Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 198–203

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01421.x

Areca nut extracts suppress the differentiation and functionality of human monocyte-derived dendritic cells

C.-C. Wang[1], T.-Y. Chen[2], H.-Y. Wu[2], T.-Y. Liu[3], T.-R. Jan[2]

[1]School of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, [2]Department and Graduate Institute of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan and [3]Institute of Environmental and Occupational Health Sciences, National Yang-Ming University, Taipei, Taiwan

Wang C-C, Chen T-Y, Wu H-Y, Liu T-Y, Jan T-R. Areca nut extracts suppress the differentiation and functionality of human monocyte-derived dendritic cells. J Periodont Res 2012; 47: 198–203. © 2011 John Wiley & Sons A/S

Background and Objective: Areca quid chewing, a major risk factor contributing to the occurrence of oral cancer and precancer, has been reported to be associated with the severity and high prevalence of periodontal diseases in areca quid chewers. As dendritic cells are critically involved in the regulation of innate and adaptive immunity in oral mucosa, the objective of the present study was to investigate the effect of areca nut extracts (ANE) on the differentiation and reactivity of dendritic cells derived from monocytes.

Material and Methods: Human peripheral blood monocytes were cultured in the presence of granulocyte–monocyte colony-stimulating factor and interleukin-4 for 7 d to generate dendritic cells. To examine the effect of ANE on the generation of dendritic cells, the monocytes were exposed to ANE throughout the 7 d culture period. In addition, the effect of ANE on the maturation of monocyte-derived dendritic cells induced by lipopolysaccharide (LPS) was examined.

Results: Monocytes cultured in granulocyte–monocyte colony-stimulating factor and interleukin-4 exhibited a typical phenotype of dendritic cells, as evidenced by the heightened expression of human leukocyte antigen (HLA)-DR, CD11c and the co-stimulatory molecules CD40, CD80 and CD86. Exposure of the monocytes to ANE did not influence the expression of HLA-DR and CD11c, but markedly attenuated the proportion of CD40-positive cells and the mean fluorescence intensity of CD86. The expression of co-stimulatory molecules in LPS-activated dendritic cells was not affected, whereas the mRNA expression of interleukin-12 induced by LPS was markedly suppressed by ANE treatment in a concentration-dependent manner.

Conclusion: These results suggest that ANE exposure interfered with the differentiation of dendritic cells from monocytes. Moreover, the functionality of mature monocyte-derived dendritic cells was attenuated in the presence of ANE.

Tong-Rong Jan, PhD, Department and Graduate Institute of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan
Tel: +886 2 33661287
Fax: +886 2 23661475
e-mail: tonyjan@ntu.edu.tw

Key words: areca nut; dendritic cell; differentiation; interleukin-12; maturation

Accepted for publication August 31, 2011

Areca nut (*Areca catechu*) chewing is a common habit in several southeastern Asian countries (1,2). It has been well documented that areca chewing is one of the primary risk factors for oral cancer and precancer, such as submucous fibrosis (OSF) (2,3). In addition, clinical studies demonstrated a high prevalence of periodontal infection in areca chewers (4). Although the etiology for areca-related oral diseases is not fully understood, several

mechanisms associated with areca nut chewing have been proposed, including oxidative damage to the oral cells, chronic inflammation in the oral cavity caused by areca nut constituents (5–8) and immune deterioration of the areca chewers (9–11).

