Ripe areca nut extract induces G₁ phase arrests and senescence-associated phenotypes in normal human oral keratinocyte

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Around 200–600 million Asians chew areca (also called betel), which contains a mixture of areca nut and other ingredients. Epidemiological evidences indicated that areca use is tightly linked to oral carcinogenesis. This study investigated the effects of ripe areca nut extract (ANE) on cultured normal human oral keratinocyte (NHOK). Acute subtoxic ANE treatment inhibited DNA synthesis and induced cell cycle arrest at G₁ phase in early passage (<4th passage) cells. This was accompanied by a slight increase in the sub-G₁ cellular fraction. O(6)-Methylguanine-DNA methyltransferase (MGMT), Hsp27 and p38MAPK was upregulated, p16 and p21 were remarkably upregulated early and declined afterwards. In contrast, the increase of dephosphorylated Rb seemed to be secondary to the episodes of p16 and p21 upregulation. To simulate the chronic areca exposure in vivo, constant ANE treatment in serial NHOK culture was performed. It resulted in a significant decrease in the population doubling, increase in senescence-associated β-galactosidase (SA-β-Gal) and decrease in cell proliferation in NHOK of late passages (≥4th passage). Induction of senescence-associated phenotypes, G₀/M accumulation and genomic instability following long-term ANE treatment were also observed in a low-grade oral carcinoma cell. ANE-treated NHOK also had a higher nuclear factor-κB (NF-κB) fraction and a lower cytotoxic IκBα level relative to the control in late passages. Moreover, electrophoretic mobility shift assay (EMSA) indicated that ANE treatment shifted the NF-κB complex from high mobility position to lower mobility position in late-passaged NHOK. ANE treatment also upregulated IL-6 and cyclooxygenase-2 (COX-2) mRNA expressions in late-passaged NHOK. In summary, our findings suggest that ANE induces the cell cycle arrest at G₁/S phase and the occurrence of senescence-associated phenotypes of NHOK. The upregulation of p38MAPK, p16, p21, NF-κB, IL-6 and COX-2 are likely to participate in the control of these impacts.

Introduction

Around 200–600 million people in South and Southeastern Asia are engaged in chewing areca (also called betel), which is a combination of an areca nut, a nut of tropic palm, and other ingredients (1,2). Areca chewing is epidemiologically related to the high incidence of oral squamous cell carcinoma (OSCC) and other lesions (3,4). OSCC has been the third most common malignancy in developing countries, consisting of ~50% of all malignancies in some nations of South Asia (1,2). Evidences also link areca use to the risk of esophageal (5) and hepatic carcinogenesis (6).

Areca nuts contain polyphenols, arecoline, arecaidine and other alkaloids (3,7). Previously, Sundqvist et al. (8) have shown that areca nut extract (ANE) is highly cytotoxic and genotoxic to cultured human oral epithelial cells. ANE was found to induce the formation of reactive oxygen species (ROS) in several types of cells (7,9,10). In addition, ANE can induce cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) production in OSCC cell lines or primary normal human oral keratinocyte (NHOK) (10,11). Recently, areca ingredients have been shown to induce the production of cytokine interleukin-6 (IL-6) by the underlying extracellular signal-regulated protein kinase (ERK) stimulation in a malignant keratinocyte (12). However, the impact of ANE on primary NHOK is still obscure.

When normal human cells gradually lose division and enter a state of permanent growth arrest, it is termed replicative senescence. Normal cells also exhibit premature senescence in cultures when treated with various treatments (13). Except for the absence of telomere erosion, premature senescence is similar to replicative senescence on many aspects (14). Premature senescence can be induced by a variety of stresses, such as DNA damage and aberrant mitogenic signaling (13). A previous report indicated that high dose of ANE could arrest malignant keratinocyte (9). Nuclear factor-κB (NF-κB) plays a central role in regulating genes in response to cellular stresses (15,16). In the inactive state, NF-κB is present in the cytoplasm as a heterotrimer consisting of p50, p65 and an inhibitory subunit of NF-κB (IκBα), κB subunits. In response to activation signals, the IκBα is phosphorylated, ubiquitinated and degraded, thus exposing the nuclear localization signals to p50 and p65. Activation of NF-κB was found to associate with senescence (17), growth arrest (18,19) and carcinogenesis (20,21) of keratinocyte. Our previous studies have shown that ANE treatment can elicit a rapid activation of ERK and c-Jun N-terminal kinase 1 (JNK1) mitogen-activated protein kinase

Abbreviations: ANE, areca nut extract; BrdU, 5-bromo-2'-deoxyuridine; COX, cyclooxygenase; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated protein kinase; IκBα, inhibitory subunit of NF-κB or subunits; IL-1α, interleukin-1α; IL-6, interleukin-6; MAFK, mitogen-activated protein kinase; MGMT, O(6)-methylguanine-DNA methyltransferase; NF-κB, nuclear factor κB; NHOK, normal human oral keratinocyte; OSCC, oral squamous cell carcinoma; PDL, population doubling level; SA-β-Gal, senescence-associated β-galactosidase; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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(MAPK) as well as NF-κB in malignant oral keratinocytes (16,22,23). Besides, ANE-modulated NF-κB activation could be the basis of COX-2 upregulation associated with areca exposure (16). In this study, chronic low-dose ANE treatment was imposed on NHOK to perceive the impacts. We identified that ANE-arrested NHOK in G1 phase and induced senescence-associated phenotypes in long-term serial culture. Moreover, upregulation of p16, degradation of IκBα and activation of NF-κB may be the likely causes.

