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Areca nut extract represses migration and differentiation while activating matrix metalloproteinase-9 of normal gingival epithelial cells

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Tseng Y-H, Chang K-W, Liu C-J, Lin C-Y, Yang S-C, Lin S-C. Areca nut extract represses migration and differentiation while activating matrix metalloproteinase-9 of normal gingival epithelial cells. J Periodont Res 2008; 43: 490–499.

Background and Objective: Areca (betel) chewing is associated with an increase in the incidence of periodontal diseases. Aberrations in matrix metalloproteinase (MMP) expression have been reported to be associated with periodontal disease. This study investigated the effects of areca nut extract on MMP activity and the phenotype of human gingival epithelial cells.

Material and Methods: Reverse transcription-polymerase chain reaction, western blotting and gelatin zymography were used to assay MMPs. Cell viability, mobility and detachment assays were performed to characterize the phenotypic impact. Confocal microscopy was employed to evaluate cell aggregation and the distribution of E-cadherin and F-actin.

Results: Treatment of gingival epithelial cells with 10 μg/mL of areca nut extract reduced its cell viability. Treatment with 5 and 10 μg/mL of areca nut extract for 24 h activated MMP-9 but not MMP-2 in gingival epithelial cells. This activation could be nuclear factor-κB dependent and was abrogated by 10 μM curcumin. Areca nut extract also reduced the migration and detachment of gingival epithelial cells. The differentiated cell–cell contact of gingival epithelial cells was markedly impaired by areca nut extract. This was accompanied by a disruption of distribution of E-cadherin and F-actin.

Conclusion: The areca nut extract-mediated activation of MMP-9 in gingival epithelial cells could signify a potential periodontal pathogenesis in areca chewers. The areca nut extract-mediated inhibition of cell viability and migration, together with the changed aggregation in gingival epithelial cells, suggests that impairment of the re-epithelization underlies the process and this, in turn, might exacerbate gingival inflammation.

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Around 200–400 million people in south Asian and south-east Asian countries are addicted to areca (1,2). Areca-associated oral squamous cell carcinoma is one of the leading cancers in these regions (1,3). It is also known that areca chewers have a higher prevalence of periodontal disease than nonareca chewers (4). In addition, areca chewers have a significantly higher risk of chronic periodontitis compared with control subjects who have never chewed areca quid (5).

Areca nuts contain polyphenols, arecoline, arecaidine and other alkaloids. Areca nut has been defined by the International Agency for Cancer Research (IARC) as a group I carcinogen, but there is only limited evidence for the carcinogenicity of any specific areca nut ingredient (6). Areca nut extract enhances the production of cyclooxygenase-2 and cytokines in human gingival epithelial cells (7) and may be associated with inflammation. Our previous studies have shown that areca nut extract elicits a rapid activation of extracellular signal-regulated kinase (ERK), mitogen-activated protein kinases (MAPKs), phosphomositide 3-kinase (PI3K)/activated human kinase (AKT) and nuclear factor-κB (NF-κB) in cultured human oral epithelial cells (1,3,8,9). Furthermore, areca nut extract-modulated NF-κB activation has been suggested as the basis of the cyclooxygenase-2 up-regulation that is associated with areca exposure (1). Recently, we have demonstrated that treatment with low-dose areca nut extract is able to induce a senescence-associated phenotype and to down-regulate involucrin in normal oral epithelial cells (3,8).

Matrix metalloproteinase 9 (MMP-9, gelatinase B) belongs to the MMP family, a set of zinc-dependent endopeptidases that are capable of degrading extracellular matrix. These endopeptidases play important roles in cell migration, tissue remodeling and in the pathogenic process of oral diseases (10,11). Research has demonstrated that the major human collagenase involved in tissue destruction in periodontitis is MMP-8 (collagenase-2) in conjunction with MMP-9 and other factors (10,12). In response to periodontal infection, MMP-9 activation has been demonstrated in the gingival epithelium (13). Increased release of MMP-8 and MMP-9 in gingival pockets, as well as of MMP-9 in plasma, has been identified in patients with periodontal disease (14). Reports have also shown that there is an association between functional *MMP-9* promoter polymorphisms and the severity of periodontitis (15,16). Interestingly, an increase in MMP-2 (gelatinase A) and MMP-9 has been noted in the saliva of areca chewers (17,18).

When cultured in a low-calcium medium, keratinocytes grow as a monolayer of polygonal cells and can be cultured for six or seven passages (3). Conversely, when a higher concentration of calcium is used, the growth of keratinocytes is arrested and the cells differentiate with a squamous morphology and aggregate into organoid colonies with intense cell–cell contact (19). The presence of links between E-cadherin and the actin cytoskeleton assure a strong and rigid adhesion between the differentiated keratinocytes (19). Down-regulation of E-cadherin in this experimental model is usually linked to a loss of differentiation. After gingival injury, re-epithelization occurs and this requires the coordination of the complex machinery that regulates the epithelial cells and allows them to migrate in order to cover the wound area; they then proliferate and differentiate to form a multilayered epithelium (20–22).

NF-κB is a transcription factor that has been linked to a number of inflammatory diseases, cancers and other diseases (23). The NF-κB family members consist of p65, p50 and three other members. In most unstimulated cells, NF-κB is complexed with an inhibitor of NF-κB (IκB), such as IκBα, in the cytosol. In response to cytokine or growth factor signaling, IκBα is phosphorylated and degraded; this allows nuclear translocation and the binding of p50/p65 to the promoter region of target genes. Curcumin is a major polyphenolic component of dietary yellow curry turmeric and it seems to have anti-inflammatory or anticancer effects (24). Curcumin is also able to interfere with the activity of NF-κB (25). The ratio of receptor activator of NF-κB ligand (RANKL) to the RANKL decoy receptor osteoprotegerin in gingival crevicular fluid can be used as an indicator of periodontal disease (26).