The major immunocompetent cells found in the subepithelial connective tissue and epithelium of OSF specimens were lymphocytes, monocytes, macrophages, human leukocyte antigen (HLA)-DR positive cells and Langerhans cells, which are the dominant dendritic cell subset within epidermis; in addition, the number of Langerhans cells is increased in the regions of OSF and oral cancer (10–12). Changes in cellular immunity and cytokine expression profile in patients with squamous cell carcinoma or OSF have been reported. For example, the frequencies of proliferating T cells in tumor tissues and peripheral blood of oral cancer patients were significantly lower than those in healthy individuals (13). In addition, mitogen-stimulated proliferation and the expression of interferon-γ and interleukin (IL)-2 by T lymphocytes were diminished in patients with head and neck squamous cell carcinoma (14). It has been further demonstrated that peripheral mononuclear cells from OSF patients secreted increased levels of proinflammatory cytokines, including IL-1, IL-6 and tumor necrosis factor-α, and a decreased level of interferon-γ in comparison with normal control individuals (15). Taken together, these findings indicate that alterations in cellular immunity and the cytokine network may play a role in the pathophysiology of areca-related oral cancer and OSF.

Dendritic cells are highly efficient and specialized antigen-presenting cells that participate in the initiation and regulation of both innate and adaptive immunity. Interstitial dendritic cells and Langerhans cells function as sentinels at the front line of defense in the oral mucosa (16). Upon the capture of antigens, the immature dendritic cells undergo maturation and migrate to lymphoid organs to induce antigen-specific T-cell responses against microorganism infection and tumor formation (17,18). The important function of dendritic cells in promoting anti-tumor immunity has led to intensive investigation of the application of dendritic cell-based immunotherapy for malignancies (19–21). The phenotype of dendritic cells changes during the maturation process. Mature dendritic cells are characterized by a decreased capability of antigen uptake, but increased expression of major histocompatibility complex and several co-stimulatory molecules, such as CD40, CD80 and CD86. The upregulation of the co-stimulatory molecules enhances the ability of dendritic cells to stimulate lymphocytes. Moreover, mature dendritic cells can produce IL-12, which promotes the development of type 1 T helper cells and subsequently enhances cell-mediated immune responses (22).

Although alterations in cell-mediated immunity in patients with OSF and oral cancer have been documented, it is currently unclear whether dendritic cells are influenced by areca nut constituents. Notably, areca nut extracts (ANE) have been shown to interfere with the phagocytic and bactericidal activity of neutrophils (23,24), suggesting an inhibition by ANE of the host defense mechanism against pathogens. We also demonstrated that ANE induced apoptosis of mouse splenocytes in culture and modulated T-cell-mediated immune responses *in vitro* and *in vivo* (25,26). These results suggest that some immune cells are sensitive targets for areca constituents. On the basis of these findings, we hypothesized that ANE may affect the differentiation and maturation of dendritic cells. To test the hypothesis, we investigated the direct effect of ANE on the differentiation and functionality of human monocyte-derived dendritic cells. We report here that exposure to ANE interferes with the differentiation of dendritic cells from monocytes and suppresses the expression of IL-12 by dendritic cells stimulated with lipopolysaccharide (LPS).

Material and methods

Reagents and ANE

All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Areca nut extracts were prepared as previously described (27). The level of endotoxin in the ANE was below the detection limit (0.05 endotoxin unit/mL) measured by an assay Kit (Kinetic-QCL®; Lonza Walkersville Inc., Walkersville, MD, USA). Reagents and antibodies used for flow cytometry were purchased from BD Biosciences (San Diego, CA, USA). Reagents and enzymes used for RT-PCR were purchased from Promega (Madison, WI, USA).

Generation of dendritic cells and ANE treatment

Dendritic cells were prepared from peripheral blood monocytes as previously described (28). Briefly, peripheral blood was collected from healthy donors upon informed consent. Mononuclear cells were separated from the peripheral blood by standard gradient centrifugation with Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden). The cells were seeded into six-well culture plates in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin (referred as complete RPMI), and cultured at 37°C in air supplemented with 5% CO_2 for 2 h. The adherent cells were collected and further cultured in complete RPMI containing granulocyte–monocyte colony-stimulating factor and IL-4 (1000 U/mL of each) for 7 d to generate dendritic cells. For some experiments, the dendritic cells obtained were left untreated (naïve) or treated with LPS (10 ng/mL) for 24 h to further stimulate the maturation of dendritic cells. To examine the effect of ANE on the differentiation of monocytes to dendritic cells, peripheral blood monocytes were left untreated (control group) or exposed to ANE (10 and 20 μg/mL) throughout the entire 7 d culture period. To investigate the influence of ANE on the functionality of dendritic cells, monocyte-derived dendritic cells were pretreated with ANE (1–20 μg/mL) for 30 min, followed by LPS stimulation for 24 h.

