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Antiapoptotic and immunomodulatory effects of chlorophyllin

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Abstract

Chlorophyllin (CHL) was earlier shown to reduce the level of intracellular ROS and apoptosis induced by ionizing radiation and 2,2′-azobis(2-propionimidinedihydrochloride) (AAPH). In the present studies, the effect of CHL on radiation-induced immunosuppression and modulation of immune responses in mice was examined. Chlorophyllin inhibited the in vitro lymphocyte proliferation induced by concanavalin A (Con A) in a dose dependent manner at doses ≥50 μM. At lower doses (10 μM) CHL significantly inhibited activation induced cell death (AICD) in Con A stimulated spleen cells. Spleen cells obtained from CHL treated mice showed an inhibition of response to Con A depending on dose of CHL and the time after its administration. Spleen cells obtained from CHL treated mice (24 h) showed lower inhibition of response to Con A following in vitro (5 Gy) as well as whole body irradiation (2 Gy). The expression of antiapoptotic genes bcl-2 and bcl-x_L was up-regulated in these cells. Chlorophyllin treatment of mice led to splenomegaly and increase in the number of peritoneal exudate cells (PEC). The numbers of T cells, B cells and macrophages in the spleen were also increased. Increased phagocytic activity was seen in PEC obtained from CHL treated mice. Most importantly, CHL administration to mice immunized with sheep red blood cells (SRBC) augmented both humoral and cell-mediated immune responses.

Keywords: Immunomodulation; Antioxidant; Proliferation; Radioresistance; Phagocytosis; Antiapoptotic

1. Introduction

During innate immune response reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by rapid uptake of oxygen by macrophages and polymorphonuclear phagocytes, activation of NADPH oxidase and other enzymes like myeloperoxidase and by activation of inducible nitric oxide synthase (Nathan and Root, 1977; Nathan and Hibbs, 1991). These ROS and RNS help in killing the invading pathogens (Babior, 1984).

ROS have been shown to be involved in activation of human peripheral dendritic cells and proliferation of lymphocytes (Fedyk and Phipps, 1994; Rutault et al., 1999; Tatla et al., 1999). It is well known that the cellular damage elicited by ionizing radiation is through generation of ROS. Lymphocytes are known to be sensitive to oxidative stress mainly because of their high mitotic potential and polyunsaturated fatty acid (PUFA) content of their cell membranes. Therefore, exposure to high doses of whole body irradiation (WBI) results in immunosuppression (reviewed in Anderson and Warner, 1976; Doria et al., 1982). Exogenous antioxidants may help in preventing radiation-induced damage by scavenging ROS. However, the influence of these antioxidants on the immune system has not been critically examined.

Some antioxidants from plants and other sources have been reported to be immunostimulatory (reviewed in Kalsi et al., 1991; De La Fuente et al., 1998; Agarwal and Singh, 1999; Desai et al., 2002; Devasagayam and Sainis, 2002). Ascorbic acid has been shown to activate phagocytic function and chemotaxis in peritoneal macrophages (Victor et al., 2000). In aged animals, the ingestion of thioproline significantly stimulated lymphoproliferative response to concanavalin A (Con A), chemotaxis, antibody dependent cellular cytotoxicity and natural killer activity of leukocytes (De La Fuente et al., 1998). On the other hand, prolif-

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eration in response to mitogens like Con A, lipopolysaccharide (LPS) and phytohemagglutinin (PHA) decreased in spleen cells obtained from Vitamin E supplemented (diet) mice (Reddy and Fernandes, 2000) and in spleen cells treated with very low concentrations trans-resveratrol (Gao et al., 2001). Gallic acid and its derivatives (Serrano et al., 1998), polyphenols (Sanbongi et al., 1997) and α-lipoic acid (Pack et al., 2002) have been reported to inhibit proliferation of human peripheral blood lymphocytes stimulated by PHA and phorbol myrstic acetate.