In view of the anatomy, the gingival epithelium is the first line of defence against oral bacteria. Aberrances in gingival epithelial cell (GES), particularly those of the junctional epithelium, could be highly associated with the invasion of periodontal pathogens and the development of periodontal diseases (27). Conversion of junctional epithelium to pocket epithelium was considered to be a hallmark of the progression from gingivitis to periodontitis (28). In this study, we identified that activation of MMP-9 was induced by areca nut extract in gingival epithelial cells and this was found to be NF-κB dependent. In addition, areca nut extract was able to inhibit the cell viability, migration and aggregation of gingival epithelial cells.

Material and methods

Cell culture and areca nut extract treatment

Sampling of gingival tissue from donors who received flap surgery to remove an impacted third molar was approved by the Institute Review Board. Gingival epithelial cells were dissociated from the tissue and grown in keratinocyte serum-free medium (KSFM®; Invitrogen, Carlsbad, CA, USA) in the presence of 0.1 mM Ca^{2+} (8). In addition, gingival epithelial cells were passaged upon reaching 70% confluence to avoid confluence-induced differentiation. Unless specified, gingival epithelial cells at passage three were used for all experiments.

OC3 is a low-grade oral cancer cell line established in our laboratory (29). It was derived from an areca chewer and was grown in a medium composed of Dulbecco's modified Eagle's minimal essential medium (Invitrogen) containing 10% fetal bovine serum and KSFM® (Invitrogen) in a 1 : 2 ratio. The OECM-1 cell line was a generous gift from Dr C-L Meng (National Defense Medical College, Taipei, Taiwan). It is an oral cancer cell line of intermediate grade. OECM-1 cells were

cultured in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum (1). SAS is a high-grade oral cancer cell line obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). It was cultured in Dulbecco's modified Eagle's minimal essential medium (Invitrogen) containing 10% fetal bovine serum and 1% L-glutamine (1). These oral epithelial cell lines were used to confirm the results of the gingival epithelial cell study. Preparation of areca nut extract from ripe areca nuts was performed as described in previously established protocols (1,3). Curcumin and dimethyl sulfoxide for dissolving curcumin were purchased from Sigma (St Louis, MO, USA).

Immunofluoresence and confocal microscopy examination

Cells grown on chamber slides were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min and permeabilized with 0.5% Triton X-100/fixative. The cells were then incubated for 4 h at 25°C with antibody to E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA), Hoechst 33258 (Invitrogen) for nuclear labeling and Rhodamine-Phalloidine (Invitrogen) for F-actin labeling (Table 1). Incubation with the E-cadherin primary antibody was followed by incubation with a 1 : 500 dilution of Alexa 488-conjugated secondary antibodies (Invitrogen) for 45 min at 25°C. Immunofluoresence was photographed on a Leica TCS NT confocal laser scanning microscope (Leica, Heidelberg, Germany) or on an Axioscope fluorescence microscope (Zeiss, Hamburg, Germany).

Trypan blue exclusion assay and growth curve

Cells were seeded on six-well plates at a concentration of 8×10^4 cells per well and cultured for 24 and 48 h. The Trypan blue exclusion assay was performed using our previously established protocols (3).

In vitro scratch wound assay

Gingival epithelial cells were cultured to 70% confluence and OECM-1 cells were cultured to 100% confluence. The cell layer on the plates was scratch wounded with a 200-μL pipette tip. The wound areas were marked with a graticule at the start time and photographed periodically until closure of the wound. Experimental groups were compared with the control groups using an inverted microscope (Nikon, Tokyo, Japan).

Table 1. Primary antibodies and fluorescent probes used in the present study

Name	Dilution	Provider
E-cadherin	1 : 500	BD Biosciences, Franklin Lakes, NJ, USA
GAPDH	1 : 20,000	Chemicon, Temecula, CA, USA
Hoechst 33258	1 : 1000	Invitrogen, Carlsbad, CA, USA
IκB	1 : 500	Santa Cruz Biotech. Santa Cruz, CA, USA
MMP-9	1 : 1000	Chemicon
Nuclear factor-κB p65	1 : 500	Santa Cruz Biotech
Nuclear factorκB p50	1 : 500	Santa Cruz Biotech
Rhodamine-Phalloidine	1 : 200	Invitrogen

Cell detachment assays

Cells were plated on 0.5 μg/mL of collagen I (BD Biosciences)-coated T25 flasks and cultured for 24 h. The cells were treated with various doses of areca nut extract for 24 h. The cells were then washed three times with 1× Hank's balanced salt solution. Next, they were treated with 0.125 mg/mL of trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) at 25°C at a constant 75 r.p.m. shaking on an orbital shaker (Kansin Instruments, Taipei, Taiwan). The detached cells were collected every 5 min up to 40 min. Cell counting was performed using a cell counter (Metertech, Taipei, Taiwan).

Reverse transcription-polymerase chain reaction

Total RNA purification, cDNA synthesis and the reverse transcription-polymerase chain (RT-PCR) reaction were performed following previously established protocols (3). The primer sequences, annealing temperature and the size of the PCR products are shown in Table 2. PCR products were resolved on a 2% agarose gel and then visualized and quantified by an imaging system (Viber Lourmat, Marne La Valle, France). The signals for *MMP-9* mRNA expression were normalized against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression.

