Calcium Glucarate Prevents Tumor Formation in Mouse Skin[1]

JAYA SINGH AND KRISHNA P. GUPTA[2]

Environmental Carcinogenesis Division, Industrial Toxicology Research Center, Post Box No.80, Mahatma Gandhi Marg Lucknow –226001, India

Objective Calcium Glucarate (Cag), Ca salt of D-glucaric acid is a naturally occurring non-toxic compound present in fruits, vegetables and seeds of some plants, and suppress tumor growth in different models. Due to lack of knowledge about its mode of action its uses are limited in cancer chemotherapy thus the objective of the study was to study the mechanism of action of Cag on mouse skin tumorigenesis. **Methods** We have estimated effect of Cag on DMBA induced mouse skin tumor development following complete carcinogenesis protocol. We measured, epidermal transglutaminase activity (TG), a marker of cell differentiation after DMBA and/or Cag treatment and [3H] thymidine incorporation into DNA as a marker for cell proliferation. **Results** Topical application of Cag suppressed the DMBA induced mouse skin tumor development. Topical application of Cag significantly modifies the critical events of proliferation and differentiation TG activity was found to be reduced after DMBA treatment. Reduction of the TG activity was dependent on the dose of DMBA and duration of DMBA exposure. Topical application of Cag significantly alleviated DMBA induced inhibition of TG. DMBA also caused stimulation of DNA synthesis in epidermis, which was inhibited by Cag. **Conclusion** Cag inhibits DMBA induced mouse skin tumor development. Since stimulation of DNA synthesis reflects proliferation and induction of TG represents differentiation, the antitumorigenic effect of Cag is considered to be possibly due to stimulation of differentiation and suppression of proliferation.

Key words: Cag; Skin tumor; Chemoprevention; TG; DNA synthesis; Skin; Transglutaminase

INTRODUCTION

Calcium glucarate (Cag), a Ca salt of glucaric acid and a dietary constituent, is present in fruits (apple, grapefruit, cherries and apricot), vegetables (broccoli, alfalfa sprouts, brussel sprouts) and seeds of some plants[1]. It has been shown that Cag can modulate -glucuronidase enzyme found in many tissues and alter the efficiency of detoxification and proliferation in different model[2]. Administration of free glucuronic acid to potentiate this system is ineffective because free glucuronic acid does not participate in detoxification. Cag forms a -glucuronidase inhibitor, glucarolactone and D-glucaro-1,4-lactone and exhibits potent antiproliferation properties *in vivo*[3]. Dietary Cag has been shown to be an effective chemopreventive agent in DMBA induced rat mammary carcinogenesis and benzopyrene induced mouse lung carcinogenesis[4].

Cag, a slow release form of glucarate was shown to be effective on inhibiting the tumor

[1] Financial support was provided by Indian Council of Medical Research, New Delhi.

[2] Correspondence should be addressed to: E-mail krishnag2@rediffmail.com Tel: +91-522-227586, Ext.313, Fax: +91-522-228227

Biographical note of the first author: Ms. JAYA SINGH, female, born in 1976, Ph.D. candidate, majoring in carcinogenesis.

Abbreviations: DMBA:7,12 –dimethylbenz(a)anthracene; Cag: Calcium glucarate; TG: Transglutaminase

0895-3988/2003
CN 11-2816

development in rodent' s[5] and different experimental models[6-8] when given in diet. Due to lack of knowledge about its mechanism of action, Cag has not been applied as a chemopreventive agent for tumor suppression and in tumor therapy. In order to make its better use, we elucidated its mode of action in mouse skin. Mouse skin model was established and had advantages over other model systems in quantifying tumor formation and progression. Here we report the effect of topical application of Cag on mouse skin tumor formation using complete carcinogenesis protocol in which a subcarcinogenic dose of carcinogen was used for tumor development. We analyzed the effect of Cag on representative tumor markers such as DNA synthesis and transglutaminase activity. Keratinocyte transglutaminase (TGK) and epidermal transglutaminase (TGe) are the major enzymes involved in the formation of cornified envelopes during terminal differentiation of epidermis[9-11]. These cornified envelopes function as the protective barrier in the skin and consist of 10-12 nm thick deposit of TG catalyzed highly cross-linked proteins.

This is the first report demonstrating the effect of topical application of Cag on complete carcinogenesis using 7,12-dimethylbenz(a)anthracene (DMBA), following long term animal bioassay and tumor markers. Earlier Cag was used as a dietary supplement but here we have used Cag topically over the mouse skin, the target tissue.

