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Roles of keratinocyte inflammation in oral cancer: regulating the prostaglandin E_2, interleukin-6 and TNF-α production of oral epithelial cells by areca nut extract and arecoline

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Betel quid (BQ) chewing is an etiologic factor of oral cancer and submucus fibrosis (OSF). Keratinocyte inflammation is crucial for the pathogenesis of cancer and tissue fibrosis. We found that areca nut (AN) extract (100–400 μg/ml) induced PGE_2 production by KB cells by 2.34- to 23.1-fold and also TNF-α production by gingival keratinocytes (GK). Arecoline (0.2–1.2 mM) elevated PGE_2 production by KB cells by 2.5- to 6.1-fold. AN extract (200–400 μg/ml) also induced IL-6 production by GK (7.5- to 8.4-fold) and KB cells. In contrast, arecoline (0.1–1.2 mM) suppressed IL-6 production by GK and KB cells, with 42–81 and 41–63% inhibition, respectively. A 48 h exposure of GK to 800–1200 μg/ml AN extract led to 37–69% cell death. Arecoline cytotoxicity to GK was noted at concentrations of 0.8–1.2 mM, which led to 28–38% cell death. AN extract (400–800 μg/ml) induced Cox-2 and IL-6 mRNA expression and also COX-2 protein production by KB cells. IL-6 (5–100 ng/ml) suppressed GK growth by 20–33%, but enhanced oral fibroblast (OMF) and KB cell growth. PGE_2 (0.05–5 μg/ml) and anti-IL-6 antibody (ab) (50–1000 ng/ml) showed little effect on GK and KB cell growth. Incubation of GK and KB cells with aspirin, anti-IL-6 ab and anti-TNF-α ab showed little effect on arecoline- and AN-induced cytotoxicity, cell cycle arrest and apoptosis. Exposure to anti-TNF-α ab slightly affected arecoline- and AN-modulated PGE_2 and IL-6 production by GK and KB cells. Arecoline- and AN-conditioned medium decreased phytohemagglutinin-mediated $CD4^+$ and $CD8^+$ T cell activation. These results indicate that BQ chewing contributes to the pathogenesis of cancer and OSF by impairing T cell activation and by induction of PGE_2, TNF-α and IL-6 production, which affect oral mucosal inflammation and growth of OMF and oral epithelial cells.

Abbreviations: AN, areca nut; ab, antibody; BQ, betel quid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GK, gingival keratinocyte; HNSCC, head and neck squamous cell carcinoma; IL-6, interleukin-6; KGM-SFM, keratinocyte growth medium; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OMF, oral mucosal fibroblasts; OSF, oral submucous fibrosis; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PG, prostaglandin; PHA, phytohemagglutinin; ROS, reactive oxygen species; RT, reverse transcription; TNF-α, tumor necrosis factor α.

Introduction

Betel quid (BQ) chewing is the fourth most popular oral habit in the world. There are about 600 000 000 BQ chewers in the world. BQ chewing shows a strong correlation with the incidence of oral cancer and oral submucus fibrosis (OSF) (1–7). Recently, keratinocyte inflammation has been shown to be crucial for tissue fibrosis and chemical carcinogenesis (8–12). Various inflammatory mediators such as prostaglandins (PGs), interleukin-1, interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) are central to these pathogenic processes (11–16). Interestingly, juxta-epithelial inflammation is a consistent histopathological observation in biopsy specimens obtained from BQ chewers with OSF and leukoplakia (1,4,5,7). Since abnormal and enduring tissue inflammation are critical to the occurrence of cancer and tissue fibrosis, we proposed that promotion of oral mucosal inflammation by BQ components is vital to the pathogenesis of oral cancer and OSF. In accord with this, we recently found that areca nut (AN) components may stimulate PGE_2 and prostacyclin production by gingival keratinocytes (GK) with concomitant induction of Cox-2 mRNA and protein production (17). Interestingly, an immunohistochemical investigation by Haque *et al*. (18) has lately found that OSF tissues express higher levels of IL-6 than comparable healthy tissues. Haque *et al*. (19) found elevated IL-6 and TNF-α production by peripheral blood mononuclear cells (PBMC) isolated from OSF patients, relative to normal healthy subjects. A TNF-α allelic polymorphism has also been shown to be linked to the occurrence of OSF (20). These results support the possible contribution of AN-induced release of inflammatory mediators in the pathogenesis of oral cancer and OSF. However, the detailed mechanisms relating to how IL-6 and TNF-α are induced by BQ ingredients should be further clarified.