Gelatin zymograph

Gelatin zymography was carried out using modifications of previously described protocols (30). Conditioned medium was mixed with sodium dodecyl sulfate sample buffer without heating or reduction to preserve enzyme activity. The samples were electrophoresed in 10% polyacrylamide gels copolymerized with 0.1% gelatin. After electrophoresis, the sodium dodecyl sulfate in gels was removed and the MMPs were renatured. The gels were then incubated with $CaCl_2$ and $ZnCl_2$, and subsequently stained with 0.1% Coomassie Blue. Clear areas that showed up on the blue background after destaining represented gelatinolytic activity.

Table 2. Primers and polymerase chain reaction conditions used in the present study

Name	Sequence (5′ – 3′)	Size (bp)	Cycle no.	Annealing temp. (°C)
G3PDH-S	TGGTATCGTGGAAGGACTCATGAC	189	20	55
G3PDH-A	ATGCCAGTGAGCTTCCCGTTCAGC			
MMP-9-S	GAGGTTCGACGTGAAGGCGCAGATG	176	32	62
MMP-9-A	CATAGGTCACGTAGCCCACTTGGTC			

A, antisense; S, sense.

In humans, the genes for gelatinase are *MMP-2* and *MMP-9*. Gelatin zymography distinguished *MMP-2* and *MMP-9* activities upon their molecular size.

Western blotting

Protein (50 μg) from the whole-cell lysate or cytosolic fraction was resolved by electrophoresis on a 10% denaturing polyacrylamide gel. The subsequent procedures were performed following previously established standard protocols (1). The primary antibodies against E-cadherin, IκB, GAPDH and MMP-9 are shown in Table 1. The secondary antibodies used in this study were conjugated to horseradish peroxidase (Chemicon). The signals were detected using the Western Lightening chemiluminescence reagent plus kit (Perkin-Elmer, Wellesley, MA, USA). The densities of the signals were measured by a densitometer (Amersham, Piscataway, NJ, USA). Quantification of the signals was achieved by normalization against GAPDH.

Electrophoretic mobility shift assay

Nuclear extracts were subjected to electrophoretic mobility shift assay analysis. The electrophoretic mobility shift assay was performed as described previously (1). Antibodies to p65 or to p50 (Table 1) were used for the supershift analysis. The radioactive bands from the dried gels were quantified by phosphoimager (Amersham).

Statistics

An unpaired *t*-test was used for the statistical analysis. The results were considered to be statistically different when the *p*-value was < 0.05.

Results

Areca nut extract treatment at the 5 μg/mL level was able to elicit morphological changes whereby the original parabasaloid cell shape was changed into an elongated or a spindle shape in ≈30% of the cultured gingival epithelial cells (Fig. 1A) The original F-actin distribution in cellular cortex and