Materials and methods

Cell culture and reagents

Sampling of gingival tissue from donors who received flap operation to remove the impacted third molar was approved by an institute review board. NHOK was dissociated from the tissue and grown in keratinocyte serum free medium (KSFM3, Invitrogen, MD) with low Ca2+ (0.1 mM) (24). In addition, cells were passaged when 70% confluence was reached to avoid the confluence-induced senescence. In KSFM, NHOK retained the feature of parabasal cells, which could be maintained for 5–7 passages (∼18–24 PD) before they reached senescence. OC3 cells, a low-grade areca-associated OSCC cell line established in our laboratory, having wild-type p53 sequence, minor genomic alterations and non-tumorigenic phenotype were cultivated with conditions used previously (23). The preparation of ANE from ripe areca nut was performed with the protocols established previously (7,10). In brief, ripe areca nuts were chopped, ground and immersed to obtain the soluble extract. This soluble fraction was lyophilized and stored at −20°C until use. NHOK and OC3 cells for serial culture experiments were trypsinized and passaged every 3 days. Cells in serial culture were subjected to ANE treatment for the whole period of time. 12-O-Tetradecanoylphorbol-13-acetate (TPA) and arecoline were purchased from Sigma (St Louise, MO). TPA was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma) as a stock. NHOK were treated with 30 ng/ml TPA for 1 or 6 h after the termination of ANE treatment.

Trypan blue exclusion assay and growth curve

NHOKs were seeded on 6-well plates at a concentration of 5 × 104 cells per well and cultured for 24 h. The assay was performed with the protocols we established previously (23). For serial cultures, the population doubling level (PDL) was calculated at each passage with the following equation: PDL = Ln(number of collected vital cells/number of plated cells)/Ln2.

5-bromo-2'-deoxyuridine (BrdU) incorporation assay

Around 5 × 105 cells were seeded on 8-well chamber slides and grown to ∼60% confluence. Following the treatment of ANE for 24 h, BrdU (BD Biosciences, Franklin Lakes, NJ, USA) was added to KSFM to get a final concentration of 10 μM, and the labeling procedure was performed for 2 h. BrdU incorporation was measured in control and ANE-treated cells following the protocols established previously (25). In each experiment, at least 1000 cells were counted and compared.

Senescence-associated β-galactosidase (SA-β-Gal) assays

SA-β-Gal assays were performed as described previously (26). In brief, cells were fixed in 2% formaldehyde/0.2% glutaraldehyde and incubated at 37°C in a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) for 24 h as described previously (26).

Combined SA-β-Gal and BrdU labeling assay

The combined SA-β-Gal and BrdU labeling assay was performed on NHOK and OC3 cells using a protocol modified from previous publication to determine the proportion of SA-β-Gal labeled and proliferating cells (27). Briefly, following the treatment of ANE for 24 h, BrdU was added to culture medium to get a final concentration of 10 μM, and the labeling procedure was performed for 2 h, then washed and stained for SA-β-Gal activity as described above. Consequently, slides were washed and stained with anti-BrdU-A using BrdU detection kit (BD Biosciences). Diaminobenzidine (DAB) was used as chromogen for detecting BrdU labeling. For each determination, at least 1000 cells were used to count the percentage of positive staining cells.

Flow cytometry

Nuclei of ANE-treated cells were collected and stained with propidium iodate, using the cycle TEST-PLUS DNA-staining kit, according to the manufacturer’s instructions (BD Biosciences). The DNA content of the stained nuclei was measured on a FACSCalibur flow cytometer, and results were analyzed using the CellQuest and ModFit LT2.0 softwares (BD Biosciences). The sub-G1 fraction was used as a measure of apoptotic percentage.