Flow cytometry

The expression of surface markers by dendritic cells was analyzed using flow cytometry. Cells were washed with an ice-cold washing buffer (phosphate-buffered saline containing 1% fetal bovine serum and 0.02% sodium azide), and then stained with fluorescein isothiocyanate-conjugated mouse monoclonal antibodies against CD11b, CD11c, CD14, CD80, CD86 and HLA-DR for 30 min on ice. For staining of CD40, cells were incubated with mouse anti-CD40 monoclonal antibodies for 60 min on ice. After washing, a fluorescein isothiocyanate-conjuated goat anti-mouse IgG secondary antibody was added. After another washing, the stained cells were analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA). The fluorescein isothiocyanate emission was detected in the FL1 channel using an emission filter of 525 nm. The data were analyzed using WINMDI 2.7 software (Scripps Research Institute, La Jolla, CA, USA). Data are expressed as the percentage of positive cells and the mean fluorescence intensity (MFI) of the stained cells.

Isolation of RNA and RT-PCR

Total RNA was isolated from cells using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) following the supplier's instructions. All RNA samples were confirmed to be free of DNA contamination, as determined by the absence of product after PCR amplification in the absence of RT. Complementary DNA was reverse transcribed using oligo$(dT)_{15}$ as primers. The PCR for IL-12 p40 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed as previously described (29). The PCR products were electrophoresed and visualized by ethidium bromide staining. Quantification was performed by assessing the optical density of DNA bands using an imaging system (BioDoc-It™; UVP, Upland, CA, USA). The expression of *GAPDH* was used as control for semi-quantification. The primers used in this study are as follows: 5′- CCAAGAACTTGCAGCTGAAG-3′ and 5′- GACACAACGGAATAGACCCA-3′ for *IL-12B*; and 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ for *GAPDH*.

Cytotoxicity assay

The cytotoxicity of ANE to monocyte-derived dendritic cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay previously described (30). Briefly, the cells (2×10^5 cells/mL) were cultured in quadruplicate (100 μL per well) in 96-well culture plates. The cells were either left untreated (naïve) or pretreated with ANE (10–40 μg/mL) for 30 min followed by stimulation with LPS (10 ng/mL) for 24 h. An MTT stock solution (5 mg/mL in phosphate-buffered saline) was then added to each well (10 μL per well) and incubated for 4 h. The formazan formed was dissolved with lysis buffer (10% sodium dodecyl sulfate in *N*,*N*-demethylformamine) overnight in the dark, and then the plates were read at 570 nm using 630 nm as background reference.

Statistical analysis

The data are expressed as means ± SEM for each treatment group in the individual experiments. Normality and homoscedasticity of data were tested by the Shapiro–Wilk test and Bartlett's test, respectively. Analysis of variance was performed by one-way ANOVA. Dunnett's two-tailed *t*-test was employed to compare treatment groups with the control group. A value of $p < 0.05$ was defined as statistical significance.

Results

Phenotypic analysis of monocyte-derived dendritic cells

Peripheral blood monocytes were cultured in complete RPMI supplemented with granulocyte–monocyte colony-stimulating factor and IL-4 (1000 U/mL) for 7 d to generate dendritic cells. The nonadherent cells were harvested and the cell surface markers analyzed using flow cytometry. It is known that dendritic cells generated under the employed protocol express high levels of CD11c and HLA-DR, but lose the expression of the monocyte marker CD14 (19,28). As expected, the cells obtained exhibited a typical phenotype of immature dendritic cells, including the upregulation of HLA-DR, CD11c and several co-stimulatory molecules, including CD40, CD80 and CD86 (Table 1). In addition, the cells were negative for CD11b and CD14, confirming that they were not moncytes/macrophages.

