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Skin tumor promotion by argemone oil/alkaloid in mice: Evidence for enhanced cell proliferation, ornithine decarboxylase, cyclooxygenase-2 and activation of MAPK/NF-κB pathway

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ABSTRACT

Consumption of argemone oil (AO) contaminated edible oil causes "Epidemic Dropsy". Previously, we have shown that AO and isolated sanguinarine possess genotoxicity and skin tumor initiating activity. Here, we evaluate tumor-promoting potential of AO/sanguinarine alkaloid and investigate the molecular mechanisms involved therein. Single topical application of AO (50–400 μl/mouse) or sanguinarine alkaloid (1.5–12.0 μmol/mouse) afforded significant increase in (i) ornithine decarboxylase (ODC) activity, (ii) uptake of [^{3}H]-thymidine in DNA, (iii) cyclooxygenase-2 (COX-2), proliferating cell nuclear antigen (PCNA) and ODC protein expressions, (iv) phosphorylation of extracellular signal-regulated kinase (ERK)1/2, c-jun-N-terminal kinase (JNK)1/2 and p38 mitogen-activated protein (MAP) kinases, (v) increased NF-κB activation and (vi) no significant increase in dark basal keratinocytes. Subsequently, when AO and sanguinarine alkaloid was tested either as complete or stage I or stage II tumor promoter in 7, 12-dimethyl benz(a)anthracene (DMBA)-initiated mice, there was enhanced tumor incidence, tumor body burden and higher % of mice with tumors, when AO (0.1 ml) or isolated sanguinarine (1.5 μmol) was tested as stage II tumor promoter. However, no tumors were found when AO or sanguinarine alkaloid was tested either as complete or stage I tumor promoter. These results indicate that AO/ sanguinarine alkaloid possesses tumor-promoting potential at stage II level involving MAPK/NF-κB pathway.

1. Introduction

Several incidences of adverse effects on human health have been reported due to consumption of contaminated mustard oil, which occupies a prominent position among the various edible oils consumed in India and its surrounding habitats (Das and Khanna, 1997). Dietary consumption of argemone oil (AO) to rats causes histopathological changes in liver, lungs, kidneys and heart suggesting these to be the target sites for toxicity (Das and Khanna, 1997). Studies indicate that AO inactivates cytochrome P-450 with concomitant enhancement of lipid peroxidation (Upreti et al., 1991a). The enhancement of lipid peroxidation has been linked to production of reactive oxygen species (ROS), which may lead to overexpression of heat shock protein (hsp-70) and decrease the defense of bio-antioxidant pool and antioxidant capacity (Das et al., 1991, 2005b; Mukhopadhyay et al., 2002; Upreti et al., 1991b).

The multistage mouse skin carcinogenesis model is an ideal system to study the biochemical alterations, changes in cellular functions and histologic changes that take place during different stages of chemical carcinogenesis (Slaga, 1989). ROS have been shown to be involved in both initiation as well as promotion stages of skin carcinogenesis, however, oxidative stress and inflammation have been reported to be closely associated with tumor promotion stage of carcinogenesis (Boutwell, 1974; Cerutti, 1985; Jang and Pezzuto, 1998). Furthermore, these ROS and free radical influence the expression of a number of genes and signal transduction pathways, such as mitogen-activated protein (MAP) kinase, nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) that are involved in regulation of cell proliferation and tumor development (Afaq et al., 2005; Astrup and Paulsen, 1981; Chun and Surh, 2004; Dhar et al., 2002; Yano et al., 2000). Moreover, studies have shown that skin application of tumor-promoting agents results in inflammatory responses, such as development of edema, hyperplasia, induction of epidermal ornithine decarboxylase (ODC), cyclooxygenase (COX) protein expression (Afaq et al., 2005; Chun and Surh, 2004; Chun et al., 2004).

