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Arecoline decreases interleukin-6 production and induces apoptosis and cell cycle arrest in human basal cell carcinoma cells

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ABSTRACT

Arecoline, the most abundant areca alkaloid, has been reported to decrease interleukin-6 (IL-6) levels in epithelial cancer cells. Since IL-6 overexpression contributes to the tumorigenic potency of basal cell carcinoma (BCC), this study was designed to investigate whether arecoline altered IL-6 expression and its downstream regulation of apoptosis and the cell cycle in cultured BCC-1/KMC cells. BCC-1/KMC cells and a human keratinocyte cell line, HaCaT, were treated with arecoline at concentrations ranging from 10 to 100 μg/ml, then IL-6 production and expression of apoptosis- and cell cycle progress-related factors were examined. After 24 h exposure, arecoline inhibited BCC-1/KMC cell growth and decreased IL-6 production in terms of mRNA expression and protein secretion, but had no effect on HaCaT cells. Analysis of DNA fragmentation and chromatin condensation showed that arecoline induced apoptosis of BCC-1/KMC cells in a dose-dependent manner, activated caspase-3, and decreased expression of the anti-apoptotic protein Bcl-2. In addition, arecoline induced progressive and sustained accumulation of BCC-1/KMC cells in G2/M phase as a result of reducing checkpoint Cdc2 activity by decreasing Cdc25C phosphatase levels and increasing p53 levels. Furthermore, subcutaneous injection of arecoline led to decreased BCC-1/KMC tumor growth in BALB/c mice by inducing apoptosis. This study demonstrates that arecoline has potential for preventing BCC tumorigenesis by reducing levels of the tumor cell survival factor IL-6, increasing levels of the tumor suppressor factor p53, and eliciting cell cycle arrest, followed by apoptosis.
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Introduction

Betel quid chewing, the fourth most popular oral habit in the world, is closely associated with a high incidence of oral leukoplakia, oral submucous fibrosis, and oral cancer. However, the mechanisms by which betel quid chewing induces oral cancer are not clear. Betel quid usually contains a piece of areca nut (AN), an inflorescence of *Piper betle*, and slaked lime, with or without *P. betle* leaves. AN contains many polyphenols and several alkaloids and many studies have demonstrated that an AN extract or its alkaloids are cytotoxic and genotoxic for several types of cells *in vitro* (Chang et al., 1998; Chou et al., 2008; Jeng et al., 2001). Arecoline, the most abundant alkaloid in AN, has been suggested as a possible carcinogen (Jeng et al., 1994, 2001, 2003). Studies have also shown that an AN extract, but not arecoline, induces keratinocyte inflammation, which can result in oral cancer (Chang et al., 2004; Jeng et al., 2003) and that arecoline can decrease interleukin-6 (IL-6) production in keratinocytes and KB epithelial cancer cells (Chang et al., 2004; Jeng et al., 2003). In addition, Chang et al. (2004) reported that arecoline elicits cell cycle deregulation in KB cancer cells. It therefore seems that arecoline is not the active ingredient in AN that causes oral cancer and that arecoline can interfere with the cell cycle progression of the cancer cell.

IL-6 is a potent, pleiotropic, inflammatory cytokine, which mediates a plethora of physiological functions, including the developmental differentiation of lymphocytes, cell proliferation, cell survival, and the amelioration of apoptotic signals (Croonquist et al., 2003; Horn et al., 2000). In addition, it has been shown to function as a growth factor in several human tumors, such as multiple myeloma, prostate cancer, and colorectal cancer (Kim et al., 2008; Vesely et al., 2007; Wegiel et al., 2008). Basal cell carcinoma (BCC) is one of the most commonly encountered neoplasms worldwide and is characterized as locally aggressive with little metastatic potential (Chu et al., 2007). Cultured BCC cells express IL-6, which plays a role in the pathogenesis of BCC (Gambichler et al., 2006; Yen et al., 1996). Moreover, overexpression of IL-6 increases the tumorigenic potency of BCC (Jee et al., 2001, 2004). The aim of the present study was to investigate whether

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arecoline decreased IL-6 expression in cultured BCC-1/KMC cells and to examine its effects on BCC cell proliferation and apoptosis.

Materials and methods

Reagents

Arecoline hydrobromide (methyl 1-methyl-1,2,5,6-tetrahydronicotinate hydrobromide), obtained from Sigma-Aldrich (St. Louis, MO, USA) at a purity greater than 99.0%, was dissolved as a stock solution in sterile double distilled water and diluted for use in serum-free medium. Recombinant human IL-6 was obtained from Peprotech (Rocky Hill, NJ, USA). Anti-human IL-6 receptor (IL-6R) neutralizing antibody was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). All other chemicals and reagents used were of analytical grade and, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell line and cell culture and growth evaluation

The stable BCC cell line BCC-1/KMC, established by Chiang et al. (1994), was maintained in RPMI-1640 medium with a moderate concentration of calcium (0.4 mM) (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Auckland, NZ) at 37 °C in a humidified 5% CO_2 incubator. The human keratinocyte cell line HaCaT was maintained in DMEM-F12 medium (Gibco BRL, Grand Island, NY, USA) containing 10% FBS at 37 °C in a humidified chamber in a 5% CO_2 incubator. To examine the effect of arecoline on cell growth, BCC-1/KMC or HaCaT cells were seeded at 5×10^4 or 1×10^5 cells/well on 24-well plates and grown overnight, then were treated with arecoline for 24 or 48 h, harvested, and viable cells counted using Trypan blue dye exclusion.

