International Immunopharmacology 9 (2009) 1401–1410

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Effects of genistein administration on cytokine induction in whole-body gamma irradiated mice

Vijay K. Singh, [a,b,*], Marcy B. Grace [b], Vaishali I. Parekh [b], Mark H. Whitnall [b], Michael R. Landauer [b]

[a] Department of Radiation Biology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20889-5603, USA
[b] Radiation Countermeasures Program, Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD 20889-5603, USA

ARTICLE INFO

Article history:
Received 28 July 2009
Received in revised form 17 August 2009
Accepted 18 August 2009

Keywords:
Cytokines
Cytokine array
Genistein
G-CSF
Luminex
Radiation injury
RT-PCR

ABSTRACT

The development of an effective pharmacological countermeasure is needed to reduce the morbidity and mortality in military and civilian populations associated with possible exposure to ionizing radiation. We previously demonstrated that a single subcutaneous (sc) administration of genistein at a non-toxic dose provided protection against acute radiation injury and that the radioprotective effects were associated with multilineage, hematopoietic progenitor cell recovery. The purpose of this study was to determine whether hematopoietic recovery was preceded by cytokine induction. In mice treated with sc genistein 24 h before irradiation (7 Gy ^{60}Co), we quantified serum cytokine levels by multiplex Luminex and also investigated a larger number of cytokines using cytokine arrays. Genistein administration stimulated serum granulocyte-colony stimulating factor (G-CSF) 4 h and 24 h after sham irradiation or γ-irradiation. Interleukin-6 (IL-6) was significantly increased in genistein-treated animals 4 h after irradiation. Because G-CSF and IL-6 are important hematopoietic factors, these results support our hypothesis that the previously observed radioprotective efficacy by genistein may be a result of early recovery of hematopoietic cells due to enhanced production of G-CSF and IL-6.

Published by Elsevier B.V.

1. Introduction

Nuclear proliferation, terrorist activity, and the distribution of nuclear and radioactive materials through underground networks make incidents involving radiation injuries increasingly likely to the military, civilians and first responders. Scenarios involving radiological hazards include nuclear detonations, covert placement of radioactive substances, and dirty bombs [1]. Radiation hazards can vary from late life pathologies to acute mortality [2,3]. Radioprotective agents are compounds that are administered before exposure to ionizing radiation to reduce its damaging effects, including radiation-induced lethality [4,5]. Radioprotectants have applications for the military as well as in clinical oncology, space travel, radiation site clean-up, and radiological terrorism [6]. During the last six decades a number of compounds of diverse structures have been considered as countermeasures for radiation [5]. Despite promising observations with various agents to date, none have been approved by the U.S. Food and Drug Administration (FDA) as a radiation countermeasure for the acute radiation syndrome.

The isoflavones are present predominantly in soybeans, and lignans, which are found in a variety of foods such as flaxseed, cereals, fruit and berries [7]. The major glycosides found in soybeans are daidzin, genistin, and glycitin. These glucose-conjugated compounds are relatively biologically inactive but upon consumption are hydrolyzed by enzymes and the gut microflora to form the active aglycone isoflavone compounds daidzein, genistein and glycitein. Genistein has a heterodiphenolic structure (4′,5,7-trihydroxyisoflavone) and has gained increased attention due to its association with beneficial effects for individuals with breast cancer, prostate cancer, cardiovascular disease, high cholesterol levels, and osteoporosis [8]. Genistein has also been shown to inhibit growth of tumor cell lines from various malignancies including breast, cervical, endometrial, prostate, ovarian, head and neck squamous cell carcinoma, lung, melanoma, leukemia, and lymphoma [9–11]. It also inhibits chemical-carcinogen-induced reactive oxygen species, oxidative DNA damage and proto-oncogene expression, topoisomerase I and II, tyrosine protein kinases, protein histidine kinase and 5α-reductase and induces G2M cell cycle arrest in some cancer cells that leads to cell growth inhibition and has antibacterial properties [12–16]. Other studies have also shown the effectiveness of genistein as a radiosensitizer in tumor cells derived from prostate and esophageal cancers [17,18].

