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Original article

EGCG, a tea polyphenol, as a potential mitigator of hematopoietic radiation injury in mice

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ABSTRACT

Agents capable of providing protection, mitigation or therapy against radiation injuries have long been of interest of radiation biologists owing to the ever expanding application of radiation in our day to day life despite the well reported ill effects of exposure. The current study investigates radiomitigating potential of EGCG (epigallocatechin gallate), a tea polyphenol with known DNMT inhibitory property, in C57 Bl/6 mice model. Treatment with 0.1833 mg/kg body weight EGCG, 1.5 h post-irradiation to lethally whole body irradiated mice rendered 45% survival for 30 days and also helped restoring the body weight of the animals. An early recovery of various hematological parameters was observed in EGCG treated animals compared to radiation alone group. Significant recovery in the number of bone marrow colony forming cells was observed in EGCG treated irradiated animals. EGCG reduced cytogenetic damage to bone marrow cells in radiation exposed mice significantly as studied by micronucleus assay without any significant affect on cell cycle distribution of the bone marrow cells. ELISA assay with bone marrow cell lysates showed EGCG as an inhibitor of HDAC activity and DNase accessibility assay showed EGCG treatment increased the accessibility of chromatin to the enzyme. The results suggest EGCG provides mitigation against radiation injury to the hemopoietic system of mice and also inhibits HDAC enzyme activity. However, further studies are required to understand its mechanism of action.

1. Introduction

Ionizing radiation has many practical applications in our everyday life such as medical imaging, radiotherapy, food sterilization, energy generation etc. The probability of some unplanned exposures like radiological accidents involving medical and industrial nuclear material, terrorist attack using radiation sources leading to disastrous consequences cannot be ignored. The enhanced level of radiation exposures, whether planned or accidental, may lead to serious health hazards. Hence there is an increasing focus on search of agents that can provide protection against the deleterious effects of ionizing radiation with no or minimum apparent toxicity [1]. Till now only Amifostine (WR2721) is approved by US FDA for its application as a radioprotetor in clinical practice only under strict medical supervision because of its several associated limitations [2]. In recent years, antioxidants and natural products have gained attention as promising radioprotective agents owing to their low toxicity [3–7], however, none of them have reached to the clinics.

Radioprotectors are effective in case of planned exposure where their administration can be planned, however during unplanned or accidental exposure, there is need to develop agents that can minimize the damage when administered during or after the exposure has occurred [8,9]. Radiomitigators are the agents which can be administered during or shortly after exposure but before the appearance of clinical symptoms of radiation exposure and reduce radiation injury. These agents can provide a way to reduce toxicity in patients undergoing radiotherapy and also to treat individuals exposed to radiation during any radiological accident or terrorist attack. Over last few years our laboratory has been evaluating several epigenetic modulators including HDAC inhibitors as potential radiomitigaive agents [9,10].

EGCG (epigallocatechin gallate), a major catechin present in green tea, has been widely studied for cancer chemoprevention. Besides this, it has also role in diabetes, Parkinson's disease,

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Alzheimer, obesity and stroke [11–14]. It also induces epigenetic changes by altering histone acetylation and DNA methylation. EGCG is an inhibitor of DNMT1, a DNA methyl transferase enzyme. The epigenetic modulation by EGCG makes it very important in the area of cancer chemoprevention; however the radiomitigative property of EGCG is not yet studied.

Current study aims at the evaluation of EGCG as a potential radiomitigative agent using mice model. Whole body survival assay and assay for changes in body weight were performed using C57 Bl/6 mice exposed to lethal dose of γ-radiation. Hematological parameters and colony forming cell assay were studied in mice irradiated at sublethal dose of 5 Gy. Changes in micronuclei frequency was studied in bone marrow marrow cells of mice irradiated with 2 Gy whole body γ-radiation and also bone marrow cell cycle analysis was performed to correlate with the changes in PCE/NCE ratio. Its HDAC inhibition activity was studied in nucleated bone marrow of irradiated mice treated with EGCG.

2. Materials and methods

2.1. Animals

6–8 weeks old C57 BL/6 male mice of average weight of 22 ± 3 g were considered for *in vivo* studies. The mice were bred in Animal House Facility of the institute and were maintained under controlled conditions of photoperiod, temperature and humidity. They were provided standard palate feed and clean drinking tap water. All animal experimentations were strictly in accordance with the Institutional Animal Ethics committee (IAEC) approval.

