by the reverse sequence.[23] Continuous exposure of the cells to genistein prior and after radiation was needed.[23]

Genistein inhibited DNA synthesis and augmented the DNA synthesis inhibition mediated by RT in PC-3 cells, and affected cell-cycle checkpoints. A greater G2/M cell cycle arrest was induced by the combined treatment than by each modality alone, which strongly correlated with alteration in expression of two cell cycle regulatory proteins, the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ and cyclin B1, resulting in decreased cyclin B1 and increase in $p21^{WAF1/Cip1}$ protein expression.[23] These findings were confirmed in other independent studies of combined genistein and RT in cancer cells.[25,26] These observations are relevant for radiosensitization of cancer cells by genistein, as cells in the G_2/M phase of the cell cycle have been shown to be more radiosensitive than cells in other phases of the cell cycle. SIF radiosensitization of PCa cells was independent of androgen receptor (AR) expression as it was observed in both PC-3 (AR-) and C4-2B (AR +) cells.[18,24]

The potentiation of RT-induced killing of cancer cells by genistein was also reported in other independent in vitro studies of human cancer cell lines, including hepatoma, esophageal, PCa, and cervical cancer cells.[25-28] Comparative studies in our laboratory tested the effect of pure genistein compared to pure daidzein and to a formulation of SIF extract from soybeans consisting of 43% genistein, 21% daidzein, and 2% glycitein.[24] Daidzein is the second major isoflavone found in soybeans and has also shown anticancer activity, whereas glycitein is a minor component. SIF was more potent than pure genistein at inhibiting cell survival and potentiating radiation-induced cell killing both in PC-3 cells and C4-2B cells. The effect of daidzein was milder than that of genistein or SIF.[24]

Increased Tumor Inhibition by SIF and RT in Metastatic PCa Tumor Model

The combined treatment of genistein and RT was tested on established prostate tumors using an orthotopic PC-3/nude mouse xenograft model.[1] Implantation of PC-3 cells in the prostate of nude mice, led to the formation of a primary prostate tumor and spontaneous metastasis to para-aortic

regional lymph nodes.[1] Mice with established prostate tumors were pretreated orally with 5 mg/day genistein followed 3 days later by prostate tumor irradiation with a single dose of 5 Gy photons and then, with continued treatment with genistein.[1] Genistein given in conjunction with RT caused significant prostate tumor growth inhibition and controlled spontaneous metastasis to para-aortic lymph nodes compared to each modality alone.[1] A significant increase in mouse survival was observed.[1] However, when pure genistein was given as a single modality treatment, an increase in spontaneous tumor metastasis to regional lymph nodes was observed.[1]

These findings were repeated in a syngeneic RM-9 orthotopic PCa model in immuno-competent C57BL/6 mice, indicating that genistein promotes metastatic spread via the lymphatic system independently of the immune system and is not due to the impaired immune system of the nude mouse model.[29] Nevertheless, in both the xenograft and syngeneic PCa models, genistein given with RT caused a marked decrease in tumor growth and lymph node metastasis, supporting a greater efficacy of the combined therapy.[1,29]

Promotion of metastasis by pure genistein was still a concern for soy-based clinical trials for cancer patients. Therefore, further in vivo studies tested a soy extract formulation of SIF containing genistein, daidzein, and glycitein compared to pure genistein and/or daidzein given as single therapy without radiation.[2,24] SIF mixture or daidzein combined with genistein did not increase lymph node metastasis, suggesting a protective effect of the daidzein in SIF mixture and promoting the use of a mixture of SIF obtained from soy extracts rather than pure genistein.[2,24] SIF given orally at 1 mg/day was as effective as genistein when combined with prostate tumor irradiation (5 Gy), causing tumor destruction as proven by histologic studies.[2] Treated prostate tumors exhibited large areas of tumor destruction replaced by fibrosis, an increase in prostate stroma, and inflammatory infiltrates, as well as numerous giant atypical cells that represent a different pattern of radiation-induced cell death in addition to apoptosis.[2] SIF serum levels measured during therapy showed significant levels of daidzein (1.6 μM) and genistein (1.7 μM), characteristic of in vivo soy metabolism.[2] In human studies, Chinese or Japanese populations, consuming 10-70 mg/day foods rich in isoflavones, had plasma concentrations of 1-4 μM SIF whereas Caucasian Americans consuming about 1 mg/day showed SIF levels of 10-30 nM.[30-32]

In all studies, including long-term survival studies up to 3 months of daily treatment with SIF, mice did not show signs of toxicity. Overall, these studies demonstrated the safety and efficacy of using SIF mixture given in conjunction with prostate tumor irradiation in preclinical PCa models.