We previously demonstrated radioprotective efficacy by genistein against γ-irradiation without toxicity or any apparent side effects in mice receiving a single subcutaneous (sc) injection 24 h prior to irradiation [19,20]. Furthermore, we observed that sc administration of genistein prior to lethal irradiation supports multilineage, hematopoietic progenitor cell recovery [21]. Protection of the bone marrow was correlated with a genistein-induced transient pause in the cell cycle where hematopoietic stem cells remained in the Go quiescent phase

* Corresponding author. Armed Forces Radiobiology Research Institute, 8901 Wisconsin Avenue, Bethesda, MD 20889-5603, USA. Tel.: +1 301 295 2347; fax: +1 301 295 0292.
E-mail address: singh@afrri.usuhs.mil (V.K. Singh).

1567-5769/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.intimp.2009.08.012

[22]. The purpose of the present study was to determine whether the genistein-induced hematopoietic stimulation is initiated by a cytokine cascade. In the experiments reported here, we present the results of genistein on induction of various cytokines in the peripheral blood. In addition, cytokine message was analyzed in the spleen and the bone marrow, both containing hematopoietic tissues in the mouse [23].

2. Materials and methods

2.1. Mice

Eight to nine-week-old male CD2F1 mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and were housed (four per cage) in an air-conditioned facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All mice were kept in rooms with a 12-h light/dark cycle with lights on from 0600 to 1800. The mouse holding room was maintained at 21 ± 2 °C with 10–15 hourly cycles of fresh air and a relative humidity of 50% ± 10%. The mice, upon arrival from the vendor, were held in quarantine for two weeks. Microbiological, serological, and histopathological examination of representative samples ensured the absence of *Pseudomonas aeruginosa* and common murine pathogens. Mice were provided certified rodent rations (Harlan Teklad Rodent Diet #8604, Harlan Teklad, Madison, WI, USA) and acidified water (with HCl, pH 2.5–2.8) *ad libitum*. All animal procedures were performed based on a protocol approved by the Armed Forces Radiobiology Research Institute (AFRRI), Institutional Animal Care and Use Committee (IACUC).

2.2. Genistein administration

Genistein was solubilized in polyethylene glycol with a molecular weight of 400 (PEG-400) on the day of the experiment by 20 s of sonication (Heat Systems-Ultrasonics Inc., Plainview, NY, USA). Genistein was obtained from Technical Sourcing International, Inc. (Missoula, MT, USA) and was confirmed by an independent laboratory to be 99.2% pure. PEG-400 was obtained from Sigma Chemical Company (St. Louis, MO, USA). Genistein was administered as a single sc injection at a dose of 200 mg/kg in a volume of 0.1 ml, 24 h before exposure to whole-body γ-irradiation when the mice were approximately 12 weeks of age. The vehicle control group received only PEG-400.

2.3. Irradiation

Mice were bilaterally irradiated in well-ventilated Lucite boxes (eight mice in each partitioned box) at a sublethal dose of 7 Gy at a dose rate of 0.6 Gy per min in the ^{60}Co γ-AFRRI radiation facility [24]. Following irradiation, mice were returned to their cages and monitored. Sham-irradiated mice were treated in exactly the same way as the irradiated animals, except that the ^{60}Co rods were not raised from the shielding water pool.

2.4. Blood and tissue collection

Mice were anesthetized with isoflurane (Abbott Laboratories, Chicago, IL, USA) in glass dressing jars before blood was collected from the abdominal vena cava. For cytokine studies, serum was prepared and stored at −70 °C until use. For pharmacokinetic studies, blood was obtained using a heparinized syringe and collected in sample tubes containing EDTA. Anesthetized mice were humanely euthanized by cervical dislocation after blood collection.

Spleens and femurs were harvested after euthanasia. Whole spleens from each mouse were homogenized in 1 ml RNA STAT-60 (Tel-Test, Inc., Friendswood, TX, USA) in a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA). Homogenized splenic suspensions were immediately frozen and kept at −70 °C until further use. The bone marrow cells were flushed from both femurs with McCoy's 5A medium (Flow Labs, McLean, VA, USA), and washed with 1× Hank's Balanced Salts Solution (ICN Biochemicals, Inc., Costa Mesa, CA, USA). Bone marrow cells were lysed in 1 ml RNA STAT-60. Bone marrow cell suspension was immediately frozen and kept at −70 °C until use.