PGs are important for the initiation, promotion and progression of chemical carcinogenesis (14). PGs can suppress the humoral and cellular immune action responsible for the killing of malignant cancer cells (14,15). On the other hand, IL-6 induces activator protein-2 mRNA and protein production by normal skin keratinocytes, an effect leading to skin inflammation, keratinocyte growth and carcinogenesis (21). IL-6 is involved in a variety of biological processes, including the immune response, inflammation and carcinogenesis by regulating the growth, survival and differentiation of target cells (22). IL-6 has also been shown to be an autocrine and paracrine factor in stimulating the proliferation of skin

keratinocytes (23–25) and the activity of oropharyngeal cancer cells (12). On the other hand, TNF-α is crucial in tumor promotion and progression via induction of tumor transformation and growth, angiogenesis, modification of matrix proteins and expression of adhesion molecules (26,27). It appears that the role of Cox-2, TNF-α and IL-6 in the oral carcinogenic processes is complex, intricate and needs to be further addressed.

Clinical studies also support the crucial roles of PGE_2, IL-6 and TNF-α in head and neck cancer (12,26,28,29). Head and neck squamous cell carcinoma (HNSCC) tissues show a higher level of COX-2 protein and 150-fold greater Cox-2 mRNA expression than healthy oral mucosa (28). Gallo *et al*. found high serum IL-6 levels in HNSCC patients (30). Using an immunohistochemical technique, universal intracellular expression of IL-6 in invasive squamous cell canicinoma of tongue, mouth floor and palate has also been reported (31). Nakano *et al*. also reported elevated expression of IL-6 and TNF-α proteins and mRNA in oral squamous cell carcinoma tissues (26). It has also been suggested that aberrant elaboration of biologically active IL-6 may contribute to altered immune status in human HNSCC patients (31). Consistently, T and B lymphocytes and macrophages have been detected in the epithelium and sub-epithelial connective tissues of OSF patients (32). Immunological abnormalities are observed in patients with OSF (7,18,19). Administration of immune milk that contains anti-inflammatory components may improve the signs and symptoms of OSF patients (33). These results may explain why a cytokine-rich oral cavity, such as in periodontal disease, may contribute to accelerated carcinogenesis, as suggested by Hong *et al*. (12). We therefore tested whether AN component induce PGE_2, IL-6 and TNF-α mRNA and protein production in primary GK and oral KB carcinoma cells. Moreover, the roles of PGE_2, IL-6 and TNF-α in regulating the biological activities of oral epithelial cells and the toxicity of AN components were also investigated.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal calf serum (FCS), penicillin/streptomycin, keratinocyte growth medium (KGM-SFM), pituitary gland extract and epidermal growth factors were from Gibco (Life Technologies BRL, Grand Island, NY). ELISA kits for TNF-α (ultrasensitive) and IL-6 measurement, recombinant IL-6 and IL-6 and TNF-α neutralizing antibody (ab) were from Biosource International (Camarillo, CA). Calf skin type I collagen, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), arecoline, aspirin and bovine plasma fibronectin were obtained from Sigma Chemical Co. (St Louis, MO). PGE_2 ELISA kits and PGE_2 were purchased from Cayman Chemical Co. (Ann Arbor, MI). Ethidium bromide, agarose and kits for reverse transcription (RT) and PCR were purchased from HT Biotech Inc. (Oxford, UK). Total RNA isolation kits were from Qiagen Inc. (Santa Clarita, CA). AN extract was prepared and weighed as previously described with slight modifications (17,34–38). Briefly, AN was cut into small pieces and lyophilized. Fifty grams of the lyophilized AN pieces were ground into fiber and powder by a motor-driven coffee blender and extracted with 1 l of ice-cold double-distilled water at 4°C for 4 h. The mixture was centrifuged at 5000 r.p.m. for 15 min and then the supernatant was filtered with Advantec filter paper (Toyo Roshi Kaisha Ltd, Tokyo, Japan). The filtrates were lyophilized and weighed before use. Specific PCR primer sets for Cox-2, β-actin (17) and IL-6 were synthesized by Genemed Biotechnologies (San Francisco, CA). Mouse anti-human COX-2 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Protein assay kits were obtained from Bio-Rad (Hercules, CA). Phycoerythrin-conjugated anti-human $CD4^+$ and $CD8^+$ and FITC-conjugated anti-human $CD69^+$ ab, IgG (isotype control) and flow cytometric reagents were obtained from Becton-Dickinson (San Jose, CA), whereas ab for γ1-FITC/γ1-PE was from Serotec Inc. (Raleigh, NC).

