Soy Isoflavones Promote Radioprotection of Normal Lung Tissue by Inhibition of Radiation-Induced Activation of Macrophages and Neutrophils

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Introduction: Radiation therapy for lung cancer is limited by toxicity to normal lung tissue that results from an inflammatory process, leading to pneumonitis and fibrosis. Soy isoflavones mitigate inflammatory infiltrates and radiation-induced lung injury, but the cellular immune mediators involved in the radioprotective effect are unknown.

Methods: Mice received a single dose of 10 Gy radiation delivered to the lungs and daily oral treatment of soy isoflavones. At different time points, mice were either processed to harvest bronchoalveolar lavage fluid for differential cell counting and lungs for flow cytometry or immunohistochemistry studies.

Results: Combined soy and radiation led to a reduction in infiltration and activation of alveolar macrophages and neutrophils in both the bronchoalveolar and lung parenchyma compartments. Soy treatment protected $F4/80^{+}CD11c^{-}$ interstitial macrophages, which are known to play an immunoregulatory role and are decreased by radiation. Furthermore, soy isoflavones reduced the levels of nitric oxide synthase 2 expression while increasing arginase-1 expression after radiation, suggesting a switch from proinflammatory M1 macrophage to an anti-inflammatory M2 macrophage phenotype. Soy also prevented the influx of activated neutrophils in lung caused by radiation.

Conclusions: Soy isoflavones inhibit the infiltration and activation of macrophages and neutrophils induced by radiation in lungs. Soy isoflavones-mediated modulation of macrophage and neutrophil responses to radiation may contribute to a mechanism of resolution of radiation-induced chronic inflammation leading to radioprotection of lung tissue.

Key Words: Radiation, Soy isoflavones, Lung inflammation, Macrophages, Neutrophils.

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The primary goal of combining a drug modality with radiation therapy is to maximize therapeutic benefit while mitigating severe off-target side effects in the treatment of patients with cancer. Radiation injury to normal lung parenchyma is a major concern in non–small-cell lung cancer (NSCLC). Radiotherapy given concurrently with chemotherapy is the conventional treatment for locally advanced NSCLC presenting as unresectable, stage III disease in approximately 50,000 Americans per year. There is an associated overall 5-year survival rate of 20%, emphasizing the need to improve the therapeutic ratio of concurrent chemoradiotherapy.[1,2] High-intensity radiotherapy could be more effective but is limited by lung tissue toxicity presenting as radiation pneumonitis that develops in up to 30% of patients after thoracic radiation.[3,4] Radiation pneumonitis is caused by an early inflammatory process triggered by damage to lung parenchyma, epithelial cells, vascular endothelial cells, and stroma. This process involves induction of proinflammatory cytokines and chemokines, which recruit inflammatory immune cells to the lung tissue resulting in pneumonitis and late fibrosis.[5–7] Early acute pneumonitis occurs by 2 to 4 months after radiotherapy, whereas late chronic pneumonitis manifests clinically over 6 to 24 months.[3,4] At late stages, radiation-induced pulmonary fibrosis results from aberrant resolution of inflammation in contrast to classic wound-healing processes.[6] These adverse events after radiotherapy affect patients' breathing and their quality of life. Various strategies to decrease the extent of pneumonitis have been investigated but need further research efforts.[8]

We previously explored a complementary approach to alleviate lung radiation toxicity using soy isoflavones, consisting of genistein, daidzein, and glycitein phytoestrogens extracted from soy beans. Although these isoflavones are similar in their chemical structure to estrogens, they have weak estrogenic activity and act as chemopreventive agents.[9–11] Our studies demonstrated that soy isoflavones have the dual capability of protecting normal lung from radiation injury and simultaneously enhancing radiation damage in the malignancy.[12,13] Soy mitigated the vascular damage, inflammation, and fibrosis caused by radiation injury to lung tissue in a lung cancer model suggesting that soy can alter the radiation-induced inflammatory response.[12,13] In naive mice,

soy isoflavones supplementation given prethoracic and postthoracic radiation protected the lungs against adverse effects of radiation including skin injury, hair loss, increased breathing rates, inflammation, pneumonitis, and fibrosis.[14] These findings in naive mice corroborated our findings in lung tumor models and provided evidence for a radioprotective effect of soy isoflavones. Importantly, soy isoflavones also sensitized cancer cells to radiation both in vivo and in vitro in preclinical tumor models of lung cancer, demonstrating a differential effect of radioenhancement on lung tumors with simultaneous radioprotection of normal lung tissue.[12,13,15]