Areca nut extracts interfered with the phenotypic differentiation of dendritic cells

To investigate the effect of ANE on the dendritic cell differentiation, ANE (10 or 20 μg/mL) were added into the monocytes cultured in complete RPMI containing granulocyte–monocyte colony-stimulating factor and IL-4 (1000 U/mL) for the generation of dendritic cells for 7 d as described above. The nonadherent cells were collected and their expression of surface markers was examined. As shown in Table 1, the presence of ANE (20 μg/mL) for 7 d markedly attenuated the percentage of CD40-positive cells compared with the untreated control cells. A slight decrease in the percentage of HLA-DR-positive and CD11c-positive cells was also noticed, but not statistically significant. In addition, the MFI of CD86 was also significantly attenuated in cells treated with ANE (20 μg/mL). The expression of the other surface markers measured, including CD11b, CD14 and CD80, was unaffected by ANE treatment.

The effect of ANE on the maturation and functionality of dendritic cells

It has been reported that the maturation of dendritic cells can be driven by LPS or proinflammatory cytokines (17). We therefore cultured monocyte-derived dendritic cells in the presence of LPS (10 ng/mL) for 24 h to further stimulate their maturation. As shown in Table 2, the percentage of

Table 1. Areca nut extracts (ANE) attenuated the expression of CD40 and CD86 during the differentiation of monocytes to dendritic cells[a]

	Percentage of positive cells (MFI[b])		
Surface markers	Control	ANE (10 μg/mL)	ANE (20 μg/mL)
HLA-DR	90.0 ± 5.2 (266.1 ± 61.3)	87.6 ± 6.2 (428.6 ± 78.3)	67.7 ± 20.3 (276.3 ± 44.4)
CD11b	3.4 ± 0.7 (26.3 ± 6.5)	3.3 ± 1.2 (25.2 ± 10.4)	6.6 ± 5.0 (38.2 ± 18.9)
CD11c	77.6 ± 17.7 (60.3 ± 16.1)	67.0 ± 27.4 (62.9 ± 17.6)	39.9 ± 36.9 (39.2 ± 14.2)
CD14	19.5 ± 10.3 (27.5 ± 4.1)	7.5 ± 3.8 (35.9 ± 8.8)	14.1 ± 1.5 (23.1 ± 3.8)
CD40	86.0 ± 6.4 (92.8 ± 8.5)	78.7 ± 13.4 (127.9 ± 30.3)	36.0 ± 10.8* (53.9 ± 11.4)
CD80	57.3 ± 14.4 (30.9 ± 5.9)	30.6 ± 16.6 (43.5 ± 3.0)	49.7 ± 18.6 (38.2 ± 12.3)
CD86	69.3 ± 8.4 (89.4 ± 8.7)	74.9 ± 7.3 (117.1 ± 7.8)	57.7 ± 13.9 (48.9 ± 10.2*)

[a]Monocyte-derived dendritic cells were generated as described in the Materials and methods section. Peripheral monocytes were exposed to ANE (10–20 μg/mL) for 7 d. Flow cytometry was used to measure the expression of surface markers.
[b]MFI, mean fluorescence intensity. Data are expressed as means ± SEM of four independent experiments.
*$p < 0.05$ compared with the matched control group.