Culture of KB oral carcinoma cells and primary gingival keratinocytes (GK)

Oral KB carcinoma cells were obtained from ATCC and cultured in DMEM supplemented with 10% FCS and penicillin/streptomycin. GK were established and cultured in KGM-SFM containing EGF and pituitary gland extract as described previously (17,35,38). GK between passage numbers 1 and 2 were used for these studies.

Effects of AN extract and arecoline on IL-6, TNF-α and PGE_2 production by GK and KB cells and concomitant cytotoxicity

Oral KB cells (5×10^5 cells) were inoculated into each well of a 6-well culture plate. GK were used when near confluence. Cells were first starved in serum-free DMEM (for KB cells) or KGM-SFM without supplement (for GK) for 24 h. The medium (1 ml) was replaced by medium containing various concentrations of AN extract or arecoline for 48 h. Culture medium was collected for measurement of IL-6, TNF-α and PGE_2 levels. Cell layers were washed three times to avoid any effects of AN components on the measurement of cytotoxicity. Finally, cytotoxicity was measured by MTT assay as described previously (17,35). Briefly, cell layers were incubated with fresh medium containing 0.5 mg/ml MTT for 2 h. The formazan produced was dissolved in 2 ml of DMSO and read against a solvent blank (DMSO) with a Dias Microwell Plate Reader (Dynatech Medical Products Ltd, St Peter Port, UK) at an optical density of 540 nm.

Chemical exposure, RNA isolation and preparation of cell lysate

Near confluent GK (in KGM-SFM without supplement) and KB cells (in serum-free DMEM) in 10 cm culture dishes were exposed to various concentrations of AN extract (100–1200 μg/ml) or arecoline (0.1–1.2 mM) for 24 h. Total cellular RNA was isolated using RNA isolation kits (Qiagen).

For western blot analysis, cell lysates of KB cells were collected as described above (17). Briefly, KB cells were exposed to AN extract (100–1200 μg/ml) or arecoline (0.1–0.8 mM) for 24 h. Cells were rinsed three times with ice-cold phosphate-buffered saline (PBS), scraped off with a rubber policeman and collected by centrifugation at 800 *g* for 5 min. Cells were dissolved in 250 μl of freshly prepared lysis buffer (10 mM Tris–HCl, pH 7, 140 mM sodium chloride, 3 mM magnesium chloride, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride, 1% aprotinin and 5 mM dithiothreitol) for 20 min on ice. The cell lysate was cleared by centrifugation at 4°C for 30 min at 10 000 *g* and employed for 12% SDS–PAGE and western blot analysis using G3PDH- and COX-2-specific ab as described previously (17). In short, equal amounts of protein (50 μg/lane) were separated by 12% SDS–PAGE (Scie-Plas Ltd, Warwickshire, UK) and transferred to PVDF membrane (MEN™; Life Science, Boston, MA) by electroblotting. The membrane was then blocked for 30 min at room temperature in blocking reagent (20 mM Tris, pH 7.4, 125 mM NaCl, 0.2% Tween 20, 5% non-fat dry milk and 0.1% sodium azide) and then incubated for 2 h with mouse anti-human G3PDH and COX-2 monoclonal ab (1:1000). Membranes were washed three times with TBST (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 10 min each and then incubated with horseradish peroxidase-labeled goat anti-mouse secondary ab for 1 h. Then the membrane was washed four times with TBST. Finally the immunoreactive bands were visualized by enhanced chemiluminescence reagent (MEN™) developed on Fuji X-ray film. Protein concentration was measured with Bio-Rad protein assay kits.