Western blot analysis

Cells were harvested and the cytoplasm and the nucleus were fractionated using NucBusterTM Protein Extraction Kit (Novagen, Darmstadt, Germany). Fifty micrograms of proteins from the whole cell lysate, cytosolic fraction or nuclear extract were resolved by electrophoresis on a 12.5% denaturing polyacrylamide gel. The subsequent procedures were performed following the standard protocols (23). The primary antibodies used include the following: anti-actin Ab (no. MAB1501; 1: 5000, Chemicon, Temecula, CA), anti-GAPDH Ab (no. MAB374; 1: 5000, Chemicon), anti-HO-1 Ab (no. SC-10789; 1: 500, Santa Cruz Biotech, Santa Cruz, CA), anti-Hsp27 Ab (MS-101; 1: 500, Lab Vision Fremont, CA), anti-Hsp70 Ab (no. 554243; 1: 1000, Lab Vision), anti-IκBα 1 Ab (c-21, no. SC-371; 1: 250, Santa Cruz Biotech.), anti-p21 Ab (EA10, no. OP64; 1: 250, Oncogene, Darmstadt, Germany), anti-p38 Ab (no. SC-7149; 1: 2000, Santa Cruz Biotech.), anti-phospho-p38 Ab (no. SC-7975-R; 1: 250, Santa Cruz Biotech.), anti-p53 Ab (DO7; 1: 1000, Novocastra, Newcastle, UK), anti-phospho-Ser15-p53 Ab (no. 9284; 1: 500, Cell Signaling, Beverly, MA), anti-Rad51 (no. 556453; 1: 1000, BD Biosciences), anti-Rb Ab (1F8 1, no. MS-107; 1: 250, Neo Markers, Fremont, CA), anti-MGMT Ab (no. 557045; 1: 1000, BD Biosciences), anti-MSH2 Ab (no. 556349; 1: 1000, BD Biosciences), anti-NF-κB p65 Ab (no. SC-109; 1: 500, Santa Cruz Biotech.), anti-NFkB p50 Ab (H-119, no. SC-7178; 1: 500, Santa Cruz Biotech.) and anti-XPA Ab (no. SC-28353; 1: 1000, Santa Cruz Biotech.). Horseradish peroxidase (HRP)-conjugated antimouse (Amersham, Piscataway, NJ) or anti-rabbit (Santa Cruz Biotech.) secondary antibody was used.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were subjected to EMSA analysis. EMSA was performed as described previously (16,23). Other than anti-p65 and p50, the antibodies used for the supershift analysis were anti-p52, anti-RelB (p68) and anti-cRel (p75) (polyclonal antibodies from Santa Cruz Biotech.). The radioactive bands from the dried gels were quantified by a phosphomager (Amersham).

RT–PCR

Total RNA purification, cDNA synthesis and RT–PCR reaction followed protocols established previously (24). The primer sequences will be provided upon request. Amplicons were resolved on a 2% agarose gel, visualized by an imaging system and quantified (Viber Lourmat, Marne La Valle, France). The signal of tested genes was normalized with the mRNA expression of GAPDH.

Statistics

Paired t-test and one-way ANOVA were used for statistical analysis. P < 0.05 was considered to have a statistically significant difference.

Results

ANE inhibits cell viability and proliferation

To investigate the effect of ANE on growth of oral keratinocytes, NHOKs in early passages (∼3rd passage) were exposed to 0, 1, 2.5, 5, 7.5 and 10 μg/ml ANE and harvested at 24, 48 and 72 h. There was a dose-dependent reduction in number of NHOKs following the ANE treatment at different time period (not shown). Treatment with ≥5 μg/ml ANE resulted in cytotoxicity. Therefore, treatments with 2.5 and 5 μg/ml ANE, which caused cytotoxicity and cytostasis, respectively, were used to evaluate the impacts of ANE on NHOKs. To test if abnormal proliferation is responsible for the reduced cell viability, BrdU was added to label cells in S phase. Exposure of NHOK cells to 2.5 and 5 μg/ml ANE for 24 h resulted in an inhibition of cell proliferation in a dose-dependent manner with an average of 20 and 43% reductions in BrdU-labeled cells relative to the control cells, respectively (not shown). The degrees of reduction in proliferation are similar to those in cell viability, suggesting that the inhibition of ANE on NHOK viability is mainly exerted by inhibiting the cell proliferation.
ANE induces G1 arrest and apoptosis

To examine how ANE affects the proliferation of NHOK, fluorescence-activated cell sorter (FACS) was performed to analyze the cell cycle status modulated by 0–5 μg/ml ANE treatment. At 24 h, a progressive decrease in S fraction in attached NHOK from ~20 to 11% was noted upon treatment with increasing dosage of ANE (Figure 1A). In contrast, the G1 fraction was increased from ~66 to 74%. No significant difference in G2/M phase was noted. The concomitant decrease in S fraction and increase in G1 phase were consistent with G1 arrest. The G1 arrest became more prominent with the ANE treatment at 48 (Figure 1B) and 72 h (Figure 1C). Trypan blue dye exclusion assay designated that all floating NHOK following ANE treatment were non-vital. The sub-G1 fraction of total NHOK was also evaluated by FACS. It showed a dose- and time-dependent increase in sub-G1 fraction in NHOK following ANE treatment (not shown). There were 1–3% and 5–7% NHOK that were apoptotic following 2.5 and 5 μg/ml ANE treatment, respectively. Apoptosis also underlies the reduction of cell viability following ANE treatment. In this study, there was 0.25 μM arecoline presenting in 10 μg/ml ANE preparation (16). NHOK treated with 0.25 or 40 μM (~160-fold) arecoline for 24 h did not cause the inhibition of proliferation or apoptosis.