Table 2. No effect of ANE on the expression of surface markers by monocyte-derived dendritic cells stimulated with lipopolysaccharide (LPS)[a]

	Percentage of positive cells			
			LPS + ANE (μg/mL)	
Surface marker	Naïve	LPS	10	20
HLA-DR	89.6 ± 8.4	87.8 ± 10.3	91.2 ± 7.4	88.4 ± 9.7
CD40	96.5 ± 1.3	97.9 ± 0.3	97.7 ± 0.9	95.5 ± 4.7
CD80	76.3 ± 2.6	87.8 ± 2.9*	85.4 ± 6.4	78.7 ± 0.5
CD83	14.4 ± 4.4	18.6 ± 6.3	22.2 ± 10.2	25.7 ± 8.4
CD86	87.8 ± 3.4	94.6 ± 1.5	87.3 ± 8.0	90.5 ± 5.6

[a]Monocyte-derived dendritic cells were pretreated with ANE (10–20 μg/mL), followed by stimulation with LPS (10 mg/mL) for 24 h. The expression of co-stimulatory molecules was determined using flow cytometry.
*$p < 0.05$ compared with the matched naïve group.

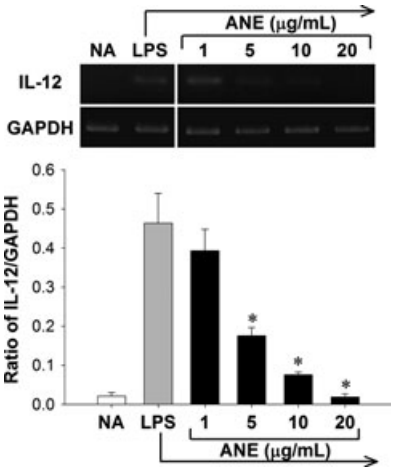

Fig. 1. Areca nut extracts (ANE) attenuated the steady-state mRNA expression of interleukin-12 (IL-12) by lipopolysaccharide (LPS)-stimulated dendritic cells. Monocyte-derived dendritic cells (5×10^5 cells/mL) were left untreated (naïve; NA), or pretreated with ANE (1–20 μg/mL) for 30 min followed by stimulation with LPS (10 ng/mL) for 24 h. The total RNA was extracted and the expression of IL-12 mRNA measured by RT-PCR. The level of mRNA of housekeeping gene *GAPDH* was used to normalize the expression of IL-12 mRNA for semi-quantification. Data are expressed as the means ± SEM of triplicate samples.*$p < 0.05$ compared with the LPS group. Results are representative of three independent experiments.

CD80-positive cells was significantly increased following LPS stimulation. However, exposure of LPS-stimulated dendritic cells to ANE (10 and 20 μg/mL) did not affect the expression of HLA-DR, CD40, CD80, CD83 and CD86 (Table 2). We next investigated whether ANE affected the functionality of dendritic cells. As IL-12 is a critical cytokine expressed by dendritic cells, the mRNA expression of IL-12 was studied. The results from RT-PCR experiments demonstrated that LPS treatment strongly induced IL-12 mRNA expression by the monocyte-derived dendritic cells stimulated with LPS (10 ng/mL; Fig. 1). Moreover, the presence of ANE (5–20 μg/mL) significantly attenuated the IL-12 mRNA expression (Fig. 1). Finally, we investigated whether ANE had a cytotoxic effect on monocyte-derived dendritic cells using an MTT assay. Our results showed that exposure of dendritic cells to ANE at concentrations up to 40 μg/mL for 24 h did not affect the cell metabolic activity (Fig. 2).

Discussion

Although it has been well documented that altered cell-mediated immunity is closely associated with the pathophysiology of areca-mediated oral diseases (9–11), little is known pertaining to the underlying mechanisms of the altered immunity. Areca ingredients have been reported to produce cytotoxicity and genotoxicity to a variety of cells, including lymphocytes, bone marrow cells and oral cells (5,6,8,31). The suppressive effects of ANE on the functionality of neutrophils and splenocytes have also been demonstrated (24,25). We therefore investigated whether ANE could interfere with the differentiation and functionality of dendritic cells. Our data demonstrated, for the first time, that ANE markedly affected the differentiation of dendritic cells, as evidenced by suppression of the expression of co-stimulatory molecules, including CD40 and