2.2. Irradiation and drug treatment

Whole body irradiation (WBI) of animals was performed using cobalt teletherapy unit (Bhabatron, Panacea Biotech) at a dose rate of 1 Gy/min (SSD adjusted to the field size of 35 × 35 cm to achieve desired dose rate). Dose of radiation delivered was based on the choice of end point under investigation, viz. survival studies were performed at a supra lethal dose of 10 Gy whole body irradiation while hematology and CFC assays were performed at 5 Gy, cell cycle, micronucleus assay, DNase accessibility and HDAC assay were performed with 2 Gy exposure where the results were expected to be evident.

Drug (EGCG, Sigma-Aldrich, USA) was administered to animals via intra peritoneal (i.p.) route in a maximum volume of 200 μl per mice.

2.3. 30 day survival assay

6 age matched male mice were considered in each group for survival against lethal 10 Gy WBI [5]. EGCG was administered at 0.1833, 0.2749, 0.3666 and 0.5499 mg/kg concentration in a final volume of 200 μl. Drug administration was done at either 1.5 or 2 h post-irradiation. All the treated animals were observed for changes in body weight on each day besides their survival for 30 days after irradiation.

2.4. Hematology

Four groups (untreated control, 0.1833 mg/kg EGCG, 5 Gy WBI and EGCG plus 5 Gy) of age matched C57 male mice (n = 3) were considered for hematological analysis. Blood (100 μl) was collected by retro orbital puncture on day 1, 7, 14, 21 and 28 in EDTA containing tubes and were subjected to hematological analysis [9] on a Celltac α MEK 6450 (Tokyo, Japan) hematology analyzer. Experiments were repeated thrice and data represented are mean ± SD.

2.5. CFC assay

Four groups of 3 mice each were considered for various treatments as described above and were sacrificed 10 days after irradiation for the collection of bone marrow cells. Lineage negative stem cells were isolated from each animal on a MACS separator using mice hemopoietic lineage depletion kit (Milteny Biotech, USA; Order no. 130-090-858). 1000 lineage negative cells from each animal were cultured in methocult media (Stem cell technologies, USA) as per manufacturer's instructions and colonies developed were counted under microscope (10–20× magnification) after 10–12 days. Results presented are mean ± SD obtained from three independent experiments.

2.6. Bone marrow cell cycle assay

Cell cycle assay based on DNA content was performed flow cytometrically using propidium iodide (PI) staining of mouse bone marrow cells. Briefly, the femur bones of mice were dissected out at 24 and 48 h after various treatments and washed with PBS. Following RBC lysis, cells were fixed with 70% chilled alcohol and stored at 4 °C for over night. Samples were then treated with 200 μg/ml RNase A for 30 min at 37 °C and 50 μg/ml of PI for another 30 min at 37 °C [15] before acquisition on a BD LSR-II flow cytometer equipped with suitable optics. At least 10,000 cells were acquired per sample and analyzed for cell cycle distribution.

2.7. Bone marrow micronucleus assay

Bone marrow smears were prepared as described earlier at 24 and 48 h after various treatments for micronucleus assay [15] from the femur bones of mice in different treatment groups (Control, EGCG, 2 Gy, 2 Gy exposed mice administered with 0.1833 mg/kg EGCG at 1.5 h after irradiation).

2.7.1. Slide staining and scoring

Slides were stained with May-Grunwald Giemsa stain diluted in Sorenson's buffer (pH 6.8) at 4:1 ratio for 35 min. Slides were washed with distilled water and air dried. Smears were observed under 40× magnification of light microscope. At least 2000 RBCs were scored from 3 to 4 slides in each group.

2.8. HDAC assay

Mice were divided into different treatment groups as described earlier and a single drug dose of 0.1833 mg/kg was used at 1.5 h post 2 Gy WBI. After 4 h of irradiation time mice were sacrificed and bone marrow cells were flushed with PBS. Nucleated bone marrow cells obtained after RBC lysis and washing with PBS were homogenized in cell lysis buffer and sonicated. 100 μg protein from each sample was analyzed for deacetylated lysine using HDAC colorimetric assay kit (K331-100; Biovision, San Francisco, CA, USA) at 405 nm as per the manufacturer's instructions and as described earlier [10]. Amount of deacetylated lysine present per μg protein was calculated from standard curve prepared using deacetylated lysine standard provided with the kit.

2.9. DNase accessibility assay

Bone marrow nucleated cells collected from mice in different treatment groups were fixed in 2 ml of 1% paraformaldehyde for 17 min. Cells were centrifuged at 1800*g* (4 °C) for 5 min and pellet was resuspended in 5 ml of sucrose buffer-A (15 mM HEPES pH-7.9, 0.32 mM Sucrose, 2 mM EDTA, 2 mM EGTA, 0.2 mM PMSF, 0.5% BSA, 0.5 mM DTT, 60 mM KCl) and kept in ice for 15 min before homogenization. 5 ml of sucrose buffer-B (15 mM HEPES, 30% w/v