ANE modulates repair enzyme expression and p38MAPK activation

ANE is an inducer for DNA damage (7) and cellular signals (16). The expression of representative repair enzymes including APE1 (for oxidative DNA damage repair), O(6)-methylguanine-DNA methyltransferase (MGMT; for direct reversal of base damage), MSH2 (for mismatch repair), Rad51 (for homologous recombination of strand break) and XPA (for nucleotide excision repair) (28); stress response proteins including Hsp27 and Hsp70 that are associated with tumorigenesis (29) as well as HO-1 (1); and signal transducers p38MAPK and ERK (16,30–32) was studied. The expression of MSH2 increased in the first 2–12 h. It was then suppressed in later period following 5 μg/ml ANE treatment (Figure 2A). A sustained increase of MGMT and activation of p38MAPK, reflected by phosphorylation of p38MAPK (pp38MAPK), in NHOK was found following 5 μg/ml ANE treatment (Figure 2B and C). However, ANE-elicited ERK activation occurred late at 24 h. Hsp27 increased within 24 or 48 h following treatment, whereas it was not the case for Hsp70 (Figure 2A and B). Slight increase of APE1 for 40% was noted in the first 2–6 h. With no change in expression level or absence of expression in control or ANE-treated NHOK being detected, the involvements of other proteins for pathogenetic changes have been excluded.

ANE affects expression of cell cycle regulators

To investigate the mechanism of G1 arrest induced by ANE, we analyzed changes in p53, p21(Waf1/Cip1/Sdi1/CAP20), p16(INK4a) and pRb, which regulate the process of G1-S transition (33). A pilot study showed no remarkable change in the expression level of aforementioned regulators in control NHOK at different time points. Figure 3A and C show the results of a representative western blot with 2.5 and 5 μg/ml ANE treatment, respectively. The expressions of p53, p21 and p16 were quantitatively normalized with actin. The dephosphorylated Rb fraction is indicated as the ratio of pRb versus total Rb (ppRb, Figure 3B and D). The most prominent change among all the tested proteins was observed in p16, which was upregulated by ~7–30-fold between 2 and 48 h. It was nearly decreased to the baseline level at 72 h. The 2.5 μg/ml ANE treatment only slightly upregulated p21 for ~2-fold between 2–48 h, whereas 5 μg/ml ANE persistently upregulated p21 by ~3–6 fold. There was a slight increase of p53 following the ANE treatment, not exceeding 2-fold. No apparent change in phosphorylation of p53 at Ser-15 was noted in the first 24 h. However, phosphorylated p53 (pp53) level decreased below the baseline at 72 h. We have observed the maintenance of dephosphorylated Rb fraction between 2 and 24 h. It was followed by a reduction of ppRb and an increase of dephosphorylated Rb fraction by ~2–5 fold at 48 and 72 h. This increase appeared secondary to the increase of p16 or p21 between 2 and 24 h. The reduction of pp53 and ppRb at 48 and 72 h could be very likely caused by the upregulation of unknown phosphatases during this period.

Fig. 1. Effect of ANE on arresting the cell cycle of NHOK at G1 phase. (A) 24 h, (B) 48 h and (C) 72 h. Cell cycle data in graphs represent the relative percentage of cells in G1 and G2/M (left axis) or S (right axis) phases in the cell cycle. Values are expressed as means ± SE from triplicate experiments. The figure shown is a representative experiment of three independent experiments.
ANE induced senescence-associated phenotypes

A previous study has shown the fibroblastoid morphological changes of NHOK induced by ANE (22). This was observed in our experiments, despite the fact that such cellular fraction is much lower since the treatment dosage of ANE was lower in this study. The serial culture of NHOK simulates the in vivo lifespan of oral keratinocytes (24). To examine whether ANE could reproduce a proliferation blockage similar to that of senescent cells, serial NHOK cultures were grown.

Phase-contrast microscopy was used to evaluate the changes associated with senescence, including enlargement, elongation and flattening in cell morphology, with cytoplasmic vacuoles and occasional multinuclei. The late-passaged control cells exhibited more senescence-associated morphologies than the early passaged control cells (not shown). In the same passage, ANE-treated NHOKs also exhibited more senescence-associated morphologies than the controls. We measured the growth curves of NHOK in the serial culture. NHOK reached

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**Fig. 2.** Western blot analysis of representative repair enzymes, stress response proteins and signal transducers in NHOK following ANE treatment. (A) and (B) Protein expression of MSH2, MGMT, Hsp27 and Hsp70 following 2.5 and 5 μg/ml ANE treatment for 0–72 h, respectively. (C) p38MAPK expression following 5 μg/ml ANE treatment for 0–72 h. Protein level was normalized with GAPDH or actin. Quantification values are shown below the illustration.