Effects of AN extract and arecoline on IL-6 and Cox-2 mRNA expression in KB carcinoma cells

Briefly, 3 μg of denatured total RNA was subjected to RT in a total volume of 44.5 μl reaction mixture containing 4 μl of random primer (500 μg/ml), 8 μl of dNTP (2.5 mM), 4.5 μl of 10× RT buffer, 1 μl of RNase inhibitor (40 U/μl) and 0.5 μl of reverse transcriptase (21 U/μl) at 42°C for 90 min. Four microliters of cDNA was used for PCR amplification in a reaction volume of 50 μl containing 5 μl of 10× SuperTAQ buffer, 4 μl of dNTP (2.5 mM), 1 μl of each specific primer and 0.2 μl of SuperTAQ enzyme (2 U/μl). Specific primers for Cox-2 were 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′. Primers for β-actin were 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-TACATGGCTGGGGTGTTGAA-3′ (17). The PCR primer sequences for IL-6 were ATGAACTCCTTCTCCACAAGCGC (sense) and GAAAGCCCTCAGGCTGGACTG (antisense) (39). The amplified DNA products were 305 bp for Cox-2, 218 bp for β-actin and 628 bp for IL-6, respectively. The reaction mixture was placed at 94°C for 5 min in the first cycle. The reaction was then run for 15–35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min with a thermal cycler (Perkin Elmer 2400; PE Applied Biosystems, Foster City, CA). Finally, the reaction was terminated following an extension at 72°C for 10 min. Amplified DNA products were subjected to 1.8% agarose gel electrophoresis and stained with ethidium bromide. The amount of amplified DNA product that showed a linear relation with respect to the input RNA was used for data presentation.

Effects of PGE_2, IL-6 and IL-6 neutralizing ab on the growth of KB cells, GK or oral fibroblasts (OMF)

Briefly 1×10^4 cells were seeded into 24-well culture plates. After 24 h, the medium was changed for serum-free DMEM (for KB cells and OMF) or KGM-SFM without supplement (for GK) or containing various concentrations of PGE_2 (0.05–5 μg/ml), IL-6 (0.1–100 ng/ml) or IL-6 neutralizing ab (250–1000 ng/ml) for 5 days. Changes in cell number were measured by the MTT assay as described previously (17,35).

Effects of IL-6 neutralizing ab on AN cytotoxicity to KB cells and GK

AN extract has been shown to be cytotoxic to oral keratinocytes at concentrations >800 μg/ml. Since AN extract obviously induced IL-6 production by oral KB cells and GK, it is interesting to know whether AN cytotoxicity is correlated with its induction of IL-6 production. We therefore used anti-IL-6 ab to neutralize the action of IL-6 to elucidate this question. Briefly, confluent GK or KB cells (5×10^5 cells/well) were exposed to 1 ml of culture medium containing IL-6 neutralizing ab (250 and 1000 ng/ml) for 30 min prior to the addition of AN extract (400 and 800 μg/ml). Cells were further incubated for 48 h. Cells were washed with PBS and cytotoxicity was measured by the MTT assay.

Effects of aspirin or IL-6 and TNF-α neutralizing ab on arecoline- and AN-induced cell cycle arrest and apoptosis of KB cells

For further elucidation of whether AN ingredients and arecoline can modulate cell cycle progression under serum-free condition, 5×10^5 KB cells were seeded and treated with AN extract or arecoline as described above for 24 h. For measurement of cellular DNA content, flow cytometric analysis was used as described previously (36,40). Briefly, floating cells and attached cells were collected and together poured into centrifuge tubes. Attached KB cells were washed with PBS and removed from the culture wells by trypsin/EDTA. Cells from two culture wells with similar exposure conditions were collected together, resuspended and fixed in 70% ice-cold ethanol containing 2 mg/ml RNase for 30 min. They were washed twice with PBS and eventually stained with propidium iodide (40 μg/ml) for 10 min at room temperature. The propidium iodide fluorescence of individual KB cells was analyzed with a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA) supplemented with an argon ion laser. The wavelength of laser excitation was set at 488 nm and emission collected at >590 nm. In total 20 000 cells were analyzed for the control and experimental samples. The percentage of cells in the sub-G_0/G_1, G_0/G_1, S and G_2/M phases were determined using standard ModiFit software and CellQuest programs. In some experiments, KB cells were treated with aspirin (25 μM), IL-6 ab (1000 ng/ml) or TNF-α ab (500 ng/ml) for 30 min prior to the addition of AN extract (400 and 800 μg/ml) or arecoline (0.8 and 1.2 mM) and co-incubated for 24 h. Cells were similarly collected for flow cytometric analysis as described above.

Effects of PGE_2 on IL-6 production by KB cells and GK

Briefly, near confluent GK or KB cells (5×10^5 cells/well) were exposed to 1 ml of culture medium without supplement or containing PGE_2 (0.05–2.5 μg/ml) for 48 h. Culture medium was collected for measurement of IL-6 production.