Our prior studies have shown unequivocal evidence for *in vivo* genotoxic potential of AO and sanguinarine alkaloid in chromosomal aberration, micronucleus test and comet assay in mouse model (Ansari et al., 2004, 2005). Further, *in vivo* DNA damaging effect of AO and sanguinarine alkaloid were correlated with their

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tumor initiating activity in a two-stage mouse skin tumor protocol (Das et al., 2005a). Though complete carcinogenic and tumor promoting activity was not observed for AO and sanguinarine alkaloid in this study, nonetheless, it was argued that AO/sanguinarine alkaloid may have tumor promoting effects at higher doses as it causes inhibition of epidermal histidase which may lead to enhanced keratin formation (Colburn et al., 1975). Since, it is well known that generation of free radicals and inhibition of epidermal histidase activities is associated with the tumor promoting action of several classes of skin promoters (Colburn et al., 1975; Perchellet and Perchellet, 1989), the objective of the present study was to evaluate tumor-promoting potential of AO and sanguinarine alkaloid and to investigate the molecular mechanism(s) involved therein.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), 1,4-dithiothreitol (DTT), 5,5′-dithio-bis-nitrobenzoic acid (DTNB), dimethylbenzanthracene (DMBA), diphenylamine (DPA), ethanolamine, 2-mercaptoethanol, methoxyethanol, ornithine, phenylmethanesulfonyl fluoride (PMSF), pyridoxal phosphate, 12-*O*-tetradecanoyl phorbol myristate acetate (TPA), and toluidine blue were purchased from Sigma Chemical Company, St. Louis, MO, USA. Tween-20 was product of E. Merck, Mumbai, India. NF-κB specific oligonucleotides were from Promega (Madison, WI). [^{3}H]–thymidine (specific activity 51 Ci/mmol) and DL-[1-^{14}C] – ornithine hydrochloride (specific activity 56 mCi/mmol) were procured from Amersham Pharmacia Biotech, Little Chalfont, UK. Other chemicals used were of the highest purity available commercially.

2.2. Collection of seeds, extraction of oil and preparation of sanguinarine alkaloid from argemone oil

Argemone mexicana seeds were procured from outskirts of Lucknow city, Uttar Pradesh, India and the oil was extracted using *n*-hexane (Tandon et al., 1993). The yield of AO from its seeds was 35% (v/w). Sanguinarine alkaloid was isolated from AO (yield 0.54%) (Das et al., 2005a).

2.3. Animals

Six–seven week old female Swiss albino mice (20 ± 3 g) derived from the animal breeding colony of Indian Institute of Toxicology Research, Lucknow, were acclimatized under standard laboratory conditions and given a commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water ad libitum. Animals were housed in plastic cages having rice husk as bedding and maintained in controlled atmosphere of 12 h dark/light, 22 ± 2 °C temperature and 50–60% humidity as per rules laid down by Animal Welfare Committee of IITR. The mice were shaved with electric clippers (Oster, WI, USA) and mice showing no sign of hair growth were used for experiments. The rationale of doses for AO and isolated sanguinarine used in the present investigation are described in detail in earlier study (Das et al., 2005a).

2.4. Assay of ornithine decarboxylase (ODC) activity

To evaluate the time dependent effect of TPA (4 nmol) or AO (0.3 ml) or sanguinarine alkaloid (12 μmol) mice received a single topical application for 2, 4, 6 and 8 h. To study the dose dependent effect, animals were treated with different doses of AO (0.1–0.3 ml) or sanguinarine alkaloid (3–12 μmol) and sacrificed after 4 h. ODC activity was determined using 0.4 ml of skin cytosolic fraction/assay tube and the release of $^{14}CO_2$ from DL [^{14}C] ornithine was measured (Verma et al., 1979). ODC activity was expressed as pmol $^{14}CO_2$ released/hr/mg protein.

2.5. Quantitation of epidermal DNA synthesis

Mice were divided into different groups and received different doses of AO (0.025–0.4 ml/mouse) and isolated sanguinarine alkaloid (1.5–12.0 μmol/0.1 ml acetone/mouse). The animals received topical application of acetone or TPA (4 nmol/0.1 ml acetone), which served as negative and positive controls, respectively. Epidermal DNA was isolated and assessment of incorporation of [^{3}H]-thymidine into DNA was carried out according to the method of Gupta and Mehrotra (1992), originally described by Smart et al. (1986).

