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A novel chemopreventive mechanism for a traditional medicine: East Indian sandalwood oil induces autophagy and cell death in proliferating keratinocytes ☆

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ABSTRACT

One of the primary components of the East Indian sandalwood oil (EISO) is α-santalol, a molecule that has been investigated for its potential use as a chemopreventive agent in skin cancer. Although there is some evidence that α-santalol could be an effective chemopreventive agent, to date, purified EISO has not been extensively investigated even though it is widely used in cultures around the world for its health benefits as well as for its fragrance and as a cosmetic. In the current study, we show for the first time that EISO-treatment of HaCaT keratinocytes results in a blockade of cell cycle progression as well as a concentration-dependent inhibition of UV-induced AP-1 activity, two major cellular effects known to drive skin carcinogenesis. Unlike many chemopreventive agents, these effects were not mediated through an inhibition of signaling upstream of AP-1, as EISO treatment did not inhibit UV-induced Akt or MAPK activity. Low concentrations of EISO were found to induce HaCaT cell death, although not through apoptosis as annexin V and PARP cleavage were not found to increase with EISO treatment. However, plasma membrane integrity was severely compromised in EISO-treated cells, which may have led to cleavage of LC3 and the induction of autophagy. These effects were more pronounced in cells stimulated to proliferate with bovine pituitary extract and EGF prior to receiving EISO. Together, these effects suggest that EISO may exert beneficial effects upon skin, reducing the likelihood of promotion of pre-cancerous cells to actinic keratosis (AK) and skin cancer.

Introduction

Ultraviolet light induces tumor promoting events in keratinocyte cells that, if allowed to proceed, will ultimately lead to the development of pre-cancerous conditions such as actinic keratosis (AK)[2] and skin tumors, including papillomas and squamous cell carcinoma (SCC). In humans, these promotion events result from constant and unavoidable exposure to the sun over many decades, a fact which highlights the need to identify new chemopreventive strategies that can be applied over a lifetime. One strategy includes topical formulations of novel natural products that are non-toxic to normal cells, but which prevent the outgrowth of

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[2] *Abbreviations used:* EISO, East Indian sandalwood oil; WASO, Western Australian sandalwood oil; AK, actinic keratosis; SCC, squamous cell carcinoma; AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's Medium; FBS, fetal bovine serum; SFM, serum-free DMEM; FDA, fluorescein diacetate; AnnV, anti-annexin V; BPE, bovine pituitary extract; EGF, epidermal growth factor; UVB, ultraviolet B; ICC, immunocytochemistry; PARP, poly ADP ribose polymerase; TBST, Tris buffered saline containing 0.1% Tween-20.

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tumors through a mechanism that targets UV-induced promotion events.

Sandalwood oils are essential oils commonly used as fragrances for body oils and incense as well as in medicines and cosmetics. Sandalwood oils have many well-known health benefits due to their anti-inflammatory and anti-septic properties, among others [1]. The principal commercial oils are steam distilled from the wood of two species of sandalwood trees: East Indian sandalwood (*Santalum album*) and West Australian sandalwood (*Santalum spicatum*). The compositions of the oils from the two types of trees are remarkably different with the fragrance and quality of East Indian sandalwood oil (EISO) considered superior to Western Australian sandalwood oil (WASO). One clear difference between the two oils is the content of α-santalol, which can vary from ~20% of the total oil content in WASO to more than 50% in EISO.

α-Santalol is one of the primary components of sandalwood oil and has been recently investigated for its chemopreventive properties. In fact, purified α-santalol, as well as sandalwood oil, has previously been demonstrated to prevent skin tumor development in mice [2–7]. Cell-based studies have found that α-santalol activates proapoptotic caspases, induces G_2/M cell cycle arrest and blocks inflammation, which may be responsible for the prevention of tumor development after UV exposure [8,9]. Although these studies identified some of the chemopreventive properties of α-santalol, little is known about the essential oil from which it was extracted and its potential value in preventing UV-induced skin cancer.

