Food and Chemical Toxicology 66 (2014) 44–55

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Topical application of Gallic acid suppresses the 7,12-DMBA/Croton oil induced two-step skin carcinogenesis by modulating anti-oxidants and MMP-2/MMP-9 in Swiss albino mice

Vimala Subramanian, Balaji Venkatesan, Anusha Tumala, Elangovan Vellaichamy *

Department of Biochemistry, University of Madras, Guindy Campus, Chennai 600025, India

ARTICLE INFO

Article history:
Received 17 September 2013
Accepted 13 January 2014
Available online 18 January 2014

Keywords:
Gallic acid
Skin cancer
Anticancer activity
DMBA/Croton oil
GST
MMP

ABSTRACT

Gallic acid (GA – 3,4,5-trihydroxybenzoic acid), a dietary anti-oxidant has been shown to inhibit cancer cell growth in *in vitro*. Herein, we investigated the *in vivo* chemo preventive activity of GA on 7,12-Dimethylbenz[a]anthracene (DMBA)/Croton oil induced two-step skin carcinogenesis in Swiss albino mice. Skin tumor incidence and tumor volume were recorded during the 16 weeks of experimental period. In addition, LDH-isozyme shift, skin collagen content, activities of matrix metalloproteinases (MMP-2/MMP-9) enzymes and enzymatic and non-enzymatic antioxidant were studied in the skin and serum of experimental mice. Tumor incidence was significantly increased in the DMBA/Croton oil induced mice (100%; $p < 0.001$) when compared to GA co-treated mice (60%; $p < 0.01$) and 5-FU treated mice (50%; $p < 0.01$). Skin collagen content, MMPs activities, LDH-isoenzymes and MMP-2/-9 expressions were increased in DMBA/Croton oil induced skin while decreased levels of enzymatic (GST, SOD, CAT & GPx) and non-enzymatic anti-oxidant (GSH) were noticed. On the other hand, GA co-treatment exhibited a significant protection by reverting back the altered levels of LDH-isoenzymes, antioxidants, collagen and MMP-2/MMP-9 activities. The results of this study indicate that topical application of GA inhibits DMBA/Croton oil induced two-stage skin carcinogenic process by modulating the antioxidants and MMPs (-2 & -9) in the mouse skin.

1. Introduction

Skin is the largest organ of the human body, and is a major target for toxic insult by a chemical mutagens and carcinogens in a day-to-day life (Simna et al., 2012). Throughout the world, the number of people affected by skin cancer is increasing due to increased exposure to environmental pollutants and UV rays (Narendhirakannan and Angeline Christie Hannah, 2013). Recent studies have also predicted that the number of people affected by this malady will be doubled by the year 2050 (Hayat et al., 2007). Although many synthetic drugs are commercially available to treat skin cancer; However, these drugs causes serious side effects to patients such as nephrotoxicity, neurotoxicity, infertility, thromboembolic complications, hair loss, nausea, and myocardial infarction (Shabaruddin et al., 2013). Hence, there is a dire need to look for more efficacious agents with lesser side effects for the prevention and eradication of skin tumors.

Number of recent evidence has shown that natural dietary antioxidants are capable of inhibiting cancer cell growth. Moreover, these natural antioxidants are reported to have the ability to enhance the activities of detoxification enzyme components such as glutathione-S-transferase (GST). The activation of GST not only neutralizes the chemical carcinogens, but also preventing them from forming a DNA adducts in the genome (Liu et al., 1992). Furthermore, it has been suggested that these natural antioxidant compounds can be used in multiple ways, either as cancer preventive agents or even as cancer therapy drugs (Thornthwaite et al., 2013).

Gallic acid (GA), a polyhydroxyphenolic compound, is abundantly present in leafy vegetables, fruits, and nuts such as gallnut, grapes, sumach, oak bark, green tea, apple peels (Atkinson et al., 2004; Chakraborty et al., 2009; Kim et al., 2006; Ng et al., 2004; Liao et al., 2012) and also present in onion and potato varieties (Albishi et al., 2013). GA exhibits variety of biological activities including antioxidant (Isuzugawa et al., 2001; Yogendra Kumar et al., 2013), anti-tumor (Agarwal et al., 2006; Liao et al., 2012; Lee et al., 2013; Locatelli et al., 2013), anti-inflammatory (Albishi et al., 2013) and anti-bacterial (Kang et al., 2008; Lee and Je, 2013). GA has been shown to inhibit gastric cancer cell growth *in vitro* by modulating the levels of MMP-2 and -9 and cytoskeletal reorganization (Ho et al., 2010). Furthermore, GA has also been