**Fig. 3.** Western blot analysis of cell cycle regulatory proteins p53, pp53, p16, p21 and Rb in NHOK, following ANE treatment. (A) and (C) Representative blots of NHOKs treated with 2.5 μg/ml and 5 μg/ml ANE, respectively, for 0–72 h. (B) and (D) Quantified protein level changes following 2.5 and 5 μg/ml ANE treatment, respectively. Bands for Rb are designated as ppRb, (hyperphosphorylated protein, top band) and pRb (hypophosphorylated protein, bottom band). Protein level was normalized with actin. Values are expressed as means ± SE from two experiments.
replicative senescence at PDL > 22, as indicated by the appearance of growth plateau. In comparison to the controls, ANE-treated NHOKs exhibited ~7 or ~12 reduction in PDL to reach replicative senescence, which was at Days 35 or 20, respectively (Figure 4A). The results suggested that ANE could modulate the senescence-associated phenotypes of NHOK. In addition, there was a significant increase in the SA-β-Gal staining cells in late-passaged NHOKs, but not the earlier ones. Treatment of ANE further increased the percentage of NHOK exhibiting SA-β-Gal staining (Figure 4B). The progressive increase of SA-β-Gal staining cells and decrease of BrdU labeling cells in control NHOK during serial passage and in NHOK following ANE treatment indicating the induction of senescence-associated phenotypes was confirmed by combined SA-β-Gal staining and decrease of BrdU labeling assay (Figure 5). In a representative NHOK with 2.5 μg/ml ANE treatment for five continuous passages, flow cytometry showed that the fraction of sub-G1, G1, S and G2/M phase was 2, 73, 12 and 12% in control cells, respectively, whereas it was 6, 76, 3 and 12%, respectively, in treated cells (not shown). In addition, morphological changes of cells also substantiated the occurrence of senescence-associated phenotypes. There was no NHOK exhibiting both SA-β-Gal staining and BrdU labeling indicating that cells positive for SA-β-Gal staining were not proliferating (Figure 5A).

**ANE induced senescence-associated phenotypes and aneuploidy in OC3 cells**

The progressive induction of SA-β-Gal activity was also observed in OC3 OSCC cell line treated with non-toxic dosages of ANE for 20 continuous passages (Figure 6A). The induction was particularly eminent following 20 μg/ml treatment for >10 passages. It was observed that the percentage of BrdU labeling proliferating cells decreased gradually in OC3 cells following 20 μg/ml ANE treatment (Figure 6B). Furthermore, OC3 cells treated with 2.5 and 5 μg/ml for 32 or 60 continuous passages exhibited a time- and dose-dependent increase in G2/M and hyperdiploid (>4N) cellular fractions (Figure 7). The induction of G2/M and aneuploidy was particularly eminent with 5 μg/ml ANE treatment for 60 passages (Figure 7F). In a representative NHOK with 2.5 μg/ml treatment for five continuous passages, 1% control cells and 2% treated cells exhibited hyperdiploidy. It is unlikely that ANE can induce aneuploidy in NHOK by such treatment owing to NHOK’s limited life span, which might render the insufficiency in treatment duration for induction of abnormalities.

**ANE affects cellular localization of NF-κB and expression of IκBα**

NF-κB has been shown as an important signaling element involved in the senescence of keratinocyte (17). To evaluate if the ANE-induced senescence-associated phenotypes are related to changes in p65, p50 and IκBα, western blot was performed on cytosolic fraction and nuclear extract of a serially cultivated NHOK following the 2.5 μg/ml ANE treatment (Figure 8A). Since p65 and p50 were the major NF-κB sub-units in NHOK (see Figure 9B for details) and their expression levels were fairly consistent, the summation of normalized p65 and p50 proteins may represent the protein expression of NF-κB. The protein changes in different passages were summarized in Figure 8B. The total expression of NF-κB protein, including that in nucleus and cytosol, was progressively but slightly increased in ANE-treated cells compared with the controls. However, there was a rapid increase in the level of nuclear NF-κB at Passage 2, followed by a reduction at Passage 3. The cytosolic IκBα was exactly opposite to the status of NF-κB in the early passed NHOK. This is interpreted as the degradation of cytosolic IκBα, conferring the nuclear NF-κB translocation at Passage 2 following the ANE treatment; and an auto-regulatory feedback of IκBα upregulation at Passage 3. In late passages, nuclear NF-κB was kept...
upregulated following the ANE treatment, whereas the cytosolic IκBα was downregulated and completely diminished at the terminal passage (Figure 8B).