SIF and RT Clinical Trial for PCa Patients

The potentiation of radiation by SIF supplementation was consistently demonstrated in vitro and in vivo in preclinical PCa models and led to the design of a clinical trial for men with early stage PCa to test the potential of SIF to improve the outcome of PCa RT.[33] Patients were randomly assigned to receive 200 mg Novasoy, which is a soy extract of SIF (Archer Daniels Midland, Decatur, IL) or placebo daily for 6 months starting on the first day of RT. Patients received external beam RT in fractions of 1.8-2.5 Gy for a total of 73.8-77.5 Gy.[33] Patients supplemented with SIF, during and after RT, showed better reduction in PSA levels and decreased incidence of urinary, gastrointestinal, and erectile dysfunction when compared to those receiving placebo.[33] These clinical observations suggest a radioprotective role of SIF for normal organs exposed to RT in addition to enhancement of RT effect on the prostate tumor.

Renal Cell Carcinoma

Increased Tumor Inhibition by SIF and RT in Metastatic Kidney Tumor Model

Although RCC was considered to be radioresistant when treated with low dose fractionated radiation, recent studies have shown that it is responsive to high fraction dose of stereotactic body radiotherapy.[34] We have investigated the effect of RT supplemented with SIF and/or with antiangiogenic drugs using the KCI-18 RCC preclinical model derived from a primary human papillary RCC tumor in our laboratory.[3] Orthotopic implantation of KCI-18 cells under the renal capsule of nude mice progress to the formation of a primary tumor in the kidney and spontaneous metastases to regional lymph nodes, lung, liver, and peritoneum mimicking the development and progression of RCC in human.[3]

Treatment of mice bearing established kidney tumors with 5 mg/day genistein given in conjunction with tumor irradiation at a single high dose of 8 Gy caused a significant tumor growth inhibition and limited metastasis. In vivo radioenhancement was in agreement with in vitro data showing a greater inhibition of KCI-18 cell division by the combined treatment.[3] In vivo, the combined treatment resulted in increased atypical giant cells similar to those seen in prostate tumors, which represent a slow death due to alterations in cell division at the level of cytokinesis.[3] As observed in our studies using genistein in the PCa tumor models, single therapy with genistein in KCI-18 RCC induced an increase the incidence of metastasis to the mesentery lining the bowel.[3] Our studies in three independent orthotopic tumor models of PCa and RCC, suggest that pure genistein could promote metastasis in animal tumor models.[1,3,29] Most animal studies have emphasized the role of genistein in the prevention of prostate cancer and mammary tumors. Only few studies have utilized genistein for the treatment of established tumors and controversial findings describing beneficial or adversary effects of pure genistein have been reported.

SIF Potentiates RT and Antiangiogenic Drugs in Metastatic Kidney Tumor Model

In additional studies with the KCI-18 RCC model, the antiangiogenic drug sunitinib was given together with SIF and RT.[35] The rationale was to cause partial destruction of tumor vasculature, regularize the blood flow; and to increase tumor oxygenation, and the access of cytotoxic SIF to tumor cells.[36] Similar to human RCC tumors, murine KCI-18 kidney tumors

displayed high vascularity characterized by abnormal vessels that are enlarged, disorganized, and leaky due to defective basement membrane.[36] Sunitinib, a small molecule receptor tyrosine kinases inhibitor, targets the receptors of angiogenic factors including vascular endothelial growth factor receptor (VEGFR)-2 and platelet-derived growth factor receptor (PDGFR)-β produced by tumor cells and associated stromal cells.[36] Sunitinib is approved for treatment of metastatic RCC but its efficacy is dose-limited due to toxicity to vasculature in normal organs.

Pilot studies, using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) in KCI-18 model, determined a suboptimal dose 20 mg/kg/day of sunitinib which mildly affected normal vessels but caused better tumor perfusion and decreased vascular permeability.[36] This sunitinib dose was selected to schedule RT (8 Gy) and SIF (1 mg/day) to investigate a triple strategy to enhance RT for RCC.[35] Pretreatment with sunitinib and SIF for 3 days followed by tumor irradiation and continued daily treatment with soy and sunitinib of kidney tumors caused almost complete inhibition of tumor growth that was confirmed histologically.[35] DCE-MRI studies confirmed that all three modalities also exert antiangiogenic effects and affect the vascular properties of tumors resulting in improved blood flow by partial destruction of tumor blood vessels.[35] SIF could play an antiangiogenic role in tumors by disrupting tumor vasculature, corroborating our previous findings on VEGF inhibition in vitro.[18] Conversely, our studies on normal kidneys by DCE-MRI and histology revealed that SIF mitigated the vascular changes induced by sunitinib alone and protected the vessels from dilatation.[35] These recent and novel findings suggest that SIF could reduce sunitinib-induced vascular damage in normal tissues and act differently in tumors vs normal tissue, in agreement with the radioprotective effects observed in the lung studies described below. Previous studies indicated that SIF inhibited apoptosis in endothelial cells[37] and have shown benefits for prevention of cardiovascular disease [19,37].