2.6. Quantitation of dark basal keratinocytes

The quantitative assessment of dark basal keratinocytes in skin of mice treated with single topical application of acetone, AO (0.1 ml) or sanguinarine alkaloid (3.0 μmol) was carried out after 24 and 48 h. The excised skin was fixed in 3% glutaraldehyde in 0.05 M scollidine buffer (pH 7.4) for 2 h at 4 °C and several blocks were embedded and sections were prepared and stained with toluidine blue as described by Klein-Szanto and Slaga (1981). The percentage of dark cells was determined by counting the total number of nucleated darkly stained epithelial cells in direct contact with the basement membrane. Only the cells of the interfollicullar epidermis were considered Klein-Szanto and Slaga (1981).

2.7. Preparation of cytosolic and nuclear lysates

Mice were topically treated with acetone or 0.1 ml AO or 3.0 μmol sanguinarine alkaloid and sacrificed by cervical dislocation at 0.5, 1 or 2 h for MAPKs and NF-κB or at 4 h for COX-2 and ODC or at 18 h for proliferating cell nuclear antigen (PCNA) expression. Epidermis from the whole skin was separated as described by Afaq et al. (2005) and was homogenized in ice-cold lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM NaF, 100 mMNa_3VO_4, 0.5% NP-40, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Mannheim, Germany). The homogenate was then centrifuged at 14,000*g* for 25 min at 4 °C and the supernatant (total cell lysate) were collected, aliquoted and stored at 80 °C. For the preparation of nuclear and cytosolic lysates, 0.2 g of the epidermis was suspended in 1 ml of cold buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.9), 2 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride] with freshly added protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). After homogenization, the homogenates were left on ice for 10 min and then centrifuged at 25,000*g* for 10 min. The supernatant was collected as cytosolic lysate and stored at 80 °C. The nuclear pellet was resuspended in 0.1 ml of the buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.9), 300 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 10% glycerol with freshly added protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). The suspension was gently shaken for 20 min at 4 °C. After centrifugation at 25,000*g* for 10 min, the nuclear extracts (supernatants) were collected and quickly frozen at 80 °C. The protein content in the lysates was measured by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) as per the manufacturer's protocol.

2.8. Western blot analysis

For western analysis, 25–50 μg of the protein was resolved on 8–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer for 1 h at room temperature followed by incubation with phospho-ERK, phospho-p38, ERK, p38, SAPK/JNK, ODC, PCNA, NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) and COX-2 (Cayman Chemical, Ann Arbor, MI) antibodies at 1:1000 dilution for overnight at 4 °C. Washed blots were detected by horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies and bands were visualized by an appropriately equipped CCD camera and chemiluminescent kit.

2.9. Electrophoretic mobility shift assay (EMSA)

Mice were topically treated with acetone or 0.1 ml AO or 3.0 μmol sanguinarine alkaloid for 1hr and sacrificed by cervical dislocation. The nuclear extract was prepared as described by Kim et al. (2005). EMSA was carried out using light shift chemiluminescent EMSA kit (Pierce, Rockford, IL) following the method outlined in the manufacture's instructions. The consensus sequences of the oligonucleotides used were 5'-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCT-GAAAGGGTCCG-5′. NF-κB-specific oligonucleotides (3.5 pmol) were end-labeled with biotin using T4 polynucleotide kinase in 10× kinase buffer. Five μg nuclear protein were first incubated with 10× gel shift binding buffer [20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris–HCl, and 0.25 mg/ml poly(deoxyinosinic–deoxycytidylic acid)·poly(deoxyinosinic–deoxycytidylic acid)] and then with biotin end-labeled consensus oligonucleotide for 35 min at 37 °C. DNA–protein complex thus formed was resolved on 6.6% DNA retardation gels and subsequently transferred on nylon membrane and bands were visualized by an appropriately equipped CCD camera and chemiluminescent EMSA kit (Pierce, Rockford, IL).

2.10. Protocol to study complete or stage I and II tumor promotion by AO and sanguinarine alkaloid

Mice were divided into 10 groups of 15 each and received the following topical treatments.

1. DMBA (120 nmol) $\xrightarrow{1\text{ week}}$ acetone (0.1 ml) $\xrightarrow{2\times/\text{week for 24 weeks}}$
2. DMBA (120 nmol) $\xrightarrow{1\text{ week}}$ TPA (4 nmol) $\xrightarrow{2\times/\text{week for 24 weeks}}$
3. DMBA (120 nmol) 1 week A.O (0.3 ml) $\xrightarrow{2\times/\text{week for 24 weeks}}$