In the current study, we investigated the effects of EISO on cultured HaCaT keratinocytes. HaCaT cells were established from adult sun-damaged skin and are well characterized as representing an initiated human keratinocyte cell line expressing mutant dysfunctional p53 and a defective NF-κB signaling pathway, both of which are commonly found in UV-initiated keratinocytes in human skin [10–12]. We investigated the use of EISO on cells irradiated with UVB light. Although UVB light comprises only 1–10% of solar UV light, UVB acts as a complete carcinogen capable of activating signaling pathways in HaCaT cells known to stimulate cell proliferation and survival, including p38, JNK, ERK and PI3-K upstream of activator protein-1 (AP-1) transcription factor activation. UVB-induced AP-1 activity has been linked to cellular proliferation and survival, and in mouse skin AP-1 activation has been demonstrated to be a major cause of skin cancer [13]. In addition, we investigated the effects of EISO on cell cycle progression and cell membrane integrity. We determined that the effects of EISO are more pronounced in proliferating cells than in quiescent cells. Loss of cell membrane integrity and expression of a prominent marker of autophagy were both clearly more prominent in cells stimulated to grow than in serum-starved quiescent cells. These findings suggest that EISO may be valuable as a topical non-destructive chemopreventive agent through selective targeting of proliferative cancer and pre-cancerous cells.

Materials and methods

Materials

EISO was provided by Santalis Pharmaceuticals, Inc. (San Antonio, TX). Annexin V antibodies, bovine pituitary extract (BPE) and epidermal growth factor (EGF) were obtained from Life Technologies/Invitrogen (Grand Island, NY). Propidium Iodide was purchased from Sigma–Aldrich (St. Louis, MO). Antibodies used for Western blot analysis of signaling proteins (phospho-ERK, phospho-p38, phospho-JNK, phospho-Akt) were all purchased from Cell Signaling Technology (Danvers, MA). All other reagents were obtained from Sigma–Aldrich.

Cells

The human keratinocyte cell line, HaCaT, was established from cells obtained from adult sun damaged skin and have been described previously [10–12]. HaCaT cells contain UV-signature mutations and express mutant dysfunctional p53 and a defective NF-κB signaling pathway, which are common findings in UV-initiated keratinocytes in human skin. However, these cells maintain normal signaling in response to UV irradiation compared to normal human keratinocytes. HCl-14 and FL-30 cells are HaCaT-derived cell lines that stably express a luciferase reporter gene driven by a portion of the human collagenase I promoter containing a single activator protein-1 (AP-1) binding site, or a full length human c-fos promoter, respectively [14,15]. The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin at 37 °C and in 5% CO_2. In experiments using growth arrested cells, the cells were cultured to 80–90% confluence and maintained in serum-free DMEM (SFM) for 24 h prior to UVB exposure. In experiments using proliferating cells, 25 μg/ml (0.25% v/v) BPE and 0.2 ng/ml EGF was added back to cells after serum starvation for 3 h prior to subsequent treatment.

EISO treatment

HaCaT cells were treated with EISO diluted to a 1000× stock concentration in DMSO. Control cells were treated in the same manner with DMSO alone. Cells were treated for 1 h prior to irradiation with ultraviolet B light. HaCaT cells were washed once in PBS and irradiated with a dose of 250 J/m^2 UVB using a bank of two FS20T12 UVB lamps (National Biological Corp., Beachwood, OH) providing a peak emission of 313 nm. Control cells were treated in the same manner and mock irradiated. Following irradiation, HaCaT cells were again washed with PBS and returned to DMEM. During the post-irradiation incubation, cells were again treated with the indicated dose of EISO.

MTS assay

An MTS assay to assess cell viability after EISO treatment was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). HaCaT cells were plated in 96 well plates and cultured and treated with EISO as indicated in triplicate. At the end of the treatment period, DMEM containing EISO was removed and replaced with 100 μl fresh SFM and 20 μl MTS substrate and incubated at 37 °C for 30 min for color development. Absorbance was read at 490 nm and triplicate values averaged. Results representative of $n = 3$.

Apoptotic analysis

HaCaT keratinocytes were serum-starved for 24 h before pretreatment with EISO (0.0005% or 0.001% in DMSO) for 1 h prior UVB irradiation. Cells were then washed with PBS and irradiated with 250 J/m^2 of UVB. Then the cells were incubated with EISO (0.0005% or 0.001%) in serum-free DMEM media for an additional 6.5 h. Floating cells were collected and pooled with adherent cells removed with trypsin. Cells were spun briefly and cell pellets incubated with Alexa 488-conjugated anti-annexin V (AnnV) antibodies and propidium iodide for 30 min at room temperature in the dark. Labeled cells were counted by flow cytometry, collecting a total of 10,000 data points per treatment condition using a FACSCanto II device (BD Biosciences).