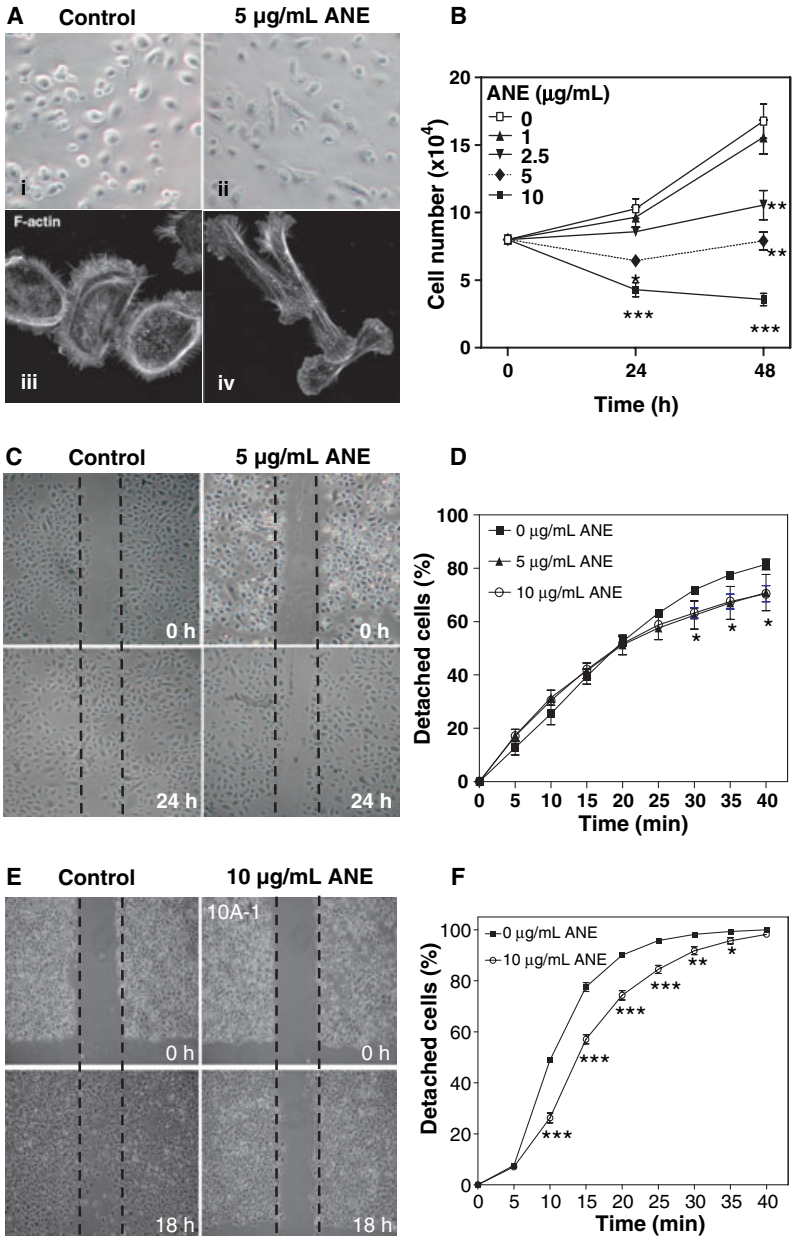

Fig. 1. Areca nut extract treatment reduces cell viability and migration. (A) Gingival epithelial cells after treatment with 5 μg/mL of areca nut extract for 24 h exhibited an increase in the cell population and elongated cell morphology. Upper panels, inverted microscopy. Lower panels, cells labelled with Rhodamine-Phalloidine and visualized with fluorescence confocal microscopy. Note the changes in morphology (i, ii) and F-actin distribution (iii, iv) of the gingival epithelial cells. (B) The survival curve of gingival epithelial cells following treatment with areca nut extract, shown by Trypan Blue exclusion. (C and E) Scratch wound assay for gingival epithelial cells and OECM-1 migration, respectively. Control gingival epithelial cells and OECM-1 migrate to nearly occupy the wound area at 24 and 18 h, respectively. Gingival epithelial cells and OECM-1, after treatment with 5 and 10 μg/mL of areca nut extract, respectively, show a significant reduction in migration. (D and F) Detachment curve of gingival epithelial cells and OECM-1, respectively. Following mild trypsinization, cells after treatment with 5 and 10 μg/mL of areca nut extract show a significant reduction in detachment compared with the controls over the assay period. Magnification of pictures, ×100. The values are expressed as means ± standard error from triplicate analyses. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. ANE, areca nut extract.

fillopodia was diminished. Lamellopodia and stress fibers were formed in the treated cells (Fig. 1A). In addition, treatment of gingival epithelial cells at passage 3 with 1–10 μg/mL of areca nut extract caused a dose-dependent reduction in cell viability. The reduction was more apparent at 48 h than at 24 h (Fig. 1B). OC3, OECM-1 and SAS oral epithelial cells with 20, 10 and 5 μg/mL of areca nut extract treatment for 24 h, respectively, exhibited viability similar to that of gingival epithelial cells treated with 5 μg/mL of areca nut extract (data not shown). Scratch wound healing assays were used to evaluate cellular migration, and the results indicated that the migration of both gingival epithelial cells and OECM-1 towards the wounded area was remarkably reduced after treatment with 5 and 10 μg/mL of areca nut extract, respectively. At 24 and 18 h after wounding, respectively, untreated gingival epithelial cells and OECM-1 cells had migrated and filled the wound space. However, only a limited number of gingival epithelial cells had migrated into the wound space in the same time period following areca nut extract treatment (Fig. 1C) and it was estimated that the rate was about 25% relative to the control gingival epithelial cells. The reduction in OECM-1 was even more obvious and very few OECM-1 cells had migrated into the wound area over the same time period following areca nut extract treatment (Fig. 1E). To resolve possible mechanisms for this retardation, detachment assays were performed. Detached cells in the culture media following mild trypsinization were collected continuously, every 5 min. Areca nut extract-treated gingival epithelial cells showed a significant reduction in detachment starting at 20 min and continuing until the end-point of the experiment (Fig. 1D). Areca nut extract-treated OECM-1 cells also showed a significant reduction in detachment and this occurred over the whole assay period (Fig. 1F). These results imply that areca nut extract-treated gingival epithelial cells or OECM-1 cells seem to exhibit a lower potential to migrate as a result of tighter cell-matrix attachment.

Gingival epithelial cells were treated with 1 mM Ca^{2+} to induce differentiation. Immunofluorescence showed that following Ca^{2+} induction for 24 h, representative gingival epithelial cells aggregated to form organoid colonies (Fig. 2Aii). Specifically, the cells differentiated with a loss of the dispersed distribution found in the controls (Fig. 2Ai). Concomitant are-