Lung Cancer

SIF Radiosensitization of Lung Cancer Cell Lines

NSCLC is the most common malignancy in the USA and the leading cause of cancer death.[38] Thoracic RT is a conventional therapy for NSCLC.[39,40] A third of patients present with unresectable stage III locally advanced disease and are currently treated by concurrent chemoradiation therapy (CCRT) using platinum-based drugs.[39,40] Unfortunately, CCRT has high rates of both locoregional and distant failure with an overall 5-year-survival rate of 20%-25%.[40,41] CCRT is associated with a significant increase in RT-induced toxicity resulting in acute esophagitis and pneumonitis that limits the radiation dose and the size of volumes treated.[42,43] To alleviate the adverse effects induced by RT in thoracic organs, lung cancer could benefit from the dual and differential activities of SIF that were observed as radiosensitization in tumors and radioprotection of normal tissues/organs in PCa and RCC.[33,35]

In vitro studies using NSCLC human cell line A549 [wtEGFR; mutKRAS] demonstrated that SIF can sensitize human lung cancer cell lines to RT doses used in NSCLC radiotherapy (2-3 Gy per fraction).[44] Potentiation of radiation-induced cell killing by SIF was synergistic in A549, corroborating our findings in PCa cells.[44] Measurement of double strand breaks (DSBs) by γ-H2AX staining showed increased and persistent DNA damage mediated by SIF and RT in contrast to RT alone, suggesting blockade of DNA repair by SIF.[44] Interference of SIF with DNA repair will be discussed below in the mechanisms of radiosensitization.

NSCLC are very heterogeneous tumors and the mutational status of oncogenes and tumor suppressor genes in NSCLC is dominated by KRAS, EGFR, and P53.[45] The effect of SIF, radiation, and cisplatin was tested in three human NSCLC cell lines which differ in the status of these oncogenes A549 (wt P53, mut KRAS, wt EGFR), H1299 (mut P53, wt KRAS, wt EGFR), and H1650 (wt P53, wt KRAS, mut EGFR). Treatment with SIF (10 μM) combined with cisplatin (Cis) (0.1 μM) and RT (2 Gy) resulted in increased cell killing in a clonogenic assay in all 3 cell lines, independent of their genotype (personal communications). DSBs measured by γ-H2AX staining showed increased DNA damage by SIF + RT + Cis that persisted at 24 hours in contrast to a decrease in RT treated cells, indicative of inhibition of DNA repair (personal communications). These findings indicate that soy isoflavones are potent anticancer agents that augment radiation-induced killing acting as radiosensitizers in human nonsmall cell lung cancer cell lines, irrespective of P53, EGFR, and KRAS mutation status.

Increased Lung Tumor Inhibition by SIF and RT

SIF and RT therapy were tested in vivo using orthotopic xenograft models of human A549 NSCLC in nude mice.[4] In an initial study, mice bearing established lung tumor nodules were treated orally with 1 mg/day SIF and 12 Gy RT delivered only to the left lung to discriminate between RT effect and SIF effect in the irradiated left vs the right lung in the same mouse.[4] SIF augmented RT-induced destruction of tumor nodules, resulting in small residual nodules containing degenerating tumor cells in the left irradiated lung compared to each modality alone.[4] SIF decreased the hemorrhages, inflammation, and fibrosis caused by radiation in lung tissue, suggesting protection of normal lung tissue.[4] In a second study in the A549 model in which the mice received full thoracic RT at 10 Gy and oral SIF, the combined therapy was tested for larger tumor burdens established in the lungs.[5] These conditions were selected to inflict greater damage to normal tissue and evaluate alleviation by SIF. SIF significantly augmented RT-induced destruction of lung tumor nodules with a 95% reduction in nodule size and up to 92% of lung tissue free of tumor, which were more pronounced than with RT or SIF alone.[5] In detectable remaining small tumor nodules, marked aberrations included the formation of large vacuoles in tumor cells and inflammatory infiltrates indicative of degenerative changes were found, as previously described in PCa and RCC studies.[5] These nodules had a much lower Ki-67 index of proliferation associated with decreased cellularity and increase in collagen matrix, probably as a result of tissue repair after tumor cell

destruction.[5] To mimic CCRT, we have treated mice with RT combined with cisplatin and SIF and observed a synergistic effect between the three modalities with greater radiosensitization and mitigation of the increased pneumonitis observed by RT and cisplatin (personal communications). Findings on mitigation of pneumonitis and fibrosis by SIF were consistently observed in all these lung experiments and will be discussed in the section on radioprotection below.