* Corresponding author. Tel.: +91 44 22202734.
E-mail address: vellaie@gmail.com (E. Vellaichamy).

http://dx.doi.org/10.1016/j.fct.2014.01.017
0278-6915/

shown to suppress cell viability, proliferation, invasion and angiogenesis in human glioma cells, *in vitro* (Lu et al., 2010; Lee et al., 2013). Lo et al. (2011) have also demonstrated that GA inhibits the migration and invasion of cultured A375.S2 human melanoma cells by inhibiting matrix metalloproteinase-2. GA derivatives such as tannic acid and ellagic acid have been shown to exhibit antitumor activities in mouse skin (Perchellet et al., 1992). GA has been shown to selectively induce apoptosis only in the pancreatic cancer cells without affecting the survival of normal cells in *in vitro* condition (Liu et al., 2012). Although several line of evidence has shown that GA inhibits tumor cells growth, migration and invasion *in vitro* (Chen et al., 2012). However, a detailed *in vivo* studies have not been carried out to examine the role of GA on tumor cell growth, invasion and on MMPs.

In this study, we have utilized a two stage *in vivo* skin carcinogenesis model, because it is a suitable model system to study both the initiation and progression phases of the tumorigenesis. The results of this study indicates that topical application of GA inhibits the skin tumor initiation and progression phases of the skin carcinogenic process by modulating the antioxidant enzyme system and MMPs (-2 & -9) in the mouse skin tissue.

2. Materials and methods

2.1. Chemicals

7,12-Dimethylbenzanthracene (DMBA) (Cat. No. D3254), and Gallic acid (Cat. No. 27645) were purchased from Sigma, St. Louis, MO, USA. Croton oil was procured from Aroma essential oils, Haryana, India. 5-Fluorouracil was purchased from Fluka Bio Chemika (Buchs, Switzerland). Primary antibodies for MMP-2 (Cat. No. sc-10736), MMP-9 (Cat. No. sc-10737) and HRP-labeled secondary antibody (Cat. No. sc-2749) were procured from Santa Cruz biotechnology, San Diego, CA, USA. Nitroblue tetrazolium (NBT), Nucleotide adenosine diphosphate (NAD^+), riboflavin and Phenazine methasulphate (PMS) were obtained from SD Fine and SRL Chemicals Ltd., India. All other chemicals used were of analytical grade, purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

2.2. Animal

Adult male Swiss albino mice, 8–10 weeks old (25–30 g) were obtained from Tamil Nadu Veterinary and Animal Science University, Chennai, India, and were housed in polypropylene cages. The animals were acclimatized for 1 week prior to the start of the experiment. Mice were fed with commercially available Gold Mohr pelleted diet supplied by Lipton India Pvt. Ltd. (Bangalore, India) and water ad libitum; maintained on standard housing conditions under controlled atmosphere with 12:12 h light/dark cycles with an ambient temperature of 25 ± 5 °C and humidity at 50 ± 10%. This study was conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and by Animal Ethics Committee Guidelines of our Institution (IAEC NO. 12/02/2012).

2.3. Experimental design

Animals were divided into five groups (10 mice/Group) as follows.

- *Group I (Control):* Mice were topically applied with acetone alone throughout the course of the experiment.
- *Group II (Induction):* Mice received topical application of single dose of DMBA (100 μg/100 μL in acetone) over the shaved dorsal area of the skin. After 2 weeks, Croton oil (1% in acetone) was applied 3 times a week for 16 weeks.
- *Group III (Co-treatment):* Mice received topical application of GA (25 mg/kg body weight per day) 10 min before the application of DMBA/Croton oil as that of Group II mice.
- *Group IV (Positive control):* Mice received topical application of 0.5% of 5-Fluorouracil (5-FU) 10 min before the application of DMBA/Croton oil as that of Group II mice (Wilgus et al., 2004; Olson and Wattenberg, 1969).
- *Group V (Drug control):* Mice received topical application of GA (25 mg/kg body weight per day) alone three times a week for 16 weeks.

During the 16 weeks of experiments, mice were observed daily for the appearance of skin papillomas and tumor volume was recorded. At the end of 16 weeks, all the mice were sacrificed and skin tissue and serum samples were collected.

2.4. Preparation of skin and tissue homogenate

The skin tissue was separated and washed several times in an ice-cold saline. Skin tissue (100 mg) was carefully chopped into fine pieces using a surgical blade. The minced skin tissue was homogenized in an ice-cold RIPA buffer, pH 7.4 with a help of homogenizer (Remi model-RQ-127A/D). Typically, the homogenizer was set at a speed of 1000 rpm, 10 strokes for 30 s for 2–3 times at 4 °C were applied to get a complete homogenate of the skin tissue. The resultant homogenate was centrifuged at 20,000 rpm for 5 min at 4 °C. The tissue debris was separated as a pellet and the tissue supernatant was pipette out. The skin tissue homogenates and serum were stored at −20 °C until further use. Protein quantification was carried out using the method of Bardford Protein Assay (Simna et al., 2012).