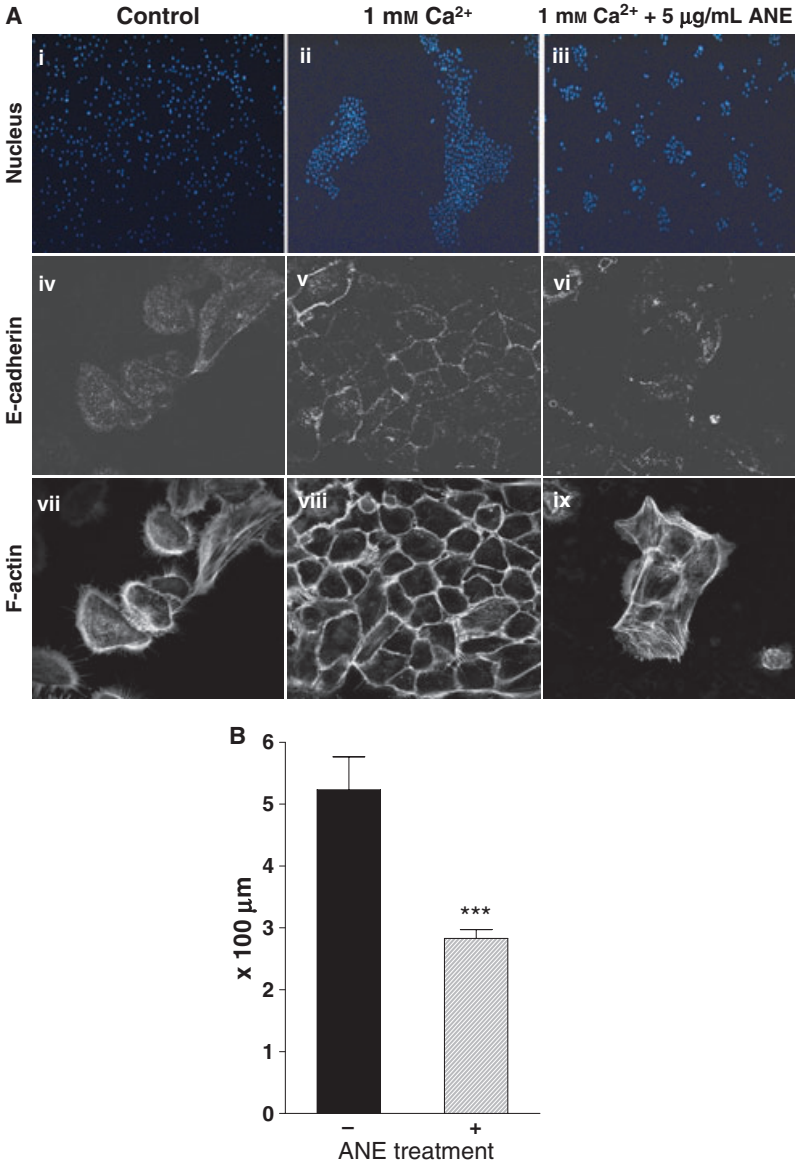

Fig. 2. Areca nut extract treatment disrupts differentiated aggregation and changes the location of E-cadherin and F-actin in gingival epithelial cells. (A) Control monolayer gingival epithelial cells with labeling of the nuclei using Hoechst 33258 (i), immunofluorescence of E-cadherin (iv) and Rhodamine-Phalloidine labeling of F-actin (vii). Fluorescence microscopy reveals that Ca^{2+} induced aggregation of gingival epithelial cells (ii), membrane localization of E-cadherin (v) and cortical condensation of F-actin (viii) were found relative to the control. Concomitant areca nut extract treatment disrupted the large and consolidated gingival epithelial cells colonies (iii), and attenuated the membrane localization of E-cadherin (vi) and the cortical condensation of F-actin (ix). i–iii, 100×; iv–ix, 400×. (B) Quantification of the colony size. The largest dimension of each colony was measured and the colonies compared in terms of size between the treated and untreated groups. The values are expressed as means ± standard error from three distinctive gingival epithelial cells lines. ***, $p < 0.001$. ANE, areca nut extract.

ca nut extract treatment reduced the size of the differentiated gingival epithelial cell colonies (Fig. 2Aiii). The largest dimension of a series of colonies was measured using a microruler. After analysis of three distinctive gingival epithelial cell lines, the dimensions of the differentiated gingival epithelial cell colonies without or with areca nut extract treatment were 523 ± 23 μm and 283 ± 14 μm, respectively, and these two values were statistically significantly different (Fig. 2B). E-cadherin was found to be localized in the cytosol in the control cells; however, in the differentiated gingival epithelial cells, E-cadherin had switched and was localized at the membrane. After areca nut extract treatment, the membrane localization of E-cadherin in differentiated gingival epithelial cells was disrupted (Fig. 2-Aiv–vi). Under these conditions, however, there was no difference in the amount of E-cadherin protein among the control cells, the differentiated gingival epithelial cells and the areca nut extract-treated differentiated gingival epithelial cells (data not shown). In the undifferentiated control cells, F-actin was found to be distributed in the fillopodia, cellular cortex and randomly scattered in the cytosol (Fig. 2Avii). This contrasted with the protein's distribution in differentiated gingival epithelial cells, where the F-actin was densely and homogenously distributed across the cellular cortex (Fig. 2Aviii). However, when treated with areca nut extract, the gingival epithelial cells showed disruption of well-defined cell–cell contact and a reduction in cortical F-actin (Fig. 2Aix).

We investigated MMP-9 expression following this treatment. RT-PCR analysis showed that treatment with 5 μg/mL of areca nut extract for 24 h significantly induced *MMP-9* mRNA expression (Fig. 3A,B). Gelatin lysis activity by MMP-9 (92 and 86 kDa) was induced following the treatment with areca nut extract (Fig. 3C,D); however, MMP-2 activity (72 and 62 kDa) was not affected by areca nut extract. In a parallel study, we have defined the increase of MMP-9 expression and activity in gingival fibroblast under the same treatment conditions (data not shown). Using gingival epithelial cells at passage three, treatment with 5 μg/mL of areca nut extract induced a two-fold increase in MMP-9 protein expression, but this was abrogated by treatment with 10 μM curcumin (Fig. 3E). To confirm that areca nut extract also induced the expression of MMP-9 protein in gingival epithelial cells other than at passage three, the same gingival epithelial cells at passage four were analyzed and gave similar results (Fig. 3E). Thus, an increase in MMP-9 expression seemed to underlie the activation of MMP-9 modulated by areca nut extract.

