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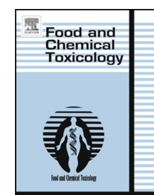

Chemopreventive efficacy of menthol on carcinogen-induced cutaneous carcinoma through inhibition of inflammation and oxidative stress in mice

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A B S T R A C T

Inflammation and oxidative stress have been implicated in various pathological processes including skin tumorigenesis. Skin cancer is the most common form of cancer responsible for considerable morbidity and mortality, the treatment progress of which remains slow though. Therefore, chemoprevention and other strategies are being considered. Menthol has shown high anticancer activity against various human cancers, but its effect on skin cancer has never been evaluated. We herein investigated the chemopreventive potential of menthol against 9,10-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation, oxidative stress and skin carcinogenesis in female ICR mice. Pretreatment with menthol at various doses significantly suppressed tumor formation and growth, and markedly reduced tumor incidence and volume. Moreover, menthol inhibited TPA-induced skin hyperplasia and inflammation, and significantly suppressed the expression of cyclooxygenase-2 (COX-2). Furthermore, pretreatment with menthol inhibited the formation of reactive oxygen species and affected the activities of a battery of antioxidant enzymes in the skin. The expressions of NF-κB, Erk and p38 were down-regulated by menthol administration. Thus, inflammation and oxidative stress collectively played a crucial role in the chemopreventive efficacy of menthol on the murine skin tumorigenesis.

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1. Introduction

Skin cancer is the most common type of cancers in the United States, with more than one million new cases being diagnosed each year, which accounts for 40% of all newly-diagnosed cancer cases (Liu et al., 2013; Stern, 2010). Traditionally, skin cancer is mainly treated by surgery, chemotherapy and radiotherapy. Surgery is the gold standard for treatment of melanoma and nonmelanoma (Wollina, 2014), and the recurrence rate after surgery with oncological safety limit has been reduced. Chemotherapy is mainly used for aggressive tumors such as melanoma (Mignot et al., 2014), while radiotherapy is used only for selected cases of nonmelanomatous tumors (Strom et al., 2014), such as elderly people with severe comorbidities, therapeutically unavailable areas. However, progress in the treatments of cutaneous carcinoma remains sluggish. Therefore, some alternative strategies are being considered, such as chemoprevention, which employs naturally derived and non-toxic compounds to prevent skin cancer. Ultraviolet (UV) rays, genetic factors, environmental carcinogens, inflammatory agents and tumor promoters, which can induce inflammation and production of reactive oxygen species (ROS), are mainly responsible for the onset of skin cancer (Chaudhary et al., 2013; Diepgen et al., 2012).

Inflammation has been causally linked to carcinogenesis, driving premalignant and malignant transformations of cells (Kuo et al., 2010). Moreover, numerous animal models, such as those of UV-induced or chemically induced skin carcinogenesis and transgenic ones, support that the development of skin cancer undergoes deregulated inflammation (Hensler and Mueller, 2013). Besides, inflammatory factors, such as cyclooxygenase-2 (COX-2), play

Abbreviations: CAT, catalase; DMBA, 9,10-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; COX-2, cyclooxygenase; ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; HO-1, heme oxygenase-1; LPO, lipid peroxidation; GSH, glutathione; MDA, malondialdehyde.

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critical roles in multistage cancer development (Azevedo et al., 2011; Tuzcu et al., 2012). Furthermore, ROS have been regarded as causative factors for mutagenesis, carcinogenesis and tumor promotion involved in the pathophysiology of many human chronic diseases (Kong et al., 2013). Topical application of some tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) for example, can cause inflammation and increase the release of ROS (Lee et al., 2012; Wu, 2006). Khan et al. reported that some antioxidant enzymes, such as heme oxygenase-1 (HO-1), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx), were involved in oxidative stress and exerted protective effects on the resulting injury (Khan et al., 2013). In many studies on skin cancer prevention, continuous inflammation and oxidative stress have been assigned as key pathological factors.

The two-stage skin carcinogenesis model, which is induced by 9,10-dimethylbenz[a]anthracene (DMBA)/TPA in female ICR mice, is an excellent *in vivo* model that well mimics multistage skin tumorigenesis mainly including initiation, promotion and progression. DMBA, a polycyclic aromatic hydrocarbon, is a procarcinogen and thus needs metabolic activation to be carcinogenic ultimately. The active metabolite and dihydrodiol epoxide, generated during the metabolic activation of DMBA, binds and causes damage to DNA, which is a rapid and irreversible process (Miyata et al., 2001). TPA, a phorbol-type tumor promoter, is a slow and reversible process (Ma et al., 2013) and is well-known to promote tumorigenesis by increasing expressions of inflammation factors and production of ROS (Fu et al., 2009; Nafz et al., 2007). In addition to physiopathological changes leading to tumor promotion, the cancer chemopreventive potential of synthetic and natural compounds can also be analyzed with this model (Bhatia et al., 2012).

NF-κB, which participates in the whole process of inflammation, is one of the most important ubiquitous redox-sensitive transcription factors that regulate the expressions of genes involved in inflammation, cell proliferation and survival. Although various signaling cascade components are involved in the promotional stage of cancer development, those that congregate with NF-κB are closely associated with tumor promotion. It is well-documented that phorbol-type tumor promoters are able to modulate the Ras signaling pathway, especially the downstream Erk and p38 ones (Chang et al., 2005; Khan et al., 2013; Kim et al., 2012).

As a naturally occurring cyclic terpene alcohol of plant origin, menthol has been used since antiquity for medicinal purposes (Farco and Grundmann, 2013). Menthol consumption has constantly been associated with reduced incidence rates of a variety of cancers (e.g. bladder and prostate cancers), due to strong anti-inflammatory and oxygen-quenching abilities (Li et al., 2009; Wang et al., 2012).