2.5. Electrophoretic separation of Lactate Dehydrogenase (LDH) Isoenzymes

LDH isoenzymes consist of five isoforms, and are encoded by LDHA and LDHB gene. The polypeptide subunits combine to form two pure types of isoenzymes which referred as H and M, LDH-1 consists of 4-polypeptide subunits of H type, while LDH-5 consists of 4-polypeptide subunits of M type and LDH-2 (H3M), LDH-3 (H2M2), and LDH-4 (HM3) consists of hybrids of H and M type subunits (Markert, 1963). The LDH isoenzymes were separated as described by Van Der Helm et al. (1962). Briefly the serum protein (10 μg) was loaded on a 1% agarose gel prepared with 25 mM Tris (pH 9.5). Tris–glycine buffer, pH 9.5 was used as a running buffer and the electrophoresis was carried out for 1 h at 100 V. After the electrophoresis, the gel was washed in 0.1 M Tris–HCl (pH 8.5). Then, gel was incubated in a LDH staining solution containing lactate (3.24 mg/ml), nicotinamide adenine dinucleotide (0.3 mg/ml), nitroblue tetrazolium (0.8 mg/ml) and phenazine methosulfate (0.167 mg/ml) dissolved in 0.01 M Tris–HCl (pH 8.5). LDH isoenzymes were separated depending on their charge and molecular weight and appeared as a clear zone. LDH-5 isoenzyme moved toward negative pole because of its net positive charge while isoenzymes move toward positive pole. The relative activity of each isoenzyme was determined by densitometry analysis.

2.6. Glutathione-S-transferase (GST), enzymatic and non-enzymatic antioxidants

Skin GST enzyme activity was measured by modified method of Habig et al., 1974. The GST activity was expressed as μmoles of 1-chloro-2,4-dinitro benzene (CDNB) conjugated per minutes per mg of protein. Enzymatic antioxidants – superoxide dismutase (SOD) activity was based on the inhibition of epinephrine–adrenochrome transition and the activity of enzyme was expressed as units/min/mg protein (Marklund and Marklund, 1974). Catalase (CAT) activity was quantified by the amount of H_2O_2 decomposed/min/mg protein (Sinha, 1972). Glutathione peroxidase (GPx) activity was estimated based on its substrate glutathione as described by Rotruck et al. (1973), and non-enzymatic antioxidant reduced glutathione (GSH) which involves the reaction of glutathione with dithio bis-nitro benzoic acid (DTNB) to give a compound which has an absorption maximum at 412 nm (Moron et al., 1979) were estimated as described previously.

2.7. Total serum activity of Lactate Dehydrogenase (LDH) and lipid peroxidation (LPO)

Serum LDH was estimated as described by King (1965), which is a ubiquitous glycolytic enzyme that catalyzes reversible oxidation of lactate to pyruvate with concomitant reduction of NAD. When the enzyme is supplied with lactate and NAD^+, the LDH catalyzed reaction starts to produce pyruvate. LDH activity was expressed as μg/dl of serum. Lipid peroxidation (LPO) was analyzed by previously described method (Okhawa et al., 1979). LPO activity was expressed as (TBARS) mmol/μg protein.

2.8. Substrate gel analysis of SOD and Catalase CAT activity

To further validate the enzymatic activity assay of SOD and CAT, substrate native gel electrophoresis was carried out. The SOD activity was detected in a native PAGE as described by the method of Beauchamp and Fridovich (1971). The CAT enzyme activity was detected in a Native PAGE as described by the method of Woodbury et al. (1971). Protein sample (30 μg/lane) was mixed with 50% glycerol and loading dye and run on the 8% Native PAGE gel constantly at 100 V, ~1 h in 4 °C. For SOD activity analysis, the gel was soaked in a Riboflavin-NBT solution (2–5 ml) and incubated at RT for 15 min in dark (Riboflavin is light sensitive). After incubation, the Riboflavin-NBT solution was removed and 2–5 ml of 0.1% TEMED was added and incubated at RT for15 min in dark. Remove the staining solution and keep the gel in light which induces super oxide synthesis. For CAT activity, the gel was extensively rinsed with double distilled water followed by incubation with 0.003% H_2O_2 for 10 min. The gel was stained with 2% potassium ferric cyanide and 2% ferric chloride solution. The clear zone in the gel indicates the CAT activity.