Mechanisms of Radiosensitization by SIF

SIF were found to be potent sensitizers of cancer cells when added to existing standard of care including RT and chemotherapy as demonstrated in human cancer cell lines in vitro and in preclinical tumor models in vivo by us and others.[6,14] Our in vitro studies in PCa, RCC, and NSCLC demonstrated that SIF can sensitize human lung cancer cell lines to RT doses used in conventional fractionated RT (2−3 Gy per fraction). SIF potentiated RT-induced tumor cell killing, by affecting the cell cycle progression, causing alterations of molecules essential for cell cycle progression and apoptosis.[23] The sensitization mechanisms involve targeting survival signaling pathways which are activated by tumor cells in response to RT or chemotherapy but are inhibited by SIF leading to increased cell killing and tumor growth inhibition. We have identified three key molecular targets including the APE1/Ref-1 DNA repair enzyme and the transcription factors nuclear factor-kappaB (NF-κB) and hypoxia inducible factor (HIF-1α), which are activated by tumor cells in their survival response to RT but are inhibited by SIF driving the tumor cells to cell death. These molecules are overexpressed in PCa, lung cancer, and others and have been implicated in radioresistance of cancer cells.[46-49]

APE1/Ref-1 and SIF Inhibition of DNA Repair Induced by RT

APE1/Ref-1, the enzyme apurinic/apyrimidinic (AP) endonuclease 1/redox factor-1 is a protein involved in DNA repair and redox activation of transcription factors NF-κB and HIF-1α.[47,50] APE1/Ref-1, is the primary enzyme responsible for recognition and incision of noncoding and mutagenic AP sites in the DNA base excision repair pathway. In addition, APE1/Ref-1 is also responsible for redox activation of multiple cellular transcription factors, including NF-κB and HIF-1α by facilitating their DNA binding via the reduction of a cysteine residue to a sulfhydryl state.[6,47,50] RT increased the nuclear expression of APE1/Ref-1, however, pretreatment with SIF inhibited upregulation and nuclear localization of APE1/Ref-1 induced by RT in PCa and NSCLC cancer cells.[17,18,24,44] This effect was reproduced in vivo in PC-3 prostate tumors clearly demonstrating the downregulation of APE1/Ref-1 by SIF and by SIF combined with RT, which were observed both by immunohistochemistry in tumor sections and Western Blot analysis of tumor nuclear extracts.[17] APE1/Ref-1 inhibition by SIF may be involved in radiosensitization, as reported in other studies where decreased APE1/Ref-1 levels in RNAi-treated human osteogenic sarcoma cells led to enhanced cell sensitization to DNA damaging agents including RT.[51]

SIF disruption of DNA repair processes was confirmed by γ-H2AX analysis. Ionizing RT causes rapid phosphorylation of the nucleosomal histone protein H2AX at Ser 139 (γ-H2AX), occurring at sites of DNA DSBs, which can be visualized as fluorescent foci by immunostaining.[52,53] Formation of γ-H2AX foci occurs within minutes after production of DSBs by RT, and the loss of γ-H2AX foci after several hours can be attributed to DNA repair enzymes. In A549 NSCLC, SIF alone or combined with RT caused an increase in γ-H2AX foci, which persisted overtime, in contrast to RT-treated cells, suggesting that SIF probably interfere with DNA repair mechanisms.[44] Another study reported that genistein targets hypermethylated Keap1 gene promoter in A549 cancer cells and genistein combined with radiation increased ROS levels by inhibiting methylation of Keap1 gene promoter and Nrf2 antioxidant activation resulting in radiosensitivity. In contrast, this antioxidant pathway is activated by genistein in normal lung fibroblasts, showing a differential effect of cancer cell radiosensitization.[54]

SIF Inhibition of NF-κB and HIF-1α Upregulated by RT

Additional critical survival pathways which are constitutively activated in cancer cells and are further upregulated by RT and chemotherapy involve the transcription factors NF-κB and HIF-1α.[6,49] These factors are responsible for promoting malignant behavior by transcription of proteins involved in cell-cycle progression, proteolysis, angiogenesis and are implicated in cancer resistance to chemotherapy and RT.[55] We showed both in NSCLC and PCa cells that SIF inhibit the activity of these factors and block their RT-induced upregulation, thereby affecting the transcription of genes involved in cancer progression.[17,18,24,44] NF-κB is constitutively activated in PCa and other cancers and correlates with tumor progression.[49,55] RT further activated NF-κB in cancer cells, as an immediate early response that functions to protect cells from apoptosis, a mechanism and could be implicated in radioresistance and cell survival.[17] In contrast to RT, genistein or SIF inhibited NF-κB DNA-binding activity by blocking the translocation of NF-κB subunits to the nucleus and inhibiting the transcription of target genes involved in the regulation of cell cycle progression in cancer cells.[17,23] Furthermore, RT-induced activation of NF-κB activity was completely inhibited by treatment of cells with genistein or SIF in PC-3 and C4-2B PCa cells, and in A549 NSCLC.[17,18,24,44] These data are in agreement with alterations in cell cycle and apoptosis mediated by SIF.[23]