Effects of TNF-α neutralizing ab on AN extract- and arecoline-induced PGE_2 and IL-6 production

Briefly, near confluent GK or KB cells (5×10^5 cells/well) were exposed to 1 ml of culture medium without supplement or containing TNF-α neutralizing ab (1000 ng/ml) and AN extract or arecoline for 48 h. Culture medium was collected for measurement of PGE_2 and IL-6 production.

Effects of cytokine-rich cell culture supernatants on $CD4^+$ and $CD8^+$ T cell activation

AN extract and arecoline may modulate the release of inflammatory mediators such as PGE_2, IL-6, TNF-α and other untested proteins from oral epithelial cells, which may potentially regulate the local immune activity or enhance the oral carcinogenic effects of carcinogens leading to oral cancer. We therefore tested the interplay between epithelial cells and immune effecter cells like $CD4^+$ and $CD8^+$ T cells. Briefly, GK and KB cells in 6-well plates were treated with AN extract or arecoline as described above for 48 h. Cell culture medium was centrifuged and the supernatants were collected for subsequent usage.

Isolation of human PBMC was performed as described previously (41). Briefly, PBMC were isolated from healthy adult volunteers by Ficoll-Hypaque centrifugation. Isolated lymphocytes (5×10^6 cells/well) were inoculated into 6-well plates in 1 ml of RPMI 1640 medium supplemented with 10% FCS and 1 ml of AN extract- or arecoline-conditioned culture medium for 24 h. In some experiments, phytohemagglutinin (PHA) (4 μg/ml) was added to induce $CD4^+$ and $CD8^+$ T cell activation for evaluation of whether conditioned medium suppressed T cell activation. After treatment, PBMC were collected into 15 ml tubes, centrifuged (1500 r.p.m., 5 min) and the supernatant was decanted. Cells were washed with 2 ml of 1% BSA/PBS and stained with CD4-PE/CD69-FITC, CD8-PE/CD69-FITC or γ1-FITC/γ1-PE for 30 min in the dark. Finally, cells were washed and resuspended in 1% BSA/PBS and subjected to flow cytometric analysis with an excitation wavelength of 488 nm.

Statistical analysis

Two or more separate experiments were performed for each case. Statistical analyses were conducted using the paired Student's *t*-test. A P value < 0.05 was considered to show a significant difference between the experimental and control groups.

Results

Modulatory effects of AN extract and arecoline on PGE_2 production by KB cells

AN extract has recently been found to stimulate PGE_2 and prostacyclin production by GK (17). Since PGE_2 may modulate the behavior of cancer cells (14,15), we further elucidated whether AN components show similar stimulatory effects on PGE_2 production by KB carcinoma cells. As shown in Figure 1a, AN extract (100–400 μg/ml) stimulated PGE_2 production by 2.34-, 17.7- and 23.1-fold relative to that of the control (average PGE_2 concentration 173 ± 22 pg/ml). At concentrations of 800 and 1200 μg/ml the stimulatory effect of AN extract on PGE_2 production by KB cells reached a plateau. An interesting question was which AN component was responsible for the induction of PGE_2 production. Arecoline, as a major areca alkaloid, was one possible contributing factor. We found that arecoline stimulated PGE_2 production by KB cells in a dose-dependent manner. As depicted in Figure 1b, 48 h exposure to arecoline (0.2–1.2 mM) stimulated PGE_2 production by KB carcinoma cells by 1.5- and 6.1-fold. Arecoline (0.4 and 0.8 mM)-induced PGE_2 production by KB cells was inhibited by 25 μM aspirin, a COX inhibitor (Table I). Further studies on the effect of arecaidine and catechin, the other two AN components, on PGE_2 production by oral epithelial cells is now in progress in our laboratory.