Quantitative real-time PCR analysis

Total RNA was isolated using TRIzol reagent according to the manufacturer's (Invitrogen, Carlsbad, CA, USA) instructions. RNA samples (2 μg) were reverse transcribed using random hexamer primers and M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) and the cDNA used for real-time PCR, performed on a Mini-Opticon™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to a published procedure (Cheng et al., 2010). Results were collected and analyzed using MJ Opticon Monitor Analysis software version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction mixture was amplified in triplicate and the results calculated based on the ΔΔCt method (Livak and Schmittgen, 2001). The cycle threshold (Ct) value for the IL-6 gene was corrected using the mean Ct value for the GAPDH gene. Relative gene expression was expressed as the fold change ($2^{-\Delta\Delta Ct}$) relative to expression in the untreated control.

Detection of IL-6 production

IL-6 released into the medium by cultured BCC-1/KMC cells was quantified using a human IL-6 ELISA kit from R&D Systems, Inc. (Minneapolis, MN, USA). BCC-1/KMC or HaCaT cells were cultured at 5×10^4 cells/well on 24-well plates in RPMI-1640 medium supplemented with 10% FBS in the presence or absence of arecoline for 24 h at 37 °C, then the culture medium was collected and centrifuged for 10 min at 5000×*g* 4 °C and the supernatants collected and stored at −80 °C for no more than one month before assay for IL-6.

Apoptosis analysis

DNA fragmentation analysis. After treatment of the cells or animals, genomic DNA was extracted from cultured cells or BALB/c mouse tumor tissue and analyzed for DNA fragmentation as described previously (Cheng et al., 2010). Tumor tissue or cultured cells were homogenized at 4 °C in lysis solution [10 mM Tris (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 0.5 mg/ml proteinase K] and incubated for 15 h at 50 °C, then nucleic acids were extracted by addition of an equal volume of phenol/chloroform/isoamyl alcohol, centrifugation for 20 min at 10000×*g* 4 °C, and harvesting the aqueous (top) layer. DNA was precipitated by addition of a 1/2 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol and centrifugation at 10000×*g* 4 °C for 5 min. After rinsing with 70% ethanol, the DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and residual RNA removed by addition of 10 μg/ml of RNase A and incubation at 60 °C for 1 h. Samples were resolved on a 1.5% Tris-acetate-EDTA-agarose gel, which was stained with ethidium bromide, and the bands visualized and photographed under short-wave UV.

Fluorescence microscopic evaluation of cell apoptosis. Apoptotic cells were detected using Hoechst 33342 staining and fluorescence microscopy. Cells were seeded at 5×10^4 per well in a 24-well plate, treated for 24 h with or without arecoline, washed twice with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde, then stained overnight in the dark at 4 °C with 10 μg/ml of Hoechst 33342 in the presence of 0.1% Triton X-100. Images were obtained at 200× or 400× magnification using a Zeiss Axiovert 200 fluorescence microscope and cells containing fragmented nuclei were designated as apoptotic.

TUNEL assay. Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assays were performed using an APO-BrdU™ TUNEL Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Briefly, the cells were incubated for the indicated time before being trypsinized, washed with PBS, fixed in 2% paraformaldehyde (pH 7.4) for 15 min, washed twice with PBS, and stored at −20 °C in 70% ethanol for 12–18 h prior to performing the TUNEL assay. After removal of the 70% ethanol by centrifugation, the cells were washed twice in wash buffer, then incubated at 37 °C for 60 min with DNA-labeling solution containing terminal deoxynucleotidyl transferase and BrdUTP. After two washes with rinse buffer, the cells were resuspended for 30 min in the dark at room temperature in a solution of Alexa Fluor® 488-labeled anti-BrdU antibody. Flow cytometric analysis was then performed using a Coulter Epics XL cytometer (Beckman Coulter, Miami, FL, USA) to quantify apoptosis. The data were analyzed using WINMDI software version 2.8 (Scripps Research Institute, La Jolla, CA, USA), a minimum of 1×10^4 cells per sample being evaluated in each case.

Detection of active caspase-3

Active caspase-3 was detected as described previously (Cheng et al., 2010). Briefly, cells were pelleted, resuspended in 1 ml of 4% paraformaldehyde, and incubated for 30 min at room temperature. The suspension was then centrifuged, the pellet washed twice with PBS, and the cells resuspended in 1 ml of 0.1% Triton X-100 and incubated for 30 min at room temperature, then washed as above. Labeling was performed by addition of 100 μl of PBS containing 5 μl of polyclonal RPE-conjugated rabbit anti-active caspase-3 antibodies (BD Pharmingen Inc., San Diego, CA, USA), incubation at 37 °C for 1 h, washing with PBS, and analysis on a Coulter Epics XL cytometer (Beckman Coulter, Miami, FL, USA). A control sample incubated with RPE-conjugated normal rabbit IgG (R&D Systems, Minneapolis, MN, USA) was run in parallel. The data were analyzed using WINMDI software

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