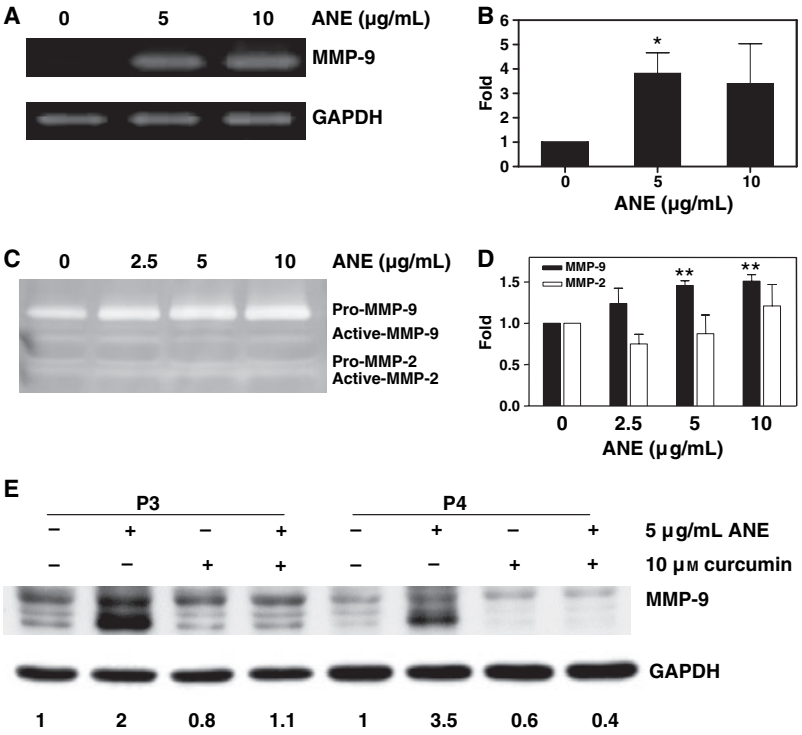

Fig. 3. Areca nut extract activates matrix metalloproteinase-9 (MMP-9) in gingival epithelial cells. (A) A representative reverse transcription-polymerase chain reaction analysis displays the induction of *MMP-9* mRNA expression following areca nut extract treatment for 24 h. (B) Quantification of three distinctive gingival epithelial cells. (C) A representative zymograph displaying an increase in MMP-9 activity following areca nut extract treatment for 24 h. (D) Quantification of three distinctive gingival epithelial cells indicates a significant increase in MMP-9 protein expression, but not in MMP-2 protein expression, following treatment with 5 or 10 μg/mL of areca nut extract. (E) Western blot analysis of gingival epithelial cells grown at passage three (P3) and passage four (P4), indicating the induction of MMP-9 expression by 5 μg/mL of areca nut extract treatment. This induction can be reversed by 10 μM curcumin. The multiple MMP-9 bands represent the proform, the latent form and the active form of the protein. The values are expressed as means ± standard error and are from three distinctive gingival epithelial cell lines. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. ANE, areca nut extract.

OC3, OECM-1 and SAS oral squamous cell carcinoma cells were treated with 20, 10 and 5 μg/mL of areca nut extract at doses ≤ 20, 10 and 5 μg/m respectively, for 24 h. Areca nut extract treatment unequivocally induced greater *MMP-9* mRNA expression in these cell lines after simulation (Fig. 4A). The induction of *MMP-9* mRNA expression in OECM-1 cells was reversed by 10 μM curcumin (Fig. 4B). Arecolin makes up 0.6% of our areca nut extract. With 50 μM arecolin treatment, a dose much higher than that present in 10 μg/mL of areca nut extract, no induction of *MMP-9* expression was noted in OECM-1 cells

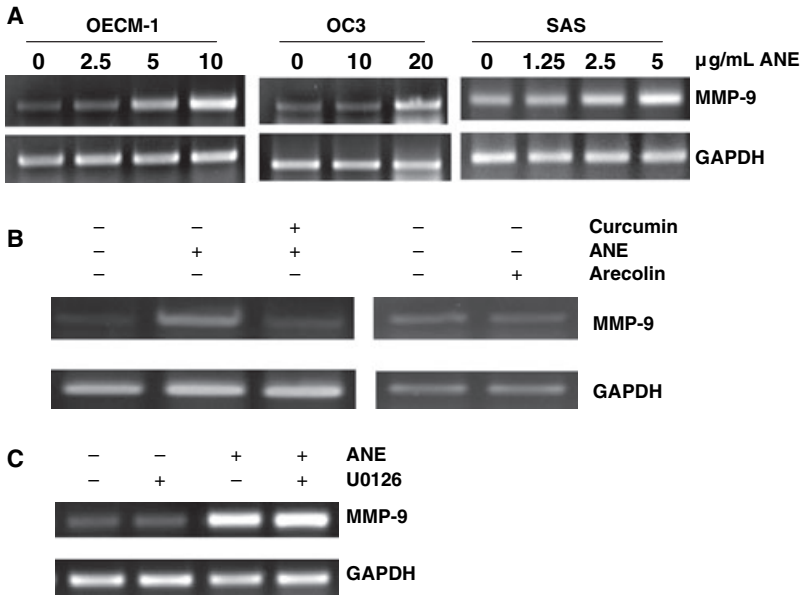

Fig. 4. Abrogation of areca nut extract-activated matrix metalloproteinase-9 (MMP-9) by curcumin. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis displays an induction of *MMP-9* mRNA expression in oral squamous cell carcinoma cells following treatment with various doses of areca nut extract for 24 h. (B) RT-PCR analysis displays an induction of *MMP-9* mRNA expression in OECM-1 cells that can be reversed by 10 μM curcumin; 50 μM arecolin did not exert such an induction effect. (C) The extracellular signal-regulated kinase (ERK) blocker, U0126, does not reverse the induction of *MMP-9* mRNA expression in OECM-1 cells. ANE, areca nut extract.

(Fig. 4B). Because areca nut extract can activate ERK (1), and ERK is known to be associated with MMP-9 regulation (31), we investigated whether ERK activation underlies the areca nut extract-induced MMP-9 up-regulation in OECM-1 cells. RT-PCR analysis showed that the ERK inhibitor, U0126, could not abrogate areca nut extract-induced *MMP-9* mRNA expression (Fig. 4C), suggesting a lack of involvement of ERK in areca nut extract-induced *MMP-9* regulation.