To further study the effects of SIF on the redox function of APE1/Ref-1 associated with NF-κB activation, PC-3 cells were transfected with APE1/Ref-1 cDNA.[17,18] Over-expression of APE1/Ref-1, caused a concomitant increase in NF-κB DNA binding activity.[17] Over-expression of APE1/Ref-1 was inhibited by SIF alone or SIF combined with RT with a corresponding decrease in the NF-κB DNA binding activity.[18] Thus, in

addition to alterations in the DNA repair activity of APE1/Ref-1, SIF also affected the redox activation function of APE1/Ref-1. These findings further confirm that SIF disrupt molecular cross-talks between APE1/Ref-1 and NF-κB which are two critical molecules essential for cell survival pathways.

HIF-1α, which is induced by hypoxia, is also a critical signaling pathway upregulated by RT-induced oxidative stress.[46] HIF-1α is responsible for the activation of more than 60 downstream target genes involved in angiogenesis, tumor growth, and invasion.[46] APE1/Ref-1 is also responsible for redox-activation of HIF-1α.[47,50] In the hypoxic response, cellular levels of HIF-1α and APE1/Ref-1 redox stabilization of the HIF-1α protein are critical for its nuclear translocation, DNA binding, and transcriptional activity.[50] RT caused nuclear translocation of HIF-1α protein that was inhibited by SIF as shown both in vitro[18] and in PC-3 prostate tumors in vivo.[2] RT- induced HIF-1α expression and DNA-binding activity were abrogated by SIF treatment of PC-3, C4-2B, and A549 cells in vitro.[18,24,44] Over-expression of APE1/Ref-1in cDNA transfected cells caused an increase in HIF-1α expression and DNA-binding activity, which were further increased by RT but inhibited by SIF.[18] This pattern was shown also for NF-κB DNA binding activity.[18] The decrease in APE1/Ref-1 mediated by SIF was associated with downregulation of HIF-1α and NF-κB transcription factors activities, thus targeting the cross-talks between these three essential survival pathways.[18] These events could play a central and pivotal role in radiosensitization of cancer cells. In agreement with our findings, recent studies have shown that tumor radiosensitivity can be increased by decreasing levels of HIF-1α.[56]

Studies on molecular events upstream to HIF-1α, showed that SIF-mediated inhibition of HIF-1α induction by RT could also involve inhibition of other signaling events.[18] Recent studies have established activation of Src and STAT3 as upstream signaling events leading to induction of HIF-1α.[57] Our results showed that RT-induced phosphorylation of Src (Tyr 416) and STAT3 (Tyr 705) and a progressive increase in HIF-1α protein expression in PC-3 cells, in correlation with an increase in APE1/Ref-1 expression.[18] This activation of Src/STAT3/HIF-1α pathway was not observed with SIF,[18] as shown by others, using genistein in PCa independent studies.[58] We found that pure genistein and the mixture of SIF showed greater inhibition of these survival molecular pathways than daidzein.[24]

In summary, these findings confirm a molecular cross-talk between APE1/Ref-1, NF-κB, and HIF-1α in SIF-mediated mechanism of radiosensitization. SIF target the dual function of APE1/Ref-1 as DNA repair enzyme and redox activator of NF-κB and HIF-1α transcription factors, blocking DNA repair and transcription of genes essential for tumor cell survival, tumor growth, and angiogenesis.