ANE induces NF-κB activation
To test if the changes described above reflect activation of the NF-κB signaling pathway, activation of NF-κB by 2.5 μg/ml ANE treatment in serial passages was assayed by EMSA. Figure 9A shows the presence of two major EMSA complexes, which bound to the NF-κB probe in nuclear extracts of the control (lanes 2, 4, 6 and 8), but these complexes were not seen in the negative control (lane 1). Besides, the expression of the lower (L) complex was generally much stronger than the upper (U) complex. The NF-κB binding activity was slightly upregulated in Passages 2–5, following the ANE treatment as reflected by the summation of U complex and L complex (Figure 9C). However, the U complex was remarkably upregulated following the ANE treatment (Figure 9A, lanes 2–9; Figure 9C). In the late-passaged NHOK, the ratio of U : L complex was increased by ~4–5-fold (Figure 9C). The specificity of NF-κB binding activities was confirmed by the competitive inhibition with an excessive unlabeled probe (Figure 9A, lanes 10 and 11) and the supershift of NF-κB binding complex with anti-p65 and anti-p50 antibodies (Figure 9A, lanes 12 and 13). To identify the subunits responsible for ANE-modulated NF-κB activation, EMSA was performed after preincubating another treated NHOK (at Passage 4) with the whole panel of antibodies against NF-κB subunits (Figure 9B). Since nearly all NF-κB signals were supershifted by antibodies of p65 or p50, but not p52, p68 and p75, p65 and p50 were considered as the major NF-κB subunits of ANE-treated NHOK. The data supported the detection of p65 and p50 subunits by western blotting in the previous experiment (Figure 8). Although both U and L complexes
Fig. 7. Long-term effects of ANE on OC3 cells. Early passage OC3 cells were continuously treated with 2.5 or 5 μg/ml ANE for 32 or 60 passages. (A and B) Controls, no treatment; (C and D) 2.5 μg/ml ANE treatment; (E and F) 5 μg/ml ANE treatment. (A, C and E) 32 passages; (B, D and F) 60 passages. Progressive increase of G2/M and hyperdiploid (>4N) cellular fractions was noted following the increase of duration and ANE dosage for ANE treatment. Values are expressed as means ± SE from triplicate experiments. The figure shown is a representative experiment of three independent experiments.

Fig. 8. Western blotting of NF-κB and IκBα in serial NHOK culture. (A) NF-κB p65 and p50 and IκBα protein expression in the cytosolic fraction (CF) and nuclear extract (NE) treated with 2.5 μg/ml ANE for serial passages as indicated. Protein loading was normalized with GAPDH. (B) Quantification of ANE-mediated NF-κB activation. Total NF-κB represented the summation of cytosolic and nuclear NF-κB. Nuclear NF-κB represented nuclear p65 plus nuclear p50. There were two episodes of NF-κB activation, early passage and late passage, and there was refractory occurrence of IκBα upregulation during early passage. P, passage.
were composed of p65 and p50 subunits, U complex might be defective in transcriptional activation because IkBa was not induced in these cells (Figure 8).

Regulation of IL-6, COX-2 and c-fos in ANE-treated NHOK

IL-6 and COX-2 are known to be induced by ANE. Interleukin-1α (IL-1α) is an important cytokine secreted by keratinocytes (34,35). Proto-oncogene c-fos expression is frequently upregulated in response to various stimuli, and c-myc expression is high in proliferating cells. In addition, IL-6, COX-2 and IL-1α were important for tumorigenesis and were NF-kB-inducible in keratinocyte (16,36,37). Therefore, we studied the potential modulation of IL-6, COX-2, IL-1α, c-myc and c-fos in serial passage of NHOK. Figure 10A shows a representative mRNA expression in a serially cultured NHOK following constant 2.5 μg/ml ANE treatment. Figure 10B illustrates the mRNA expression profile of theses genes in two serial cultures of NHOK. There was a gradual decrease in the IL-6 and COX-2 mRNA expressions in serial cultures of NHOK. However, ANE mediated the upregulation in their mRNA expressions in comparison with controls. The upregulation became rather prominent in the late passages. There was no change in IL-1α mRNA expression level. A progressive increase in c-fos mRNA expression was observed in controls; it was downregulated by ANE treatment in late-passaged NHOK. A remarkable downregulation in c-myc mRNA expression was noted in ANE-treated NHOK at the terminal passage.

TPA is a well-known tumor promoter; it evokes higher increases in IL-6 and c-fos expressions of photoaged normal human epidermal keratinocyte than those that are younger (35). We observed that, under the process of senescence, the late-passaged control or ANE-treated NHOK still maintained the ability for TPA induction, as indicated by the regulation of mRNA expressions of IL-6, COX-2 and c-fos (Figure 11). The data suggested that the occurrence of ANE-mediated senescence-associated process on NHOK could maintain the ability on promoter induction.