MATERIALS AND METHODS

Animals

Female Swiss albino mice from the inbred colony of ITRC, Lucknow were used. Animals were 4-6 weeks old and weighed between 10-12 g. They were fed with synthetic pellet diet and water ad libitum. The mice were shaved in the interscapular region using surgical clippers two days before the experiment and only those in the resting phase of hair growth were selected for the study.

Chemicals

Calcium glucarate (Calcium salt of D-saccharic acid), 7,12-dimethylbenzanthracene, putrescine and N,N-dimethyl casein were obtained from Sigma Chemicals Company, St Louis, MO, USA, [^{3}H]-putrescine (sp.act.25 Ci/mmol) was from Amersham, Arlington Heights. [^{3}H]-thymidine (sp. Act.17000 mCi/mmol) was obtained from BARC Mumbai; India. GF/C Filters were from Whatman International Company. All other chemicals used were obtained from commercial sources.

Chronic Animal Bioassay

Chronic animal bioassays were followed as reported earlier in complete carcinogenesis protocol[8]. Animals were divided into groups consisting of 15 animals each. All treatments were applied topically in the interscapular region of shaved back. 5 g DMBA was applied twice weekly.

Immediately after the application of DMBA, Cag was applied in the same area topically at a dose of 1 mg, 3 mg or 6 mg. Acetone was used as a vehicle in all the treatments. Controls were treated with acetone. For the measurement of tumorigenesis we counted the number of tumors/animal, tumor bearing animals, % tumorigenicity and % of tumor bearing animals at the end of the experiment. Experiments were terminated when 100% tumorigenesis was attained or when the animals were ill. Animals were inspected regularly for baldness, acne, sickness, poor health, growth or ulceration. Animals of the respective control groups were

similarly inspected.

Assay for Transglutaminase (TG) Activities

Animals were treated with DMBA at the doses and time mentioned. Transglutaminase activity was assayed in epidermal homogenate. Epidermis was separated from skin by following the method of freezing/thawing[12] and then homogenized in 8 mL of homogenizing media consisting of 0.25 mol/L sucrose, 1 mmol/L EGTA and 1 mmol/L tris HCl at a pH 7.4. Epidermal transglutaminase activity was estimated as described earlier in the filter paper assay by Lorand *et al*.[13]. In brief the reaction mixture contained 5 mmol/L $CaCl_2$, 3.8 mmol/L DTT, 500 g casein, 1.2 mmol/L putrescine and 3 Ci of [^{3}H]-putrescine. Reaction was started by the addition of 100-200 g enzyme protein. After 30 min of incubation at 37°C reaction was stopped by putting an aliquot of reaction mixture on GF/C filters. Filters were washed twice with 10% trichloroacteic acid (TCA) and once with 5% TCA and ethanol:acetone (1:1). Radioactivity bound to filters was counted using toluene based scintillation fluid containing PPO and POPOP. Enzyme activity was calculated in terms of pmoles [^{3}H]-putrescine incorporated per mg prot. per hour.

Protein Estimations

Protein estimations were done according to Lowry's method using bovine serum albumin as standard[14].

Determination of DNA Synthesis

Animals were treated with DMBA and/or Cag for 16 h at the doses mentioned in the legends. One hour prior to killing, 30 Ci [^{3}H]-thymidine/mouse was administered intraperitoneally in saline. DNA synthesis was followed by measuring [^{3}H]-thymidine incorporation into mouse epidermal DNA as mentioned[15] and was expressed in terms of % of control.

Animal Treatment for the Acute Study

Animals were divided into groups consisting of 2-3 animals each. All treatments were given topically in the interscapular region of the shaved back. DMBA was given once at a dose of 5 or 50 g. Immediately after the application of DMBA, calcium glucarate was applied in the same area at a dose of 1 mg, 3 mg or 6 mg to different groups. Acetone was used as a vehicle in all the treatments. Animals were sacrificed at the time mentioned elsewhere.

RESULTS

Effect of Cag on DMBA Induced Tumor Development

Cag inhibited the tumor formation induced by DMBA. The onset of tumors was delayed by 6 mg dose of Cag. The cumulative number of tumor/animal was also reduced from 60-62 to 12-15 in presence of 6 mg dose of Cag. In the positive control group 5, 100% tumorigenesis was attained in about 25 weeks of DMBA application whereas there was only 50%-60% tumorigenesis at this time point in groups 6, 7, and 8 receiving 1 mg, 3 mg or 6 mg of Cag/ animal respectively together with DMBA (Fig. 1). We did not observe 100% tumorigenesis in group 8 until the 30th week of experimentation when in groups 6 and 7 tumorigenesis was still 53% and 46%. Though the difference was not found in the onset of tumors as in

groups 6 and 7, tumors started appearing on the 12th week of experimentation. The number of tumors in groups 5, 6, 7, and 8 was significantly different. No ill effect of Cag was observed in groups 6, 7, or 8 but animals in Cag treated groups were healthy as shown by their better hair growth and weight (Table 1) .