Radioprotection of Normal Tissues by SIF

Our clinical and preclinical studies presented cogent evidence of radioprotective effects of SIF in irradiated organs. Clinical studies with SIF supplements given to PCa patients undergoing fractionated RT reported radioprotection of normal organs surrounding the prostate, as detailed above.[33] Preclinical studies of SIF combined with RT and/or sunitinib in RCC models confirmed decreased vascular damage in normal kidney, suggesting a protective role of SIF.[35] SIF radioprotective observations in lung tumor models were particularly impressive and were expanded further by developing methodology to document and study SIF effects on normal tissues.[4,5]

Lung Radioprotection by SIF

In lung cancer, RT-induced lung tissue toxicity often presents as radiation pneumonitis, which is an interstitial pulmonary inflammation that develops in up to 30% of patients after thoracic radiation.[43,59,60] Pneumonitis impedes breathing functions by the pathological progression of normal lung tissue to fibrotic tissue causing shortness of breath and potentially requiring the use of oxygen.[43,59-61] Radiation pneumonitis is caused by an early inflammatory process triggered by damage to lung parenchyma, epithelial cells, vascular endothelial cells, and stroma that involves induction of proinflammatory cytokines and chemokines, which recruit inflammatory immune cells in the lung tissue.[62-64] This acute early pneumonitis can progress to a chronic inflammation mediated by cyclical phases of cytokines, chemokines, and growth factors released in the tissue microenvironment.[62-64] These complex events culminate in the later stage of lung fibrosis which is due to excessive accumulation of collagen and other extracellular components.[61,63,65] These adverse events of radiotherapy affect patients' breathing and their quality of life.[59,60,66]

In the A549 NSCLC mouse model, inflammation/pneumonitis caused by RT in lung tissue was already evident at 4 weeks after high doses of 10-12 Gy RT.[4,5] Pneumonitis was manifested by thickened alveolar septa caused by heavy inflammatory infiltrates due to damage to alveolar structures and vessels, as seen by hemorrhages.[4,5] These alterations were markedly reduced in lungs of mice treated with SIF and RT.[4,5] The extent of pneumonitis following single and combined therapy was quantitated by morphometric measurements of the thickness of alveolar septa on H&E stained lung tissue sections and confirmed a marked decrease in pneumonitis following SIF and RT treatment.[5] Extensive studies to detect fibrosis, using Masson's Trichrome stained lung tissue sections, showed that RT caused a prominent increase in collagen fibers supporting the vessel walls and bronchiole walls.[5] RT-induced fibrosis in bronchovascular bundles was mitigated by SIF.[5] Thus, we demonstrated that SIF clearly mitigated the extent of pneumonitis and fibrosis in lung tissue of mice treated with RT, indicating a radioprotective role for irradiated lung.

Pneumonitis has also been associated with vascular damage induced by RT, which plays an important role in the development of RT-induced pulmonary toxicity and pulmonary hypertension.[67] Extensive hemorrhages were observed in irradiated lungs but less in SIF and RT treated-lungs.[4,5] To quantify and

validate this effect, lung sections were stained by immunofluorescence of the basement membrane of vessels using collagen, endothelial and pericyte-specific antibodies that allowed for visualization of vessel abnormalities.[5] Irradiated lungs showed a greater number of abnormal vessels with interruptions and projections in the basement membrane compared to fewer damaged vessels in lungs treated with SIF and RT, suggesting a protective role of SIF in vascular damage caused by RT in the lung tissue.[5] In conclusion, in the preclinical A549 lung tumor model, we have demonstrated that SIF exert a differential effect of radiosensitization on lung tumors with simultaneous radioprotection of normal lung tissue.

Studies by other investigators using naïve mice and rats (not-bearing tumors), confirmed our findings on the role of genistein, the most active component of SIF, as a radioprotector of RT-induced damage in lungs.[64,68-70] RT-induced pneumonitis was documented by 2-4 months after RT following single dose or fractionated radiation.[64,68-70] Endpoints to assess pneumonitis included measurement of DNA damage in lung cells by micronucleus assay, macrophage activation, cytokine expression, and lung function by measuring breathing rate.[64,68-70] These studies reported that genistein and/or EUK-207 superoxide dismutase catalase mimetic delayed and suppressed radiation increased breathing rate, decreased pneumonitis, and fibrosis.

To further explore the role of SIF in moderating adverse effects of RT in lung tissue, we conducted studies in naïve mice, in parallel to the studies in mice-bearing lung tumors.[71] Mice were treated with oral SIF 1-5 mg/day and 12 Gy RT administered to the left lung.[71] Hair loss and skin injury were observed in the irradiated area by 3-5 weeks after RT in mice treated with RT alone but this effect was mitigated in mice treated with SIF and RT.[71] These findings could be valuable in decreasing radiation-induced dermatitis, which might progress from mild erythema to moist desquamation and ulceration.[72] RT-induced dermatitis is still observed mostly in breast and head and neck cancer patients because of the proximity between the skin and the target volume.[73]

Our long-term studies of breathing rates in naïve mice showed that SIF blocked the increase in breathing rate induced by RT by 4 months after 12 Gy lung RT.[71] Histologic observation of lung tissues from naïve mice confirmed that SIF protected normal lung structures against RT-induced inflammation, damage, and fibrosis.[71] Overall, our findings in naive mice provided further evidence that SIF can protect against RT-induced injury to normal lung tissue[71] and corroborated our findings in the lung tumor models.[4,5] as well as those reported in independent studies.[64,68-70]