NF-κB binding activity in the gingival epithelial cells was induced following treatment with 2.5–10 μg/mL of areca nut extract treatment for 2 h (Fig. 5A). The specificity of NF-κB activation was confirmed by competitive inhibition with excess unlabeled probe and the presence of supershifts, which indicate an affinity between NF-κB and its antibodies. On treatment with 20 μg/mL of areca nut extract for 1 h, which resulted in no cytotoxicity, cytosolic IκB was significantly reduced in OECM-1 and SAS cells, indicating activation of NF-κB in these cells (Fig. 5B). Treatment with 10 μM curcumin reverted areca nut extract-reduced IκB expression in OECM-1 cells (Fig. 5C). Nuclear factor-κB activation seemed to underlie the areca nut extract-induced MMP-9 expression in gingival epithelial cells and OECM-1 cells.

Discussion

Areca chewing is tightly linked with a high prevalence of various epithelial diseases, including precancer and oral squamous cell carcinoma in Asians (2). Signaling elements, including ERK, Jun N-terminal kinase, p38MAPK, PI3K/AKT, cyclooxygenase-2 and NF-κB, have been shown to be activated by areca nut extract in oral epithelial cells (1,3,8,9,32). This study further identifies the activation of MMP-9 by areca nut extract in gingival epithelial cells. We also demonstrated that the activation of NF-κB and the increase in MMP-9 expression seem to be possible mechanistic factors in epithelial diseases. It has become clear that MMP-8 and MMP-9, as well as other MMPs, are important in periodontitis (10). Smith *et al.* (13) recently showed that the activation of MMP-9 in gingival epithelium occurs as a response to periodontal infection. The level of MMP-9 has been found to be increased in the saliva of areca chewers (18). Our findings suggest a unique pathogenic role for MMP-9 in areca chewers. It is possible that periodontal diseases might be better controlled if oral MMP levels are monitored. Furthermore, the design of individualized therapy using MMP blockers (10,14,33) is a possible approach to treatment. The RANKL/osteoprotegerin system may impair periodontal health through regulating NF-κB (34). The down-regulation of MMP-9 mediated by curcumin suggests that these compounds could act as potential adjuvants for the control of periodontal diseases. Although MMP-2 is also increased in the saliva of areca chewer, (17), and this enzyme could be important for periodontal pathogenesis (35), we did not find the induction of MMP-2 by areca nut extract in gingival epithelial cells. Sawicki *et al.* (36) demonstrated the differential MMP activities in dermal epithelial cells and dermal fibroblasts, with MMP-9 expression in the former and MMP-2 expression in the latter. Our preliminary studies confirmed the presence of such variation in gingival epithelial cells and gingival fibroblasts.

Areca chewing is epidemiologically linked to the prevalence of periodontal diseases (4,5). Areca nut extract enhances the production of interleukin-6 and interleukin-8 from peripheral blood mononuclear cells (8). It also regulates alkaline phosphatase and RANKL in osteoblasts (34). Our results, together with those of previous reports, indicate that areca nut extract may exert a tremendous impact on various target cells during periodontal pathogenesis. Because the areca nut extract concentration in areca chewer's saliva has been estimated to be at the mg/mL level (Dr K-W Chang, unpub-