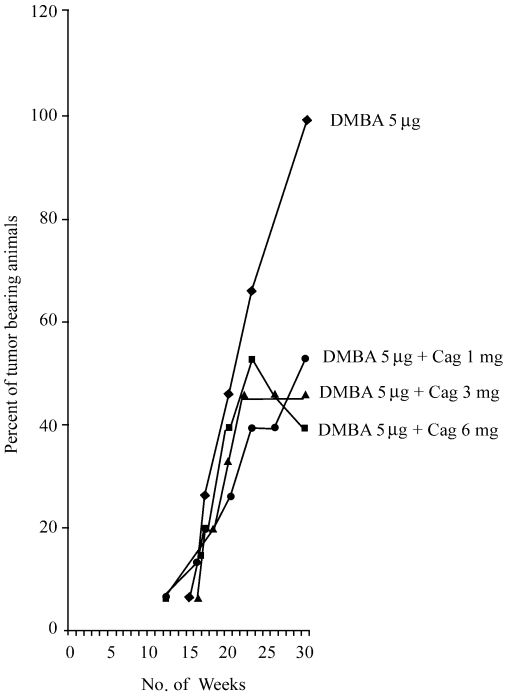

FIG. 1. Animals were treated with 5 g Dose of DMBA and/or 1 mg, 3 mg, 6 mg dose of Cag twice weekly. Controls received acetone alone. Each group consisted of 15 animals.

TABLE 1

Effect of Cag on DMBA Induced Tumorigenesis

Group	Treatment	Onset of First Tumor (Weeks)	% Tumorigenesis on 30th Week	No. of Tumor per Animal	No. of Animal Bearing Tumor/ Initial No. of Animals
1.	Control	-	-	-	-
2.	Cag 1 mg	-	-	-	-
3.	Cag 3 mg	-	-	-	-
4.	Cag 6 mg	-	-	-	-
5.	DMBA+DMSO	14th	100*	60-62	15/15
6.	DMBA+Cag 1 mg	11th	53.3*	21-25	8/15
7.	DMBA+Cag 3 mg	11th	46.6***	11-15	7/15
8.	DMBA+Cag 6 mg	16th	40***	10-12	6/15

* Big Tumors, ** Tumor size small like acne, *** Acne like tumors, **** Animals were healthy with small acne like tumors.

Note. Animals were treated with 5 g of DMBA, twice weekly. Cag was applied at a doses of 1 mg, 3 mg, or 6 mg alone or together with DMBA.

The most important and remarkable observation in this experiment of DMBA induced tumorigenesis was that the growth of tumors was retarded tremendously in Cag treated groups 6, 7, and 8 as compared to that of group 5, the positive control group. Besides this, some tumors in groups 6, 7, and 8 remained, like acne till the end of the experiment. The animals receiving Cag alone at the doses of 1 mg, 3 mg, or 6 mg/animal did not show any sign of tumor development. On the contrary, they were very healthy. Acetone treated control animals in group 1 did not show any spontaneous tumors.

Effect of Cag on DMBA Altered Transglutaminase Activity

In order to check the effect of Cag on TG induction, we first established the effect of DMBA on TG activity. For that we tried to find the optimum dose of DMBA and time of exposure by doing experiments for dose response and time course.

1. To determine the response of different doses, DMBA at doses ranging from 5 g to 50 g per animal was given topically. TG activity was estimated after 4 h of the application. As shown in Fig. 2, DMBA decreases the TG activity at all doses used but it appears that 50 g dose gives the maximum inhibition of TG activity.

2. The result shown in Fig. 2(a) indicates that the inhibition of TG by DMBA is dose-dependent. Further in order to see if this inhibition was a function of time, a time course study was carried out. For that animals were exposed to 50 g DMBA for different time periods ranging between 1, 2, 4, 8, or 16 h. Results showed that DMBA inhibited TG activity at all the time points but 4 h exposure time appeared to be optimum as TG inhibition was maximum at this time point (Fig. 2(b)).

3. To check the effect of Cag on DMBA reduced TG activity, Cag was administered together with DMBA (50 g/5 g) at the doses mentioned. Cag clearly increased the DMBA- inhibited TG activity in a dose-dependent manner (Fig. 2(c)). Cag alone had no effect on TG activity. Cag induced TG activity at all the doses applied but 6 mg dose was found to be most effective in inducing the TG activity.