Despite the evident anti-inflammatory effect of menthol, its role in prevention and treatment of skin cancer has seldom been referred hitherto. Thereby motivated, we investigated whether menthol had chemopreventive efficacy on carcinogen-induced skin cancer and tried to elucidate the potential mechanisms involved. The DMBA/TPA-induced mouse skin carcinogenesis model was used in order to test the preventive effects of menthol on promotional changes during neoplastic development. In the present study, we evaluated the effects of topically applied of menthol on DMBA/TPA-induced tumor promotion biomarkers such as skin hyperplasia, proliferation, and expression of COX-2. We also examined the activities of a battery of antioxidant enzymes and alterations in the levels of glutathione (GSH) and lipid peroxide (LPO). Furthermore, the expressions of NF-κB, Erk and p38 were also detected to clarify the possible mechanisms of action.

2. Materials and methods

2.1. Chemicals and reagents

12-O-tetradecanoylphorbol-13-acetate (TPA) and 9,10-dimethylbenz[a]anthracene (DMBA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); menthol (purity >99%) was purchased from Xi'an Helin Biological Engineering Co. (Xi-an, Shanxi, China); MDA assay kit (Cat. No. S0131); GSH assay kit (Cat. No. S0052); CAT assay kit (Cat. No. S0051); GR assay kit (Cat. No. S0055) and GPx (Cat. No. S0058) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China); 1-chloro 2,4-dinitrobenzene (CDNB) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of highest purity and commercially available.

2.2. Animals

Eight week old female ICR mice (25–30 g), free from infection, were obtained from the Slac Animal Inc. (Shanghai, China). Throughout the experiments mice were maintained in plastic cages at 21 ± 2 °C on a 12h light/dark cycle with free access to food and water. Animal welfare and experimental procedures were performed strictly in accordance with the care and use of laboratory animals, and the related ethics regulations of our university. All possible efforts were made to minimize the animals' suffering and to reduce the number of animals used.

2.3. In vivo *skin tumorigenesis*

The dorsal skin, which was on the back area of female ICR mice, was shaved 3 days before the commencement of the experiment, and only those in the resting phase of the hair cycle were selected (Dhawan et al., 1999). The mice were randomly divided into four groups (control; DMBA/TPA; DMBA/TPA/menthol (20 mg/kg); DMBA/TPA/menthol (80 mg/kg)) (n = 12). The model and DMBA/TPA/menthol groups were treated on their shaven backs with single topical application of DMBA (60 µg), which was dissolved in 0.2 mL of acetone, and the normal group was treated only with vehicle (0.2 mL of acetone). One week after initiation with DMBA, the model and DMBA/TPA/menthol groups were treated topically with TPA (4 µg) twice a week for 20 weeks. Menthol (20 and 80 mg/kg) was applied topically onto the shaved area of dorsal skin twice a week, which began in the week when TPA was topically applied and terminated at the 20th week (Lai et al., 2007). Body weights of the animals were recorded initially, then at 1 week intervals and at the time of death. Papillomas appearing on the skin were recorded every week during the experimental period, with only those with diameter >1 mm considered as positive. Experiments were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine and strictly performed according to the NIH Guide for the Care and Use of Laboratory Animals.

2.4. Preparation of skin/tumor lysates

The excised dorsal skin (2 cm × 2 cm)/tumors from each group were cleaned to remove the extraneous materials with a sharp scalpel blade in ice-cold phosphate buffered saline. The pooled skin/tumors of each group were processed to prepare cytosol and membrane as described by Kundu et al. (2006) with minor modifications. Briefly, the skin/tumors were minced and homogenized in ice-cold lysis buffer (10 mM HEPES, pH 7.9; 10 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT, 0.1% NP-40) and 1 mM each of freshly prepared pepstatin, leupeptin and aprotinin, and then centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatant was collected as cytosolic fraction and aliquoted in 1 mL tubes. The pellet was processed for membrane fraction, resuspended in buffer containing 50 mM HEPES (pH 7.9), 0.1 mM PMSF and 0.1% SDS and 1% NP-40, and centrifuged for 10 min at 14,000 *g*. The supernatant was collected and aliquoted in 1 mL tubes (Kundu et al., 2006). All aliquots were stored at −80 °C. The protein content in each sample was assayed by the Bradford's method (Bradford, 1976) using bovine serum albumin as standard.

2.5. Immunohistochemistry

2.5.1. Tissue processing

Animals of all groups were sacrificed by cervical dislocation 1 h after the last application, and dorsal skin tissue (2 cm × 2 cm) was processed for sub-cellular fractionation. A piece of skin was preserved in 10% neutral buffered formalin for histological observation. Homogenates (10%) were prepared in chilled phosphate buffer (0.1 M, pH 7.4) using Polytron homogenizer (Kinematica, Inc., Switzerland). The homogenized tissue was centrifuged at 12,000 *g* for 15 min at 4 °C to obtain the supernatant that was taken to estimate the activity and expression of antioxidant enzyme (Khan et al., 2013). The dorsal skin of mouse was prepared for immunohistochemical analysis of COX-2 and PCNA expressions. The skin was fixed in 10% formalin and then embedded in paraffin. Five-micrometer-thick sections were cut and mounted on silanized glass slides. The section was deparaffinized in xylene and dehydrated in gradient concentrations of ethanol. The deparaffinized section was then heated and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To block non-specific binding, each section was treated with 3% H_2O_2 for 20 min and then with blocking solution (1% non-fat skimmed milk) for 30 min. The section was incubated with monoclonal anti-COX-2 and anti-PCNA antibodies at room temperature for 2 h, respectively, and then incubated with secondary antibodies at

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