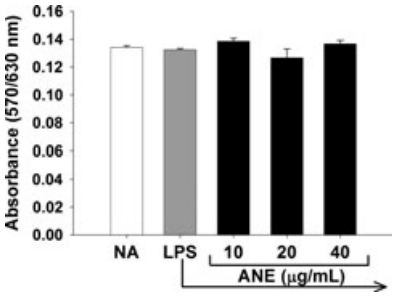

Fig. 2. The metabolic activity of LPS-stimulated dendritic cells was not affected by ANE treatment. Monocyte-derived dendritic cells (2×10^5 cells/mL) were left untreated (naïve; NA), or pretreated with ANE (10–40 μg/mL) for 30 min followed by stimulation with LPS (10 ng/mL) for 24 h. The metabolic activity of viable cells was determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Data are expressed as the means ± SEM of quadruplicate cultures. Results are representative of three independent experiments.

CD86, in monocyte-derived dendritic cells (Table 1). Exposure to ANE did not, however, influence the expression of CD11c, a marker for dendritic cells derived from the myeloid lineage (32). Moreover, the ANE-exposed cells remained CD14 negative. These data suggest that the presence of ANE did not affect the development of dendritic cells under the experimental conditions employed; however, the functional differentiation of dendritic cells was influenced.

In periodontal tissues, the Langerhans cells and interstitial dendritic cells serve as a bridge to connect innate and adaptive immunity against bacterial challenges (16,33). In addition, an increased number of Langerhans cells in periodontitis and areca-mediated oral diseases has been reported, implicating a role for Langerhans cells in the pathophysiology of the diseases (10–12,33,34). At present, the identity of the precursors of Langerhans cells in the oral cavity is unclear. It has been suggested that local monocytes might be the precursor of oral mucosal Langerhans cells that become mobilized and mature in response to inflammatory cytokines and pathogen-associated molecules (33). It is well established that dendritic cells are key professional antigen-presenting cells participating in cell-mediated immunity against pathogens and tumor cells. One of the primary mechanisms by which dendritic cells upregulate the host cell-mediated immunity is the activation of antigen-specific T cells. During the process of antigen presentation to T cells, co-stimulatory molecules expressed on dendritic cells provide crucial activation signals that supplement the primary signal delivered via the engagement of the T-cell receptors by presented antigens (17). Notably, we revealed that exposure of monocytes to ANE for 7 d attenuated the subsequent expression of CD40 and CD86 by dendritic cells derived from the monocytes. On the basis of these results, we speculate that the co-stimulatory signals delivered by dendritic cells to activate T cells may be attenuated following ANE exposure. As dendritic cells play a pivotal role in the initiation of both innate and adaptive immunity, the present results suggest a potential mechanism that may account for the deregulated cell-mediated immunity in patients with areca-associated oral diseases, which has been previously reported in many clinical studies (9,11,15,35).

We also investigated the influence of ANE on the maturation and functionality of monocyte-derived dendritic cells. Our results showed an increased percentage of CD80 expression in dendritic cells stimulated with LPS for 24 h (Table 2), confirming the well-known effect of LPS to enhance dendritic cell maturation (19). However, the expression of surface antigens, including CD40, CD80, CD83 and CD86, was not affected by ANE treatment. These data are somewhat surprising, as they are inconsistent with the attenuation of CD40 and CD86 expression in monocyte-derived dendritic cells exposed to ANE for 7 d. Although the underlying mechanisms for these contrasting effects are currently unclear, it is apparent that a longer time of exposure (i.e. 7 d) to ANE may be required to cause effects on the differentiation of dendritic cells. In addition, because ANE disrupted the differentiation of dendritic cells from monocytes, but not the further maturation of dendritic cells stimulated with LPS, it is possible that the effect of ANE is influenced by the stage of differentiation of dendritic cells.