Esophagus Radioprotection by SIF

Acute esophagitis is an early manifestation of RT toxicity to normal esophageal tissue resulting in difficulties in swallowing, eating, and hydration.[42,74,75] It can be dose-limiting in RT for lung cancer, and head and neck cancer. In particular, NSCLC patients treated with CCRT present with a high frequency and severity of esophagitis. RTOG trials reported that 95% of these patients developed some level of radiation esophagitis (grade 1-4) and 33% of patients experienced severe esophagitis (Grade ≥ 3) peaking within the first or second month of RT.[42,75] Therefore, esophageal radioprotection by SIF at 1-5 mg/day was tested in naïve mice receiving high doses of 10 or 25 Gy thoracic RT.[74] Histology studies on esophageal tissues performed at time points from 1-16 weeks after RT revealed that SIF reduced early and late effects of radiation injury in several esophageal tissue layers.[74] RT-induced alterations increased with time and dose including hypertrophy of basal cells in mucosal epithelium, damage to smooth muscle cells in muscularis mucosae, and disruption of collagen fibers in lamina propria connective tissue associated with leukocyte infiltration.[74] SIF limited the extent of tissue damage induced by RT suggesting that SIF could be administered as a supplement to thoracic RT to decrease the incidence and severity of esophagitis in cancer patients.[74]

Cardiac Radioprotection by SIF

Cardiotoxicity has been reported as a late effect of RT in clinical analyses of thoracic RT for cancers such as locally advanced NSCLC, esophageal cancer, left-sided breast cancer, and lymphoma.[76-78] When targets are in close proximity to the heart, incidental RT can result in subacute and chronic cardiac complications including coronary artery disease, ischemia, congestive heart failure, and myocardial infarction, which can manifest several months to years after RT.[79,80] In our kinetics murine studies, histologic examination of heart tissue showed late effects of damage to arteries and myocardium caused by RT that was detectable by 16 weeks after 10 Gy thoracic RT.[81] SIF supplementation to RT reduced damage to the artery walls and radiation-induced fibrosis in the myocardium.[81] Giving thoracic RT in conjunction with SIF could be a good strategy to reduce heart toxicity and improve overall survival.[81]

Our studies on major thoracic organs showed evidence of radioprotection by SIF of tissues in the lungs, esophagus, and heart. SIF could be used as a safe, nontoxic complementary strategy that simultaneously increases radiation effectiveness on the malignancy while reducing damage, pneumonitis, and fibrosis in normal tissues in the field of RT.

SIF Modulation of RT-Induced Inflammatory Responses

SIF reduced RT-induced inflammation in normal tissues in preclinical and clinical studies, suggesting an immune-based mechanism that could be studied in lung models. RT-induced lung tissue toxicity is caused by an early inflammatory process triggered by RT damage to lung parenchyma, epithelial cells, vascular endothelial cells, and stroma.[62-64] This process probably occurs as a result of immune responses to tissue injury and involves induction of proinflammatory cytokines and chemokines, which recruit inflammatory immune cells to the lung tissue resulting in pneumonitis and late fibrosis.[62-64] We have demonstrated

both in tumor models and naïve mice that SIF can reduce the extent of inflammatory infiltrates, vascular damage, and fibrosis caused by RT in the lungs, esophagus, heart and kidneys, which indicates that SIF modulate immune responses triggered by tissue injury.[4,5,35,71,74,81]

Measurements of cytokines in lung homogenates of mice treated with 10 Gy thoracic RT and SIF confirmed that SIF decreased RT-induced inflammatory cytokines including IL-6, TNFα, IL-1β, and IFN-γ,[5,82] as found in other studies.[63,83] These cytokines activate macrophages and are involved in the acute/chronic pulmonary inflammation triggered by RT.

Further studies in naïve mice treated with SIF and 10 Gy thoracic RT examined the cellular and molecular mediators of RT-induced inflammatory responses in lung tissues and their alterations by SIF supplementation.[84] We focused on macrophages and neutrophils, which are recruited to sites of tissue injury after RT and are two major culprits of inflammation.[85] Lung macrophages are involved during early and late phases of tissue injury after RT, and macrophage activation could contribute to the pathogenesis of RT-induced lung injury.[85] In our studies, RT caused macrophage activation shown as an increase in the number and size of macrophages both in the bronchoalveolar space and lung parenchyma compartments whereas SIF consistently decreased the frequency and size of macrophages after RT.[84]