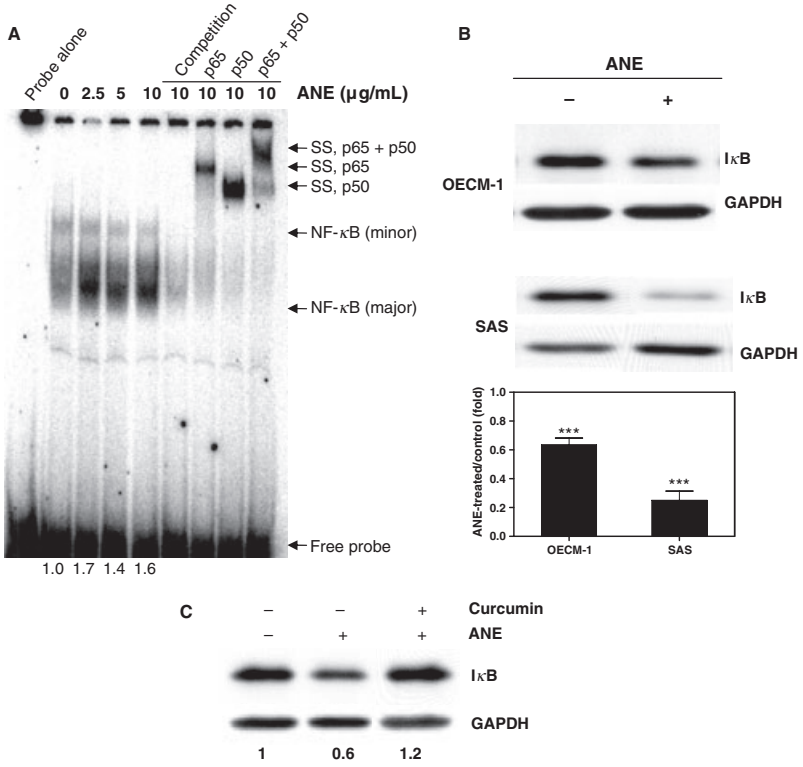

Fig. 5. Abrogation of areca nut extract-activated matrix metalloproteinase-9 (MMP-9) by curcumin through blocking NF-κB. (A) Electrophoretic mobility shift assay analysis of gingival epithelial cells shows areca nut extract-modulated NF-κB activation. Each nuclear extract was incubated with a ^{32}P-labeled probe containing the NF-κB binding site to measure NF-κB activity. Cells without areca nut extract treatment show basal NF-κB activity. A nuclear extract of gingival epithelial cells after treatment with 10 μg/mL of areca nut extract in the absence or presence of an excess of unlabeled competitor DNA (labeled as competition) was used to confirm the specificity of the experiments. These were also pre-incubation with antibodies against p65 or p50 and this was followed by standard electrophoretic mobility shift assay. Supershifts are indicated by SS. The relative NF-κB activities are shown at the bottom of the picture. (B) Representative western blot analysis of cytosolic IκB in OECM-1 (upper panel) and SAS (middle panel) cells following treatment with 20 μg/mL of areca nut extract for 1 h. Quantification of IκB protein expression from three independent experiments is illustrated in the lower panel. This indicates a significant decrease in cytosolic IκB protein expression in cells after treatment with areca nut extract. (C) Western blot analysis shows that an inhibition ofIκB expression by areca nut extract can be reversed by 10 μM curcumin in OECM-1 cells. The values are expressed as means ± standard error from triplicate analyses. ***, $p < 0.001$. ANE, areca nut extract.

lished data), the doses used in the present study are similar to those present *in vivo*. In areca nuts, arecolin is the main alkaloid component and catechin is the main phenolic component. Because a dose of arecolin higher than that present in areca nut extract was unable to induce MMP-9 in OECM-1 cells, the role of areca nut extract catechin in mediating MMP-9 activation requires further investigation. Furthermore, the vast majority of areca chewers are also tobacco smokers or tobacco chewers (1) and it is known that cigarette smoke is able to induce MMP-9 expression in lung epithelial cells (25). Liu *et al.* (37) demonstrated that a higher level of MMP-8 expression occurs in the periodontal tissues of smokers than in those of nonsmokers. Individuals who combine areca and tobacco use may therefore have an increased risk of periodontal damage because of the synergism in MMP activities between the habits, which may enhance oral inflammation and osseous degradation.

Epithelial cell migration is an important event during wound repair and disease progression (20–22). MMPs, including MMP-9, have been shown to be an important part of the machinery controlling cell migration (5,38,39). It is known that pro-inflammatory cytokines are able to activate focal adhesion kinase, which then activates MMP-2 and MMP-9 and gives rise to cell migration (14). We investigated if the activation of MMP-9 is associated with gingival epithelial cell migration. The results of the migration assays, namely the wound-repair model and the detachment assay, were in agreement and indicated that areca nut extract reduced gingival epithelial cell migration and increased attachment between gingival epithelial cells and the matrix. The results imply that the inhibitory effects modulated by areca nut extract may exceed the stimulatory effect derived from MMP-9 activation during gingival epithelial cell migration. It has been noted that the repair process of dermal keratinocytes is controlled by the balance between the positive ERK and p38 MAPK signals and the negative PI3K/AKT signal (20). Our previous study showed rapid PI3K/AKT activation at 0.5–2 h and a delayed ERK activation at 24 h in normal oral epithelial cells following treatment with areca nut extract (8). As a result, it is possible that the reduction in gingival epithelial cell migration during the first 24 h following areca nut extract treatment is a consequence of areca nut extract-mediated PI3K/AKT activation. Increased cell–matrix adhesion has been shown to be regulated by the integrin-linked kinase pathway (40). It is therefore the pathogenetic mechanisms implicated in the increase in gingival epithelial cell–matrix attachment modulated by areca nut extract that requires further exploration.

Because E-cadherin/actin affects cell–cell adhesion, and such linkage is particularly strong during the maintenance of keratinocyte differentiation (19), we were interested to establish whether areca nut extract also affected E-cadherin, actin and differentiation. By increasing the calcium concentration in the medium, an induction in

gingival epithelial cell differentiation without a change in E-cadherin expression was noted. These findings are in agreement with a previous study (41). Interestingly, the acquisition of membranous E-cadherin and cortical F-actin, which are responsible for maintaining cell–cell contact and intercellular adhesion, were extensively impaired by areca nut extract. The consolidated organoid gingival epithelial cell aggregates were also significantly dispersed. Our previous study has shown that disruption of the cornified envelope of oral epithelial cells occurred through the down-regulation of involucrin by areca nut extract (8). Epithelial wound healing involves complicated events, including proliferation, migration and differentiation (20). Our evidence demonstrates that areca nut extract interrupted these cellular phenotypes, which suggest that areca chewing may prolong the duration or enhance the severity of periodontal diseases and this is caused by compromised re-epithelization.

This study provides novel clues demonstrating that areca nut extract exerts effects by activating MMP-9, loss of detachment and disrupting the re-epithelization of gingival epithelial cells. Except for a more rapid loss of cellular detachment being induced in the OECM-1 cell line, areca nut extract exerts other influences on OECM-1 similar to those in gingival epithelial cells. Gingival epithelium serves as a protector against the destruction of pathogens to other periodontium (28,42). Factors that perturb the structural integrity or pathogenic virulence-host defense homeostasis in the gingival epithelium may be advantageous for the progression of periodontal destruction (27,43). MMP-9 activity and disruption in re-epithelization induced by areca nut extract may disturb the structural protection and delay the healing of gingival epithelium following physical or inflammatory injuries (20–22), which might facilitate the invasion and damage of pathogens and the progression of periodontal diseases. Our findings may provide functional clues to support the higher propensity of periodontal diseases in areca chewers (4,5). However, it would be important to see such genotypic and phenotypic disruptions in the gingival epithelium, particularly in the junctional epithelium (28,42) of areca chewers.

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