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BP-13

REDUCTION OF RADIATION-INDUCED TOXIC EFFECTS *IN VIVO* BY APIGENIN

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KEYWORDS: Apigenin, Post-irradiation, NF-kappa B, Cytokine

INTRODUCTION

The potential use of flavonoid compounds, found in many of the vegetables and fruits consumed by humans, as protectors/mitigators against oxidativedamage and inflammation induced by toxic agents, such as radiation. One flavonoid of special interest is apigenin (4′,5,7-trihydroxyflavone). The first evidence for the radioprotective effects of apigenin was reported from an *in vitro* study conducted in our laboratory. In that study, we found a reduction in the frequency of micronuclei in cultures of human lymphocytes treated with varying concentrations of apigenin prior to irradiation. However, the beneficial effects of apigenin given *after* irradiation have not been reported. With increasing concern about the possibility of radiological terrorism and/or a nuclear accident, it is important to determine the potential beneficial effects of apigenin given *after* radiation exposure to optimize strategies for the use of apigenin in protecting individuals who may be the victims of such events. We measured levels of activated NF-κB and also selected pro-inflammatory cytokines known to be regulated by NF-κB in bone-marrow-derived macrophages (BMDMs) that were obtained from BM collected (the target cell for radiation-induced myeloid leukemia) of mice receiving apigenin at various concentrations 3 hr after irradiation. BMDMs are primary macrophages derived from BM cells cultured *in vitro* in the presence of growth factors. The BMDMs were selected for measuring levels of activated NF-κB and related pro-inflammatory cytokines because macrophages are one of the key inflammatory cells that respond to micro-environmental signals after exposure of cells to harmful agents.

MATERIALS AND METHODS

Chemicals Apigenin (≥95% purity by HPLC), heat-inactivated fetal bovine serum (FBS), sodium chloride (NaCl), Tween 80, Dulbecco's minimal essential medium (DMEM), L929 conditioned medium (LCM), penicillin 10,000 units/mL/streptomycin 10,000 μg/mL (P/S), and glutamine (29.2 mg/mL) were purchased from Invitrogen (Carlsbad, CA, USA).

Animals Male CBA/CaJ mice, 8–10 weeks old. They were allowed two weeks to acclimate prior to irradiation (at 10–12 weeks old, with an average body weight of 25 g). The animal rooms were maintained with the light cycle of 12 hr light/12 hr dark, at 21 ± 2 °C, with 10–15 hourly cycles of fresh air and a relative humidity of 50 ± 10%.

Irradiation and apigenin treatment There were four groups of male CBA/CaJ mice, 10–12 weeks old at exposure (20 mice per group), given an acute whole body dose of 0 or 3.0 Gy of ^{137}Cs γ rays. A stock solution of 10 mg/mL apigenin was prepared in 0.9% NaCl–Tween 80 (9:0.1, v/v). Either 0, 10, 20, or 40 mg of apigenin per kilogram (kg) of body weight (bw) was given IP in a single dose at 3 hr after the mice had received 0 or 3 Gy of ^{137}Cs γ rays. Mice receiving no radiation (0 Gy) or no apigenin (vehicle only) served as sham controls. At day 3 or day 10 post-irradiation, BM cells were collected from each treatment group (5 mice in each group) for analyses. The body weight of each mouse was also recorded at the initiation of the experiment and before sacrifice.

Collection of BM cells for the analyses of NF-κB, including NF-κB related pro-inflammatory cytokines At each harvest time for each treatment group, we collected BM cells from each mouse by flushing both femurs and tibiae with 10 mL of DMEM. We cultured these cells at a concentration of 2×10^6 BM cells/mL of DMEM supplemented with 30% LCM, 20% heat-inactivated FBS, 1% P/S, and 1% glutamine. We used one 100-mm dish containing 12 mL of complete DMEM per mouse. Hence, a total of 24×10^6 BM cells were required per mouse for obtaining BMDMs for measuring the levels of activated NF-κB and related pro-inflammatory cytokines. Tissue-culture dishes for obtaining BMDMs were incubated at 37 °C in an atmosphere of 5% CO_2. BMDMs were collected at day 7 after culture initiation for the measurement of activated NF-κB and selected NF-κB-regulated pro-inflammatory cytokines.

Measurement of levels of activated NF-κB, TNF-α and IL-1β Seven days after initiating *in vitro* culture, adherent BMDMs were harvested for the measurements of NF-κB activation and selected NF-κB-regulated pro-inflammatory cytokines (TNF-α and IL-1β). Nuclear extract kits were used for obtaining cytosolic and nuclear fractions. Activated NF-κB was measured in the nuclear fraction; while the expression of cytokines was determined in the cytosolic fraction. Protein contents were measured by the Bradford assay. A commercially available ELISA kit, in which the activated form of the transcription factor is selectively captured onto an immobilized oligonucleotide sequence, was used to measure the

levels of activated NF-κB. Likewise, ELISA kits purchased from Biosource (Invitrogen, Carlsbad, CA) were used for determining the expression levels of TNF-α and IL-1β. Levels of activated NF-κB and cytokines were measured using a microplate spectrophotometer (Molecular Devices) at 450 nm and were performed in duplicate wells for each BMDM sample from each treatment group.