Effect of Cag on DMBA Altered [^{3}H]-thymidine Incorporation

The status of DNA synthesis in mouse epidermis following topical treatment with DMBA (5 g or 50 g) alone or in presence of Cag (6 mg) was determined by measuring the incorporation of [^{3}H]-thymidine into epidermal DNA. Epidermal DNA synthesis in animals treated with DMBA showed a significant increase by 40%-80% as compared to the non-treated controls skin. Interestingly, Cag significantly inhibited the DMBA promoted stimulation of DNA replication, suppressing the effect to nearly control level (Table 2).

TABLE 2

S. No.	Treatment	cpm/ g DNA(±s)
1.	Control	56.18±4.5
2.	DMBA 5 g	85.3^a±7.0
3.	DMBA 5 g + Cag 6 mg	60.1^b±5.0
4.	DMBA 50 g	98.8^a±7.5
5.	DMBA 50 g + Cag 6 mg	70.4^c±5.5

Note. Effect of Cag on DMBA altered [^{3}H]-thymidine incorporation in mouse epidermal DNA. Animals were topically exposed to DMBA (5 g or 50 g). 6 mg dose of Cag was used 1 h before sacrificing the animals. 30 µCi of [^{3}H]-thymidine was given intraperitoneally in saline. Epidermal extract was prepared from 3-4 mice and [^{3}H]- thymidine incorporation was done as mentioned. $^a P<0.005$, $^b P<0.1$, $^c P<0.01$.