We have reported that supplementation of soy isoflavones with thoracic irradiation mitigates radiation-induced inflammatory cytokines, infiltration of inflammatory cells, and fibrosis,[12–14] but the cellular mediators of radioprotection remain unclear. In this study, we investigated the role of macrophages and neutrophils in the mitigation of radiation-induced inflammatory events by soy isoflavones in lung tissue. Macrophages are recruited as a first response to radiation-induced damage in the tumor microenvironment or in normal tissues.[16] Macrophages play distinct roles in the early versus late stages of inflammatory response.[17–19] Monocytes can differentiate into functionally different macrophage subsets. Inflammatory cytokines (tumor necrosis factor-α [TNF-α], granulocyte-macrophage colony-stimulating factor, interferon-γ) generate classically activated M1 macrophages that mediate acute inflammation and participate in Th1 reactions.[20] M2 macrophages can be activated by interleukin (IL)-4, IL-13, IL-10, transforming growth factor-β, or immune complexes, participate in Th2 and Treg reactions, and promote tumor growth and fibrosis.[21,22] M1 predominates during acute inflammation, and then switches to M2 during the wound-healing phase at later stages.[21] We tested whether soy influences macrophage skewing to M1 or M2 subsets, and if this altering of macrophage phenotypes could dictate normal lung response to radiation-induced damage.

Activation and infiltration of neutrophils are a hallmark event in the progression of acute lung injury[23] and have been shown to be involved in radiation-induced alveolitis.[24] Therefore, the effect of soy isoflavones on infiltration and activation status of neutrophils was studied after radiation to the lungs. Our findings suggest that soy can inhibit the infiltration and activation of macrophages and neutrophils induced by radiation in lung parenchyma. Radiation induced a proinflammatory M1 macrophage phenotype in lungs, while mice receiving soy isoflavones and radiation switched to an anti-inflammatory M2 macrophage subtype. These data indicate that soy isoflavones modulate the cellular mediators of the inflammatory response induced by radiation.

MATERIALS AND METHODS

Mice

Female BALB/c mice (Harlan, Indianapolis, IN), 5 to 6 weeks old, were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animal protocol was approved by Wayne State University Institutional Animal Care and Use Committee.

Soy Isoflavones

The soy isoflavone mixture G-4660 used is a pure extract of 98.16% isoflavones from soybeans consisting of 83.3% genistein, 14.6% daidzein, and 0.26% glycitein (manufactured by Organic Technologies (Conshocton, OH) and obtained from the National Institutes of Health [NIH], Bethesda, MD). The soy isoflavone mixture was dissolved in DMSO and mixed with sesame seed oil at a 1:20 ratio just before treatment to facilitate gavage and avoid irritation of the esophagus by DMSO.[12–14]

Lung Irradiation

Radiation was delivered to the whole lung. Three anesthetized mice, in jigs, were positioned under a 6.4-mm lead shield with three cut-outs in an aluminum frame mounted on the radiograph machine for selective irradiation of the lung, as previously described.[13] The dose rate was 101 cGy/minute, and half value layer was 2 mm Cu. Photon irradiation was performed at a dose of 10 Gy with a Siemens Stabilipan X-ray set operated at 250 kV, 15 mA with 1-mm copper filtration at a distance of 47.5 cm from the target.

Experimental Design

Mice were pretreated with oral soy isoflavones for 3 days at a dose of 5 mg/day (250 mg/kg). Then, the lung was selectively irradiated with 10 Gy. Soy treatment was continued at 5 mg/day for 10 days and then switched to a lower dose of 1 mg/day (50 mg/kg), given 5 days a week for up to 18 weeks, based on previous studies.[12,25] We have reported that these doses of soy isoflavones result in plasma levels comparable with those measured in Asian populations consuming foods rich in soy isoflavones (1–4 μM).[14] At different time points, mice were either processed to harvest bronchoalveolar lavage (BAL) fluid for differential cell counting and lungs for flow cytometry or immunohistochemistry studies.

Analysis of Immune Cells by Differential Cell Counting in BAL Fluid and Flow Cytometry on Single-cell Suspension from Lungs

BAL was performed before lung resection at 1, 8, 12, and 18 weeks after irradiation. Cells were loaded onto slides using a cytospin centrifuge and stained using a DiffQuik staining kit (IMEB Inc., San Marcos, CA,). Differential cell counts of leukocyte subsets were performed by counting at least 300 nucleated cells.[26]

After collection of BAL fluids, the same mice provided the lungs for flow cytometry studies. Lungs were digested with 0.4 mg/ml collagenase IV, and red blood cells were lysed. Lung single-cell suspensions were incubated with Fc receptor-blocking antibody (eBioscience, San Diego, CA) before staining. For morphological characterization of leukocytes, $CD45^+$ cells were sorted by fluorescence-activated cell sorting (FACS) using a BD FACSVantage SE. $CD45^+$ cell subsets were gated according to cell size and granularity. Cell subsets obtained from each gate were spun onto slides using a cytospin and stained using a DiffQuik staining kit. To determine immunophenotype, cells were immunostained using a 5-color fluorophore combination of