Statistical analyses Differences in the levels of NF-κB and cytokines for each treatment group at each time-point were determined statistically with a non-parametric Mann–Whitney test.

RESULTS

Levels of NF-κB in BMDMs Fig. 1a and b illustrates highly significant increases in levels of activated NF-κB in BMDMs obtained at day 3 ($p < 0.05$, Fig. 1a) and day 10 ($p < 0.01$, Fig. 1b) from BM cells of mice receiving a single dose of 3 Gy of ^{137}Cs γ rays without apigenin treatment, as compared to NF-κB levels in the sham controls (vehicle only, No Rad, No AP). Importantly, there was a significant reduction in the levels of activated NF-κB in BMDMs obtained from BM cells collected at both time-points post-irradiation from exposed mice receiving apigenin treatment, relative to the level from exposed mice without apigenin treatment, except in samples collected at day 3 from irradiated mice receiving the lowest concentration of apigenin at 10 mg/kg bw (Fig. 1a). However, there was no concentration-dependent mitigation by apigenin. It should be noted that the level of NF-κB in BMDMs of mice receiving only 3 Gy of ^{137}Cs γ rays was higher at day 10 post-irradiation than that found at day 3 post-irradiation.

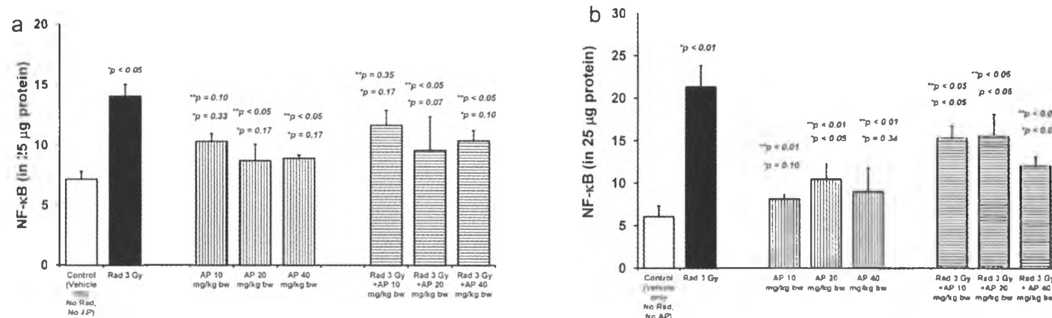

Fig. 1. NF- κB levels in BMDMs on days 3 and 10, respectively, after γ-irradiation of mice with 3 Gy. Apigenin (AP) was given 3 hr post-irradiation.

Levels of the NF-κB-related pro-inflammatory cytokines (*i.e.* TNF-α and IL-1β) in BMDMs

Fig. 2a and b shows significantly reduced levels of TNF-α in BMDMs in irradiated mice receiving apigenin treatment ($p < 0.01$), compared to those in mice receiving radiation only, at day 3 (Fig. 2a) and day 10 (Fig. 2b). There were significantly higher levels of TNF-α in all treatment groups (except mice receiving apigenin only) at day 10 post-irradiation, as compared to those detected at day 3 post-irradiation. Although all three concentrations of apigeningiven to miceafterirradiation showed anti-inflammatory effects at both harvest time-points, there was no concentration-dependence. The levels of TNF-α in mice receiving apigenin only (apigenin 20 or apigenin 40 mg/kg bw) were as low as those in the sham-control (vehicle only) group at both days 3 and 10. Hence, the resulting data indicate that administration of apigenin is not only safe but also provides beneficial effects. Fig. 5a and b shows significantly decreased levels of IL-1β in BMDMs obtained at day 3 (Fig. 3a) and day 10 (Fig. 3b) from mice receiving apigenin (at all concentrations, except 10 mg/kg bw of apigenin at day 10) after exposure to radiation, compared to IL-1β levels in irradiated mice without apigenin treatment. Further, there was no difference in the levels of IL-1β in sham controls (vehicle only) and non-irradiated mice receiving apigenin only (at all three concentrations).

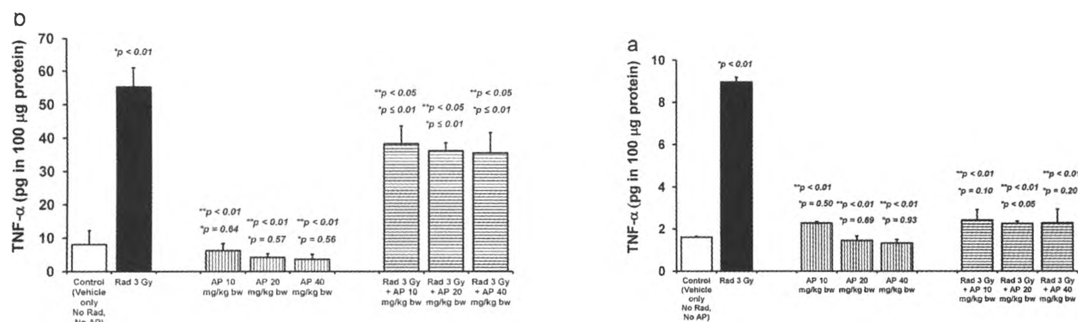

Fig. 2. TNF- α levels in BMDMs on days 3 and 10, respectively, after γ-irradiation of mice with 3 Gy. Apigenin (AP) was given 3 hr post-irradiation.