Chlorophyllin is a water-soluble mixture of sodium-copper salts of green plant pigment, chlorophyll. CHL is widely marketed for a variety of dietary and medicinal uses (Kephart, 1955; Young and Bergei, 1980). Recently, it has also been shown to occur naturally in a constituent of traditional Chinese medicine (Chiu et al., 2003). It has chemopreventive, antimuatgenic and anticarcinogenic properties (Guo et al., 1995; Park et al., 1995; Sugie et al., 1996; Egner et al., 2001; Ong et al., 1989). It may bind the carcinogen or mutagen thus reducing its bioavailability (Dashwood et al., 1998). Chlorophyllin exhibited protection against radiation and chemical induced cytogenetic damage (Abraham et al., 1994; Madrigal-Bujaidar et al., 1997; Santosh Kumar et al., 1999). It was found to inhibit radiation induced single strand breaks in plasmid pBR322 DNA (Santosh Kumar et al., 1999). Apart from this, it has direct radical scavenging ability as shown by electron spin resonance spectroscopy (Santosh Kumar et al., 2001). In cellular and animal models CHL acted as antioxidant as well as prooxidant depending on time of administration and dose (Santosh Kumar et al., 2004). More importantly, CHL has been successfully used as a cancer chemopreventive agent in human population residing in certain parts of China who are at high risk of aflatoxin B1 (a hepatocarcinogen) exposure (Egner et al., 2000; Egner et al., 2001; Egner et al., 2003). There are no reports on modulation of immune function in vivo by CHL, though inhibition of IFN-γ secretion by LPS activated spleen cells in vitro has been reported (Yun et al., 2005). In the present studies, we have examined the role of CHL as an immunomodulator and for modification of radiation induced immunosuppression.

2. Materials and methods

2.1. Chemicals

Chlorophyllin, RPMI1640, fluorescein isothiocyanate (FITC) isomer I-celite, propidium iodide (PI) and lipopolysaccharide (LPS) were obtained from Sigma Chemical Company, USA. Fetal calf serum (FCS) was obtained from GIBCO BRL. Con A was obtained from Calbiochem, USA. Carboxy fluorescein diacetate succinimidyl ester (CFSE) was procured from Molecular Probes, The Netherlands. Phycoerythrin (PE) conjugated anti-mouse CD3, anti-mouse CD14 and anti-mouse CD19 antibodies and FITC conjugated anti-mouse CD4 and anti-mouse CD8 antibodies were procured from BD pharmingen USA. mRNA capture kit and single tube RT-PCR kit were obtained from Roche, Germany. Oligonucleotides for PCR were purchased from Numex Biochemicals, India. Tritiated thymidine was obtained from Board of Radiation and IsotopeTechnology, Vashi, Navi Mumbai, India. All other chemicals were purchased from reputed local manufacturers. Fresh sheep blood was aseptically collected from jugular vein and was immediately diluted in Alsever's solution and stored at 4 °C and used as a source of sheep red blood cells (SRBC).

2.2. Animals

Eight to ten-week-old inbred BALB/c mice, weighing approximately 20–25 g and reared in the animal house of Bhabha Atomic Research Centre were used. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissections of small animals were strictly followed.

2.3. Irradiation schedule

All in vivo and in vitro gamma irradiations were carried out using a junior theratron teletherapy machine. The dose rate as estimated by chemical dosimetry was 43 cGy/min. Animals were placed in perspex boxes and exposed to radiation. In all the experiments, mice were sacrificed 24 h after irradiation.

2.4. Treatment with CHL

For in vitro experiments, CHL was added in culture medium at the time of initiation of the cultures to get a final concentration of 30 nM to 500 μM. In ex vivo or in vivo experiments, CHL was dissolved in RPMI medium and different amounts of CHL (50–200 μg/gram body weight (gbw)) were injected i.p. to BALB/c mice.

2.5. Proliferation assay

Spleen cells were obtained by squeezing the spleen through a nylon mesh in a petri plate containing RPMI medium. The RBC were lysed by brief hypotonic shock. Lymphocytes were stained with CFSE (20 μM, 5 min, 37 °C) and washed three times using ice-cold RPMI medium containing 10% FCS (complete medium, CM). Two million lymphocytes were stimulated with Con A in presence of CHL (2–100 μM) and were cultured for 72 h at 37 °C in 2 ml CM in a 95% air/5% CO_2 atmosphere. Untreated cells served as a control. The cell proliferation was measured by dye dilution in a FACS vantage flowcytometer (Becton Dickinson Immunocytometry Systems, USA). The fluorescence intensity of CFSE decreases to half with each cell division. Percent daughter cells that had undergone more than four divisions during 72 h culture was determined using Cellquest® software. In some experiments lymphocytes (2×10^6/ml) were stimulated with Con A (for 72 h) in presence of CHL (2–100 μM) in 96 well plates and were pulsed with ^{3}H thymidine (1 μCi/well, specific activity 6500 mCi/mM) and were further cultured for 16 h (Shankar et al., 1999). Cells were harvested on glass fiber filters using a Multimash-2000 harvester (Dynatech Laboratories Inc., USA). The incorporated activity was counted in a liquid scintillation counter.