While ANE did not affect the expression of surface markers by monocyte-derived dendritic cells stimulated with LPS, the mRNA expression of IL-12 by the dendritic cells was remarkably suppressed. To rule out the possibility that ANE-induced suppression of IL-12 mRNA is mediated by nonspecific cytotoxicity, we employed an MTT assay to determine the influence of ANE on dendritic cell metabolic activity. The results showed that ANE at concentrations up to 40 μg/mL were not cytotoxic to dendritic cells (Fig. 2). Interleukin-12 is one of the major cytokines expressed by activated dendritic cells (19). Interleukin-12 induces a broad range of biological effects, including the upregulation of the development of type 1 T helper cells (36), thereby enhancing cell-mediated immunity. Hence, in addition to the inhibition of dendritic cell differentiation, ANE-mediated attenuation of IL-12 expression by dendritic cells could be another critical feature of areca-mediated immunotoxicity.

In summary, the present study demonstrated, for the first time, the suppressive effect of ANE on the differentiation and functionality of dendritic cells. Given the critical role of dendritic cells in cell-mediated immunity, our data suggest that ANE-mediated downregulation of dendritic cell differentiation and IL-12 expression may be a critical mechanism contributing to the immune deterioration reported in patients with areca-associated oral diseases.

Acknowledgements

This work was supported by grants NSC98-2320-B-002-036-MY3 from the National Science Council, Executive Yuan, Taiwan. We would like to thank Professor Jen-Pei Liu, Professor Ching-Ho Wang and consultants of The Consulting Center of Statistics and Bioinformatics in the College of Bio-Resources and Agriculture, National Taiwan University, for their help with statistical analysis.