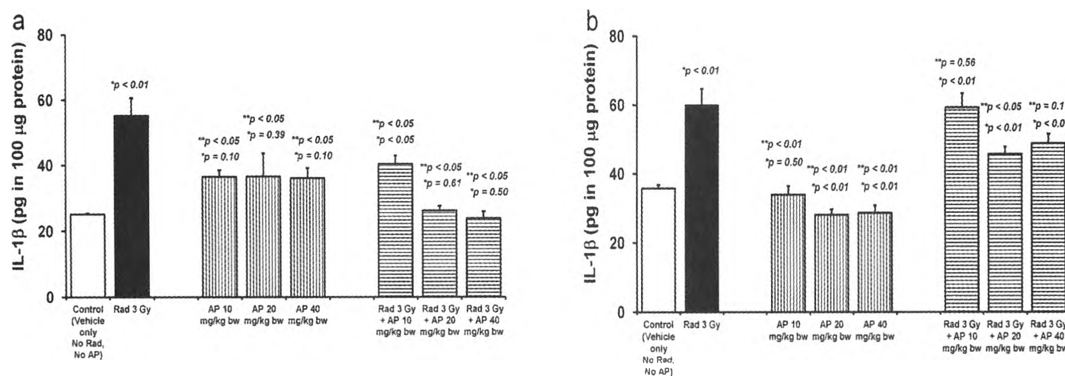

Fig. 3. IL-1 β levels in BMDMs on days 3 and 10, respectively, after γ-irradiation of mice with 3 Gy. Apigenin (AP) was given 3 hr post-irradiation.

DISCUSSION

Our data present evidence for the significant efficacy of apigenin in counteracting radiation-induced inflammatory responses when administered to mice *after* a whole-body exposure to radiation. Inflammation is one of the major physiological and potentially detrimental effects of exposure to moderate and high doses of radiation. It has been well recognized that NF-κB is a crucial transcription factor that plays an important role in regulation of the inflammatory. It also is known that NF-κB mediated cytokine production is a complex system leading to pro-inflammatory cytokines [1] (such as TNF-α [2] and [3], or IL-1β [4]) can activate NF-κB, leading in turn to yet further increased expression of NF-κB-regulated pro-inflammatory cytokines. Hence, the observation of high levels of activated NF-κB, TNF-α, and IL-1β in BMDMs of irradiated mice with or without apigenin treatment at day 10 post-irradiation, compared to the levels of the inflammatory biomarkers detected at day 3 post-irradiation, may reflect such positive feedback mechanisms in the NF-κB signaling pathway. Although there was a significant reduction in the levels of activated NF-κB, TNF-α, and IL-β collected at day 10 from irradiated mice receiving apigenin (all three concentrations), relative to the corresponding biomarker levels in irradiated mice not receiving apigenin, the levels of these proteins were still significantly higher than those of the sham-control (vehicle only, No Rad, No AP) group, indicating that apigenin treatment achieved partial, but not complete, reversal of these components of the pro-inflammatoryresponse following radiation exposure. Our data indicate that 20 and 40 mg/kg bw of apigenin (which are 7.5- and 3.75-fold lower than the LD_{50} value of apigenin by IP injection to mice consistently provided more beneficial effects than 10 mg/kg bw. To optimize the use of apigenin as a mitigative or therapeutic agent for radiation exposure, administration of multiple doses of apigenin should be tested in future studies in combination with higher doses of radiation, a longer interval (12–24 hr) between irradiation and apigenin treatment, and using other routes of apigenin administration for potential applications in clinical and field settings (such as intravenous, intramuscular, or subcutaneous injection, and oral ingestion). Further, neuroprotective effects of apigeninn, presumably mediated by suppression of inflammation, have been reported, both in *in vitro* and in *in vivo* [5] studies. These findings expand the repertory of reported beneficial effects of apigenin on several cell types, making apigenin an ideal radiation countermeasure agent.

CONCLUSION Results indicated significant reductions ($p < 0.01$ or <0.05) in the levels of activation of NF-kappa B in BMDMs collected from gamma-irradiated mice that received apigenin was suppressed at both harvest times. Further, the levels of pro-inflammatory cytokines in gamma-irradiated mice treated with 20 or 40 mg/kg body weight apigenin were significantly lower than those in mice receiving radiation only ($p < 0.01$ or <0.05) even at day 10 post-irradiation. Our studies demonstrate the mitigative/therapeutic effects of apigenin given to mice after irradiation.

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