Differential effects of AN extract and arecoline on IL-6 and TNF-α production by GK and KB cells

IL-6 is the other inflammatory mediator that may contribute to keratinocyte inflammation (11,13). A recent study revealed that salicylate and indomethacin (two COX inhibitors) reduced the synthesis of IL-6 by MDA-MB-231 and Hs578T breast cancer cells. Exogenous PGE_2 increases IL-6 production by these cells (42). It is therefore reasonable to speculate that AN extract may stimulate IL-6 production partly mediated by activation of COX. As expected, AN extract stimulated IL-6 production by GK at concentrations ranging from 100 to 1200 μg/ml. As shown in Figure 2a, at concentrations of 200 and 400 μg/ml AN extract induced IL-6 production in GK 7.5- and 8.4-fold over that of the untreated control (with an average medium IL-6 concentration of 48.3 ± 8.9 pg/ml). However, this stimulatory effect of AN was not due to its arecoline content. As shown in Figure 2b, arecoline showed only mild stimulatory effects on IL-6 production by GK at a concentration of 0.2 mM. At concentrations of 0.4–1.2 mM arecoline showed a marked suppressive effect on IL-6 production by GK, with 42–81% inhibition. Similarly, AN extract also augmented IL-6 production by KB carcinoma cells. Maximal stimulatory effects were found when the concentrations of AN extract were 200 and 400 μg/ml, with 14- and 13.4-fold

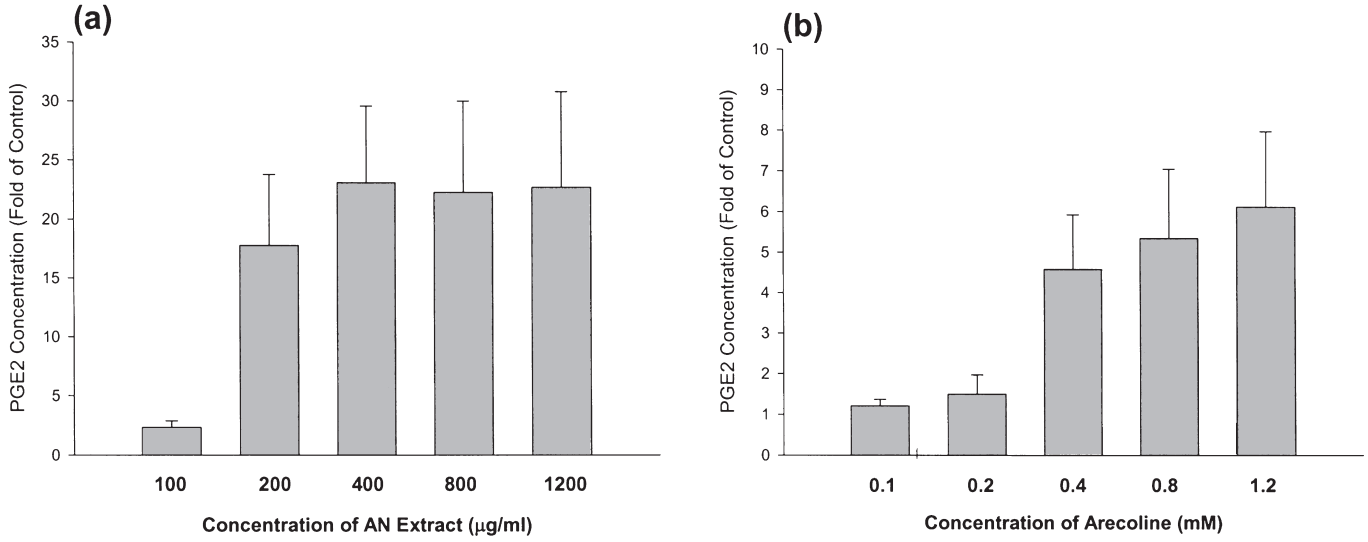

Fig. 1. Effects of AN extract and arecoline on PGE_2 production by KB cells. Results are expressed as fold of control. (**a**) KB cells were treated with 100–1200 μg/ml AN extract for 48 h ($n = 4$); (**b**) KB cells were treated with 0.1–1.2 mM arecoline for 48 h (control = 173 ± 22 pg/ml, $n = 7$).