2.5. RNA extraction

Total RNA of each sample was extracted according to the RNA STAT-60 manufacturer's protocol with a published modification [25]. The quality of RNA was tested by agarose gel electrophoresis and ethidium bromide staining to visualize the 28 S and 18 S RNA subunits under a UV transilluminator. RNA was quantified and characterized for purity by UV spectrophotometric analysis of A260 and A260/A280 ratio, respectively.

2.6. Reverse transcription reaction

The total RNA was converted to complementary DNA (cDNA) by a reverse transcription step with Moloney murine leukemia virus (M-MLV) reverse transcriptase, OligodT primer, RNase inhibitor, and dNTP with random hexamer primers using the Qiagen Omniscript reverse transcription kit (Applied Biosystems, Inc., Foster City, CA, USA). Reverse transcription of 2 μg total RNA was carried out using a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). Samples of cDNA were stored at −20 °C until tested.

2.7. Primers and probes

Pairs of primers based on published sequences were used. Amplicon lengths were set between 70 and 110 bp. To avoid co-amplification of contaminating genomic DNA, primers were (where possible) designed to span different exons or intron–exon boundaries. All primers and hydrolysis probe sequences were designed with the Beacon Designer (Premier Biosoft International, Palo Alto, CA, USA). BLAST (Basic Local Alignment Search Tool) was used to confirm gene specificity of the primers and probes. All primers and probe sets were designed for optimal RT-PCR efficiency and to avoid secondary structure and primer dimers. Fluorophores for probe labeling were chosen to minimize overlaps in their emission spectra. Sequences and melting temperature (Tm) of three oligonucleotides (forward primer, reverse primer and probe) for each cytokine are presented in Table 1.

Table 1
Oligonucleotide primer sequences for murine cytokines used in this study.

Target	Name	Sequence 5′-3′	T_m °C
G-CSF	G-CSF-F	5′-CCCACCTTGGACTTGCTTCA-3′	62 °C
	G-CSF-R	5′-GCCACCCCTAGGTTTTCCAT-3′	62 °C
GM-CSF	GM-CSF-F	5′-TTT CCT GGG CAT TGT GGT CTA-3	62 °C
	GM-CSF-R	5′-AAG GCC GGG TGA CAG TGA T-3′	60 °C
IFN-γ	IFN-γ-F	5′-TCA AGT GGC ATA GAT GTG GAA GA-3	66 °C
	IFN-γ-R	5′-CTG GCT CTG CAG GAT TTT CAT-3′	62 °C
TPO	TPO-F	5′-ACATCTCGCCCGGAGCTT-3′	58 °C
	TPO-R	5′-AAGGCTTGGAGAAGGAGGAAGT-3′	66 °C
IL-2	IL-2-F	5′-CCT GAG CAG GAT GGA GAA TTA CA-3′	68 °C
	IL-2-R	5′-CGC AGA GGT CCA AGT TCA TCT-3′	64 °C
IL-3	IL-3-F	5′ CCT GGG ACT CCA AGC TTC AA-3′	62 °C
	IL-3-F	5′-GAC AAT AGA GCT GCA ATT CAA CGT-3′	68 °C
IL-4	IL-4-F	5′-GACGCCATGCACGGAGAT-3′	58 °C
	IL-4-R	5′-GCCCTACAGACGAGCTCACTCT-3′	70 °C
IL-6	IL-6-F	5′-AGT CGG AGG CTT AAT TAC ACA TGT T-3′	70 °C
	IL-6-R	5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′	66 °C
IL-10	IL-10-F	5′-GCC CAG AAA TCA AGG AGC ATT-3′	62 °C
	IL-10-R	5′-TGC TCC ACT GCC TTG CTC TTA-3′	64 °C
IL-12	IL-12p40-F	5′-TGA GAA CTA CAG CAC CAG CTT CTT-3′	70 °C
	IL-12p40-R	5′-CTT CAA AGG CTT CAT CTG CAA GT-3′	66 °C
18 S	18 S-F	5′-AGG-AAT-TCC-CAG-TAA-GTG-CG-3′	60 °C
	18 S-R	5′-GCC-TCA-CTA-AAC-CAT-CCA-A-3′	56 °C