Discussion

Chronic areca chewing has been strongly linked to oral pathogenesis. Evidences indicated that ANE was a genotoxic substance (7–9,24). A low concentration of ANE was used in this study to dissect the phenotypic impacts on target NHOKs. We identified that ANE could mediate the inhibition of cell growth. Furthermore, G1 arrest seems to be underlying such cytoinhibition. In cells, G1 phase of the cell cycle is responsible for...
growth factor-dependent signals (33). Dysregulation of G1 phase is advantageous to tumorigenic cell growth; as such, G1 regulation is frequently disrupted in cancer through the impairments of regulators. Cultivated NHOK maintained proliferative characteristics of parabasal cells and had a minimal p16 expression. Kang et al. (38) have demonstrated that the onset of limited NHOK growth was accompanied with a remarkable induction of p16. p16 is an inhibitor of Cdk4/6, the cyclin D kinases that initiate the phosphorylation of the Rb, and arrests cells in G1 phase. We identified the rapid and drastic p16 upregulation following the ANE treatment. Since this upregulation occurred early and sustained through 48 h, it is probably a major reason for the G1 arrest. The mechanisms by which the cellular responses are triggered are not understood. It is postulated that ROS elicited by ANE could be an important trigger (7).

p21 is involved in the assembly of cyclin D–Cdk4/6 and plays a key role in cell cycle arrest at the G1 checkpoint in response to DNA damages. Following an ANE treatment for cytostasis, p21 is rapidly and sustainedly upregulated other than p16. Thereby, the upregulation of p21 could also be an important element underlying the G1 arrest. DNA damages can activate p53 to protect cells by inducing target genes. The molecules activated by p53 induce apoptosis, cell arrest and DNA repair to conserve the genome (33). However, the activation of p53, reflected by the amount of protein or the phosphorylation level of Ser-15, was not a case of ANE treatment. Therefore, ANE damage may bypass the surveillances of p53 and undergo genetic pathogenesis predisposing tumorigenesis. Our data also suggested that the activation of p21 by ANE is p53-independent. Cyclin D–Cdk4/6 phosphorylates Rb, which drives the cell cycle through late G1 phase. The late increase of dephosphorylated Rb at 48 h could be secondary to the p16 or p21 upregulation prior to 48 h. Our study suggested that p16 and p21 are important in modulating cell arrest for the protection of areca-mediated cell damage in NHOK. The frequent impairments of such genes in areca-associated OSCC might substantiate the fact that keratinocyte lacking such genomic protection may confer tumorigenic propensity (39, 40). For cytostatic ANE treatment (5 μg/ml at 24 h), >40% NHOK was arrested in the proliferation stage, whereas only ~5% NHOK cells underwent apoptosis. This could be derived from the consequent effects of extensive cell arrest or mild cytotoxicity exerted by ANE (7, 41).

In response to DNA damages, activation of repair or stress response systems might trigger cell cycle arrest or apoptosis (28). Previous study has specified that the oxidative DNA damage resulted from ANE (7). In NHOK, a slight increase in APE1 expression for a short period that might respond to oxidative DNA damage was found following ANE treatment. Moreover, more sustained increase of MGMT, a repair enzyme for removal of alkylating DNA adduct, in response to higher dose ANE was noted. Whether this implicates that ANE ingredients might also induce alkylation lesion deserves further characterization. Interestingly, MSH2 expression increased during the first 12 h of treatment, then remarkably decreased at 48 h and 72 h. Deficiency in MSH2 is prone to mutator phenotypes and genesis of colorectal carcinoma (42). It is possible that a higher dose and longer duration of ANE exposure could trigger more prominent MSH2 suppression that warrants a susceptibility of oral carcinogenesis. Implication of Hsp27 in tumor progression and resistance to chemotherapy...
further substantiates the fact that the upregulation of Hsp27 evoked by ANE could be beneficial for neoplastic progression (29). Unlike the vast majority of MAPks that elicit cell survival, p38MAPK has been identified for negative regulation of cell survival and growth (43). Such process implies the activation of p53, and stabilizes of p21 or p16 that leads to cell cycle arrest or premature senescence (30–32). We identified a rather high and sustained activation of p38MAPK in NHOK following ANE treatment, which could be a potential element underlying ANE-induced cell cycle arrest and activation of negative regulators.

NHOK enters the terminal proliferation arrest state of senescence following ∼20–24 PDL in the defined medium (38). We pursued the pathological role of ANE in the irreversible growth of NHOK in serial culture and identified the increase in SA-β-Gal level and decrease in BrdU labeling of ANE-treated NHOK. Besides, ANE treatment reduced the PDL of NHOK in a dose-dependent manner. The effects of ANE for chronic exposure in inducing senescence-associated phenotypes were also observed in OC3 cells, a cell with unlimited lifespan. Intriguingly, with low-dose ANE treatment for long duration, OC3 cells gradually exhibited accumulation of G2/M fraction and prominent genetic instability reflected by the remarkable genesis of hyperdiploid cells. The process simulates the conversion from a low-grade malignancy to a high-grade malignancy. It is not known whether aneuploid cells arose from cells resistant to ANE exposure or emerged from senescent population. For the increase in G2/M fraction, further dissection to elucidate a G2 arrest or increase of mitosis is necessary. Since the increase of G2/M and generation of aneuploidy occurred concordantly, we postulate that long-term ANE treatment may impair G2/M check, leaving unrepaired cells for mitosis that finally resulted in mitotic abnormalities or aneuploidy (44). For this instance, our findings might propose important evidences supporting the roles of ANE in oral carcinogenesis. Alternatively, evidences should be provided to highlight the fact that the hyperdiploidy being detected represents the genesis of binuclear OC3 cells during the senescence induced by ANE.