References

1. Gupta PC, Warnakulasuriya S. Global epidemiology of areca nut usage. *Addict Biol* 2002;**7:**77–83.
2. IARC. Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. *IARC Monogr Eval Carcinog Risks Hum* 2004;**85:**1–334.
3. Nair U, Bartsch H, Nair J. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. *Mutagenesis* 2004;**19:**251–262.
4. Ling LJ, Hung SL, Tseng SC *et al*. Association between betel quid chewing, periodontal status and periodontal pathogens. *Oral Microbiol Immunol* 2001;**16:**364–369.
5. Bagchi M, Balmoori J, Bagchi D, Stohs SJ, Chakrabarti J, Das DK. Role of reactive oxygen species in the development of cytotoxicity with various forms of chewing tobacco and pan masala. *Toxicology* 2002;**179:**247–255.
6. Chang MC, Ho YS, Lee PH *et al*. Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. *Carcinogenesis* 2001;**22:**1527–1535.
7. Chen CL, Chi CW, Liu TY. Hydroxyl radical formation and oxidative DNA damage induced by areca quid in vivo. *J Toxicol Environ Health A* 2002;**65:**327–336.
8. Jeng JH, Wang YJ, Chiang BL *et al*. Roles of keratinocyte inflammation in oral cancer: regulating the prostaglandin E2, interleukin-6 and TNF-alpha production of oral epithelial cells by areca nut extract and arecoline. *Carcinogenesis* 2003;**24:**1301–1315.
9. Chang MC, Chiang CP, Lin CL, Lee JJ, Hahn LJ, Jeng JH. Cell-mediated immunity and head and neck cancer: with special emphasis on betel quid chewing habit. *Oral Oncol* 2005;**41:**757–775.
10. Chiang CP, Wu HY, Liu BY, Wang JT, Kuo MY. Quantitative analysis of immunocompetent cells in oral submucous fibrosis in Taiwan. *Oral Oncol* 2002;**38:**56–63.
11. Haque MF, Harris M, Meghji S, Speight PM. An immunohistochemical study of oral submucous fibrosis. *J Oral Pathol Med* 1997;**26:**75–82.
12. Kurihara K, Hashimoto N. The pathological significance of Langerhans cells in oral cancer. *J Oral Pathol* 1985;**14:**289–298.
13. Laad A, Kode J, Chavan S, Rao R, Fakih AR, Chiplunkar S. Limiting dilution analysis of proliferating and cytotoxic lymphocytes in the peripheral blood and tumours of oral cancer patients. *Eur J Cancer B Oral Oncol* 1996;**32B:**337–342.
14. Heimdal JH, Aarstad HJ, Klementsen B, Olofsson J. Disease stage related in vitro responsiveness of peripheral blood T-lymphocytes in patients with head and neck carcinoma. *Acta Otolaryngol* 1998;**118:**887–891.
15. Haque MF, Meghji S, Khitab U, Harris M. Oral submucous fibrosis patients have altered levels of cytokine production. *J Oral Pathol Med* 2000;**29:**123–128.
16. Azuma M. Fundamental mechanisms of host immune responses to infection. *J Periodontal Res* 2006;**41:**361–373.
17. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;**392:**245–252.
18. Pierre P, Turley SJ, Gatti E *et al*. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* 1997;**388:**787–792.
19. Banchereau J, Briere F, Caux C *et al*. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;**18:**767–811.
20. Osada T, Clay TM, Woo CY, Morse MA, Lyerly HK. Dendritic cell-based immunotherapy. *Int Rev Immunol* 2006;**25:**377–413.
21. O'Neill DW, Adams S, Bhardwaj N. Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood* 2004;**104:**2235–2246.
22. Ebner S, Ratzinger G, Krosbacher B *et al*. Production of IL-12 by human monocyte-derived dendritic cells is optimal when the stimulus is given at the onset of maturation, and is further enhanced by IL-4. *J Immunol* 2001;**166:**633–641.
23. Hung SL, Chen YL, Wan HC, Liu TY, Chen YT, Ling LJ. Effects of areca nut extracts on the functions of human neutrophils in vitro. *J Periodontal Res* 2000;**35:**186–193.
24. Hung SL, Cheng YY, Peng JL, Chang LY, Liu TY, Chen YT. Inhibitory effects of areca nut extracts on phagocytosis of Actinobacillus actinomycetemcomitans ATCC 33384 by neutrophils. *J Periodontol* 2005;**76:**373–379.
25. Wang CC, Liu TY, Wey SP, Wang FI, Jan TR. Areca nut extract suppresses T-cell activation and interferon-gamma production via the induction of oxidative stress. *Food Chem Toxicol* 2007;**45:**1410–1418.
26. Wang CC, Lin HL, Wey SP, Jan TR. Areca-nut extract modulates antigen-specific immunity and augments inflammation in ovalbumin-sensitized mice. *Immunopharmacol Immunotoxicol* 2010;**33:**315–322.
27. Liu TY, Chen CL, Chi CW. Oxidative damage to DNA induced by areca nut extract. *Mutat Res* 1996;**367:**25–31.
28. Romani N, Gruner S, Brang D *et al*. Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994;**180:**83–93.
29. Snijders A, Hilkens CM, van der-PouwKraan TC, Engel M, Aarden LA, Kapsenberg ML. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *J Immunol* 1996;**156:**1207–1212.
30. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;**65:**55–63.
31. Kumpawat K, Deb S, Ray S, Chatterjee A. Genotoxic effect of raw betel-nut extract in relation to endogenous glutathione levels and its mechanism of action in mammalian cells. *Mutat Res* 2003;**538:**1–12.
32. Chicha L, Jarrossay D, Manz MG. Clonal type I interferon-producing and dendritic cell precursors are contained in both human lymphoid and myeloid progenitor populations. *J Exp Med* 2004;**200:**1519–1524.
33. Cutler CW, Jotwani R. Antigen presentation and the role of dendritic cells in periodontitis. *Periodontology 2000* 2004;**35:**135–157.
34. Muller HK, Dandie GW, Ragg SJ, Woods GM. Langerhans cell alterations in cutaneous carcinogenesis. *In vivo (Athens, Greece)* 1993;**7:**293.
35. Das SN, Khanna NN, Khanna S. In vivo and in vitro observation of cellular immune parameters in squamous cell carcinoma of the oral cavity and its correlation with tumor load and prognosis. *Cancer Invest* 1986;**4:**207–216.
36. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003;**3:**133–146.