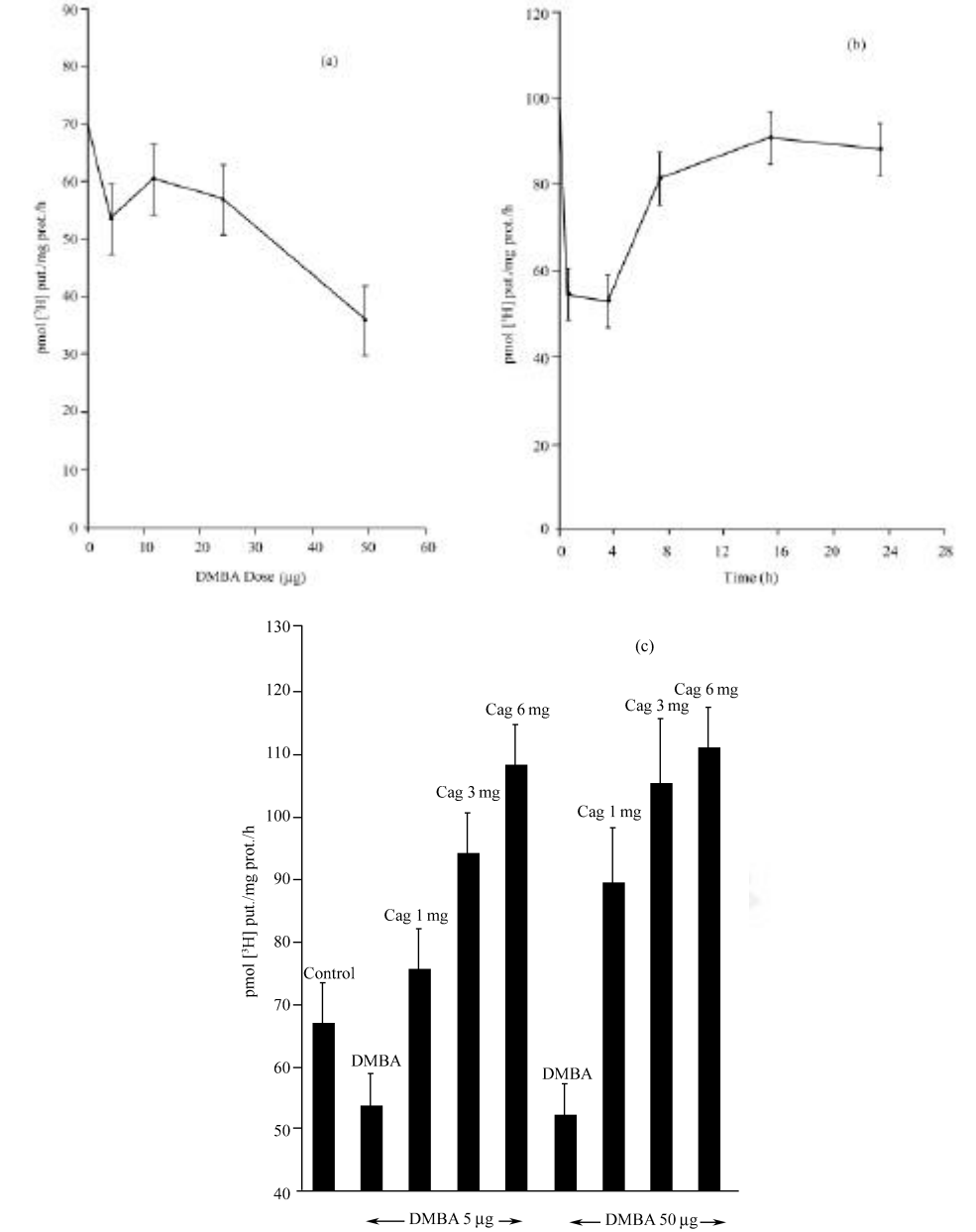

FIG. 2. (a) Dose dependent effect of DMBA on mouse skin TG activity. Mice were treated with different doses of DMBA as mentioned and enzyme activity was measured at 4 h after DMBA treatment. (b) Time dependent effect of DMBA on mouse epidermal TG activity. Mice were exposed to 50 g dose of DMBA for different time periods as mentioned and enzyme activity was measured as mentioned. (c) Dose dependent effect of Cag on DMBA induced mouse epidermal TG activity. Two different doses of DMBA were used as indicated. Mice were treated with 1 mg, 3 mg, or 6 mg dose of Cag as indicated along with DMBA. Animals were killed at 4 h after DMBA application and enzyme activity was measured as mentioned.

Each point represents the mean of triplicate determination of enzyme activity ±SE. Values are significantly different from their respective controls. $P<0.02$.

DISCUSSION

The credibility of chemoprevention as an approach to the control of cancer has been greatly enhanced. The appropriate use of a chemopreventive agent ultimately depends on the understanding of its mechanism of action[16]. Cag is a safe, non-toxic component of fruits and vegetables and is also present in human body. Cag supports glucuronidation, the major detoxification pathway in the body' s primary defense against cancer causing agents. Cag has been shown to be effective on inhibiting the development of cancers in laboratory animals. Its mode of action as a tumor suppressive agent has not yet been identified. The only known activity to date is the inhibition of -glucuronidase, which is exerted through the formation of D-glucaro-1-4, lactone, a potent -glucuronidase inhibitor. It was reported that in mouse skin dietary glucarate acted as inhibitors of phorbol ester-mediated tumor promotion and could cause the regression of pre-existing benign tumors[17, 18]. However there is no indication of mechanistic aspects. There is no report showing inhibition of tumor development by DMBA induced complete carcinogenesis either. We showed that topical application of Cag could inhibit DMBA induced tumor development. There was no ill effect with the topical application of Cag. Instead animals remained healthy with better fur growth. This is the first report showing the effect of topical application of Cag on DMBA induced complete carcinogenesis. This suppression might be due to the induction of transglutaminase enzyme activity, which was greatly elevated by Cag. The effect depended on the doses of Cag used.

A chemopreventive agent that decreases cell proliferation markedly reduces the susceptibility to cancer. Cell proliferation and DNA synthesis are clearly essential for tumor development. It has been shown that the state of DNA synthesis in the target tissue at the time of carcinogen application plays an important role in tumorigenesis[19]. Cag is a better agent for cancer control as it prevents the changes caused by complete carcinogen. On the basis of our studies of TG activity and [^{3}H]-thymidine incorporation, we can say that Cag affects both proliferation as well as differentiation stages which might contribute to its antitumor activity. Since it is non-toxic, it can be used in a better way with the lower doses of other known anticancer drugs, which produce cytotoxicity at their effective concentration. The activation of TG is thought to be responsible for the formation of intercellular cross-linked protein polymers that constitute the major component in an apoptotic cell[20]. Importance of TG in neoplastic cells is that, decreased TG is the requirement for rapidly proliferating tissue as reduced amounts of E-(glutamyl)-lysine have been reported in transformed cells[21]. Hence its increase in TG provides the molecular architecture necessary to the non proliferating or differentiating state. TG appears to be involved in cell growth, differentiation and cell death (apoptosis), an important mechanism of cell elimination[22]. TG induces protein cross linking, and one might anticipate that the expression of high levels of tissue TG inside a cell might be associated with a profound inhibition of proliferative activity. Possibly, inhibition of DMBA induced tumor formation by Cag might be due to the induction of TG activity and inhibition of DNA synthesis. More studies in this direction are in progress in our laboratory.

ACKNOWLEDGEMENTS

We are grateful to the Director of Industrial Toxicology Research Centre, Lucknow for his taking interest in this study.

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(*Received August* 8, 2002 *Accepted October* 8, 2002)