Table I. Effect of aspirin on arecoline-induced PGE_2 and IL-6 production by KB cells ($n = 3$)

Chemicals	PGE_2 (pg/ml)	IL-6 (pg/ml)	MTT reduction (% of control)
Control	231 ± 11	299 ± 5	100
Arecoline (0.4 mM)	425 ± 68	69 ± 6	75 ± 1.5
Aspirin (25 μM)	146 ± 39	320 ± 32	97 ± 2.1
Arecoline (0.4 mM) + aspirin (25 μM)	304 ± 67	68 ± 3	76 ± 1.5
Arecoline (0.8 mM)	726 ± 33	52 ± 2	40 ± 2.7
Arecoline (0.8 mM) + aspirin (25 μM)	280 ± 52	48 ± 2	26 ± 7.1

Data for IL-6 and PGE_2 are presented as means ± SE (pg/ml). Cell number was presented as percent MTT reduction of control (100%) as assayed at OD_{540}.

increases in IL-6 production, respectively (Figure 2c). IL-6 production by KB cells was also inhibited by arecoline. Even at concentrations of 0.1 and 0.2 mM, arecoline markedly inhibited IL-6 production by 41 and 63%, respectively (Figure 2d). Arecaidine, the other areca alkaloid, showed little effect on IL-6 production by KB cells (data not shown). Aspirin showed little protective effect against arecoline-mediated IL-6 production inhibition (Table I). TNF-α production by GK was also altered after treatment with AN extract. As shown in Figure 2e, the basal level of TNF-α in the culture medium of untreated GK was ~3.9 pg/ml. Exposure of GK to AN extract (200 and 400 μg/ml) elevated the TNF-α concentration to 7.2 and 19.9 pg/ml, respectively. On the other hand, arecoline showed little effect on TNF-α production by GK under these experimental conditions (Figure 2f). The TNF-α level in the culture medium of KB cells was below the detection limit of the ELISA kit (data not shown).

Concomitant cytotoxicity of AN extract and arecoline on GK and oral KB cells

Since the cytotoxicity of AN components has been shown to be dependent on cell density, concentrations of chemical and exposure time (43), it is critical to evaluate the cytotoxicity of AN extract and arecoline on oral epithelial cells under these experimental conditions. As shown in Figure 3a, a 48 h exposure of GK to 100–200 μg/ml AN extract produced no marked cytotoxicity. Further elevating the AN concentration to 800 and 1200 μg/ml resulted in 37 and 69% cell death, respectively. Arecoline at concentrations below 0.4 mM also lacked marked cytotoxicity on GK. At concentrations of 0.8 and 1.2 mM arecoline led to 28 and 38% GK cell death, respectively (Figure 3b). Similarly, exposure of KB carcinoma cells to AN extract for 48 h decreased the cell number by 24–42% at concentrations ranging from 400 to 1200 μg/ml (Figure 3c). A 48 h exposure of KB cells to 0.4, 0.8 and 1.2 mM arecoline led to 22, 56 and 95% cell death, respectively (Figure 3d). Aspirin, however, showed little effect on arecoline-induced cytotoxicity to KB carcinoma cells (Table I).

Effects of AN extract and arecoline on IL-6 and Cox-2 mRNA expression in GK and KB cells

Differential effects of AN components and arecoline on PGE_2 and IL-6 production may be due to their regulation of Cox-2 and IL-6 mRNA expression in oral epithelial cells. As indicated in Figure 4a, the expression of Cox-2 and IL-6 was generally low in untreated cells. Exposure of KB cells to AN extract (400 and 800 μg/ml) markedly elevated Cox-2 and IL-6 mRNA expression (Figure 4a). These results generally conform to the measurement of PGE_2 and IL-6 production in the culture medium. On the other hand, arecoline decreased IL-6 production by KB cells with 24 h exposure. Only a faded band of amplified IL-6 (628 bp) PCR product was noted when RNA was isolated from untreated cells. When treated with 0.1–1.2 mM arecoline, no marked PCR product was noted on agarose gels (Figure 4b). Unexpectedly, arecoline (0.1–1.2 mM) showed little effect on Cox-2 mRNA expression within 24 (Figure 4b) or even 48 h of exposure (data not shown).

Effects of AN extract and arecoline on COX-2 protein production by KB cells

Cell lysate was further subjected to western blotting for elucidation of whether induction of Cox-2 mRNA expression by AN extract and arecoline was related to COX-2 protein production. As shown in Figure 5a, AN extract (200–1200 μg/ml) stimulated COX-2 protein production by KB cells in a dose-dependent fashion following 24 h exposure. This result is