Recent evidences have indicated that NF-κB was involved in senescence of keratinocyte (17). We found that activation of NF-κB followed the occurrence of senescence-associated process induced by ANE. NF-κB activation can induce the reciprocal upregulation of IκBα, and the subsequent reduction in the NF-κB activity (36). Such regulation is similar to findings in SAS OSCC cells treated with 5 μg/ml ANE (16). The molecular mechanisms of such regulation deserve further study. We observed that early passaged NHOK maintained such homeostasis even following the ANE treatment. However, late-passaged NHOK seemed to have poor maintenance of such homeostasis. The findings might suggest that, following ANE treatment, late-passaged NHOK may enter a state.

Fig. 11. IL-6, COX-2 and c-fos mRNA expressions in ANE-mediated premature senescence in response to TPA stimulation. (A) NHOK at P2–P7 treated with TPA for 1 or 6 h resulted in increases in IL-6, COX-2 and c-fos mRNA expressions. NHOK with 2.5 μg/ml ANE treatment resulted in upregulation of IL-6 and COX-2 and downregulation of c-fos. ANE-treated NHOK followed by TPA treatment enhanced ANE-mediated IL-6 and COX-2 upregulation, as well as the reversal of c-fos downregulation. (B) and (C) Quantification of the TPA-induced mRNA expression in ANE-treated NHOK relative to controls. (B) 1 h TPA treatment. (C) 6 h TPA treatment. P, passage.
of worse homeostasis that is prone to regulatory imbalance and disease susceptibility. EMSA analysis revealed that late-passaged NHOK had a higher proportion of NF-kB complex in the upper mobility position following the ANE treatment. Our studies showed that NF-kB in nuclear extract was not degraded and therefore excluded the possibility of forming degraded NF-kB complex in lower mobility position (detailed western blot analysis not shown). Some nuclear proteins that bind to NF-kB can alter the activity of NF-kB and affect its EMSA pattern (45). It is also possible that the protein bindings mediated by ANE disrupted the transcription activity of NF-kB, which might inhibit the reciprocal upregulation of IkBα. The significance of complex shifts in premature senescence needs to be clarified. NF-kB is involved in multiple phenotypes in addition to the senescence of keratinocyte (17–21). Thereby, whether it plays an oncogenic or anti-oncogenic role in keratinocyte is still controversial. Our study provided scenarios that ANE, a carcinogenic substance that activates NF-kB through the degradation of IkBα, could be associated with the premature senescence of target NHOK. Activation of NF-kB has been implicated in the development and the progression of OSCC (20,21). Strategies that mediate NF-kB abrogation may reveal its potential against areca-associated tumorigenesis.

NF-kB is involved in the regulation of multiple target genes, which include IL-6, COX-2, IL-1α, MMP9, cyclin D1, and so on (36,37). COX-2 is frequently overexpressed in areca-associated OSCC (10). Its overexpression in neoplastic cells has been shown to enhance malignant phenotypes. IL-6 has been linked with the migration of keratinocyte (46) and the increase of growth and invasion in OSCC (47). Suppression of IL-6 renders the chemosensitivity and radiosensitivity of OSCC cells (48). COX-2 and IL-6 can be upregulated by areca ingredients (10–12,49). However, the induction of c-fos in oral keratinocyte following the treatment of areca ingredients has been controversial (12,50). We identified the downregulation of COX-2 and IL-6 and upregulation of c-fos in the replicative senescence process of NHOK. In contrast, the reversal regulation of these genes was observed in ANE-treated NHOK. Such discrepancies were particularly prominent in late-passaged NHOK. Our data confirmed that ANE can mediate the upregulation of COX-2 and IL-6, and implicated that oral keratinocytes following chronic exposure of ANE is advantageous for neoplastic phenotypes possibly by the upregulation of COX-2 and IL-6. The finding of c-fos down-regulation is consistent with the remarkable decrease in cell proliferation following ANE treatment. Moreover, tumor promoter TPA can increase the mRNA expressions of IL-6, COX-2 and c-fos in NHOK underlying ANE treatment. It further suggests that ANE-treated NHOK may retain the responses to certain carcinogenic insults (35).

ANE has pleiotropic effects on many biological systems. Our findings implied for the first time that p38MAPK, p16, p21 and NF-kB were activated by areca in NHOK, which could be linked to senescence-associated phenotypes induced by ANE. The mechanisms directing the activation need to be elucidated. The findings in ANE-mediated COX-2 and IL-6 upregulation in serial NHOK culture could substantiate the potential of areca-exposed oral mucosa on neoplastic transformation. Anti-NF-kB strategy might be advantageous in the prevention or treatment of areca-associated neoplastic or senescent processes (15).


Received March 25, 2005; revised January 19, 2006; accepted January 27, 2006.