Alveolar macrophages activated by RT could be involved in pulmonary inflammation. Analysis of F4/80$^+$CD11c$^+$ alveolar macrophages and F4/80$^+$CD11c$^-$ interstitial macrophages, isolated from lung tissues, revealed that SIF protected F4/80$^+$CD11c$^-$ interstitial macrophages which are known to play an immune-regulatory role and are decreased by radiation.[84] Macrophages have demonstrated plasticity in response to stressors in tissues that range from M1 proinflammatory to M2 immunosuppressive phenotypes.[86,87] M1 macrophages produce nitric oxide synthase 2 (NOS2) and generate reactive NO species promoting inflammation.[86] M2 activated macrophages produce arginase-1 (Arg-1) which generates L-ornithine from arginine which is a precursor of proline, known to enhance collagen synthesis, thus promoting tissue repair and resolution of inflammation.[87] SIF decreased the levels of NOS2 expression while increasing Arg-1 expression in lung tissues in contrast to RT, suggesting a switch from a proinflammatory M1 macrophage to an anti-inflammatory M2 macrophage phenotype.[84] SIF also prevented the influx and activation of neutrophils in lung tissues that occurred after RT in agreement with a marked decrease of the neutrophil activation marker myeloperoxidase.[84] These findings suggest that SIF act by inhibiting RT-induced infiltration and activation of macrophages and neutrophils, a process that could be essential in the resolution of radiation-induced chronic inflammation leading to radioprotection of lung tissue.

To reduce RT-induced inflammation, immunosuppressive mechanisms induced by SIF could also involve myeloid-derived suppressor cell (MDSCs) as regulators of inflammation.[88] At an early time point of 1 week after RT, we found that CD11b$^+$ MDSCs expressing Arg-1 were decreased by RT in lung tissue but were maintained by SIF treatment in irradiated lungs.[82] Arg-1 was predominantly expressed by CD11b$^+$Ly6-C^{low}Ly6G$^+$granulocytic MDSCs (gr-MDSCs).[82] Arg-1 expression in gr-MDSCs was reduced by RT and preserved by SIF combined with RT treatment.[82] Overall Arg-1 expression was persistently increased at early and late time points in lung tissues treated with both SIF and RT, and could be associated with SIF inhibition of RT-induced NF-κB activation and transcription of proinflammatory cytokines demonstrated in our studies.[82] In support of our findings, other studies showed that Arg-1 could attenuate the function of iNOS, inhibit NF-κB activation, and inflammatory cytokines in vitro and decrease macrophage infiltration and inflammation in vivo, in a rabbit model of atherosclerosis.[89] MDSCs played a critical role in the resolution of acute inflammation and tissue repair caused by spinal cord injury.[90]

We have shown that SIF modulate immune inflammatory responses triggered by RT injury to normal tissues. SIF inhibit RT-induced immune cell activation of macrophages and neutrophils, promote Arg-1 vs NOS2 expression which interfere with signaling events and transcription and release of proinflammatory cytokines caused by RT-induced reactive oxygen species. These cellular and molecular mechanisms tilt the balance from RT-induction of inflammatory responses to SIF-mediated anti-inflammatory immune responses, which could contribute to the mitigation of chronic inflammation and SIF radioprotective effects in the lungs.

Conclusions

We have explored SIF as a new biological approach to favorably modify clinical responses to RT. Our studies demonstrated that SIF radiosensitized cancer cells and exerted radiosensitizing effects in preclinical orthotopic models of PCa, RCC, and NSCLC. The mechanisms of radiosensitization, studied both in vitro in cancer cells and in vivo in the tumor models, indicated that SIF targeted signaling survival pathways upregulated by RT, including DNA repair and transcription factors, ultimately driving cancer cells to death. Conversely, radioprotection of normal tissues and organs was mediated by SIF supplementation to RT and was consistently observed in RCC and lung preclinical models, and in a PCa clinical trial. In lung models, SIF mitigated adverse events caused by RT, including pneumonitis, fibrosis, and vascular damage. SIF also alleviated esophagitis and cardiotoxicity caused by thoracic irradiation. Investigation of inflammatory responses triggered by RT-induced injury in normal lung tissues revealed that SIF acted by inhibiting macrophage and neutrophil activation, decreasing the release of proinflammatory cytokines and promoting molecular mediators of anti-inflammatory responses. SIF modulation of inflammatory processes could result in the resolution of RT-induced chronic inflammation leading to radioprotection of normal tissues. Both effects of

radiosensitization and radioprotection mediated by SIF were confirmed by other investigators in independent studies. These findings support the use of SIF as a complementary and nontoxic approach to enhance RT therapeutic efficacy by increasing the response of the tumor whilst simultaneously relieving normal tissue toxicity in cancer patients.

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