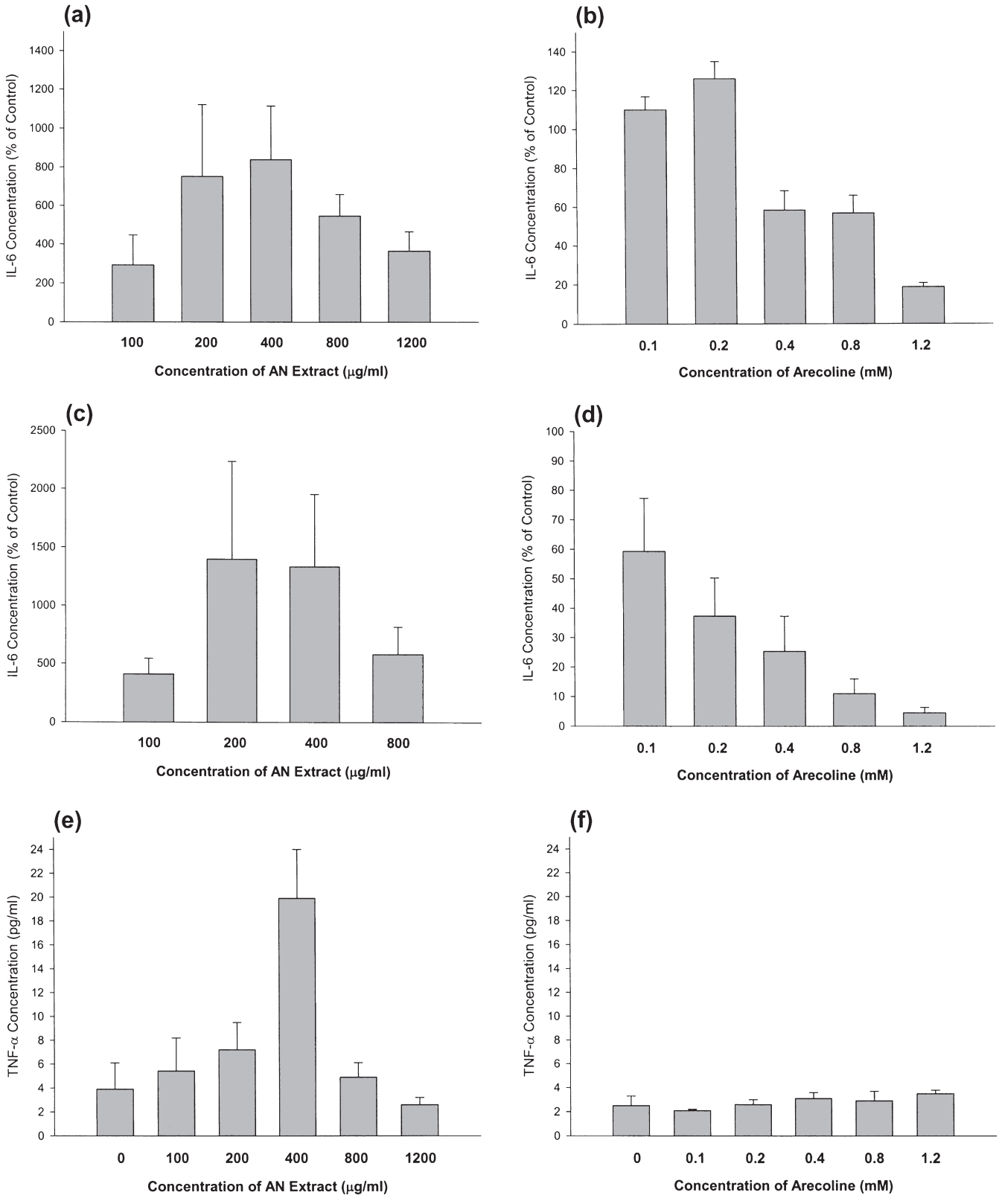

Fig. 2. Effects of AN extract and arecoline on IL-6 and TNF-α production by GK and KB cells. IL-6 production was measured by enzyme-linked immunosorbent assay. Results are expressed as fold or percentage of control. (**a**) GK were treated with 100–1200 μg/ml AN extract for 48 h ($n = 4$); (**b**) GK were treated with 0.1–1.2 mM arecoline for 48 h ($n = 5$); (**c**) KB cells were treated with 100–1200 μg/ml AN extract for 48 h ($n = 4$); (**d**) KB cells were treated with 0.1–1.2 mM arecoline for 48 h ($n = 4$); (**e**) TNF-α level of GK after exposure to 100–1200 μg/ml AN extract for 48 h ($n = 3$); (**f**) TNF-α level of GK after exposure to 0.1–1.2 mM arecoline for 48 h ($n = 3$).

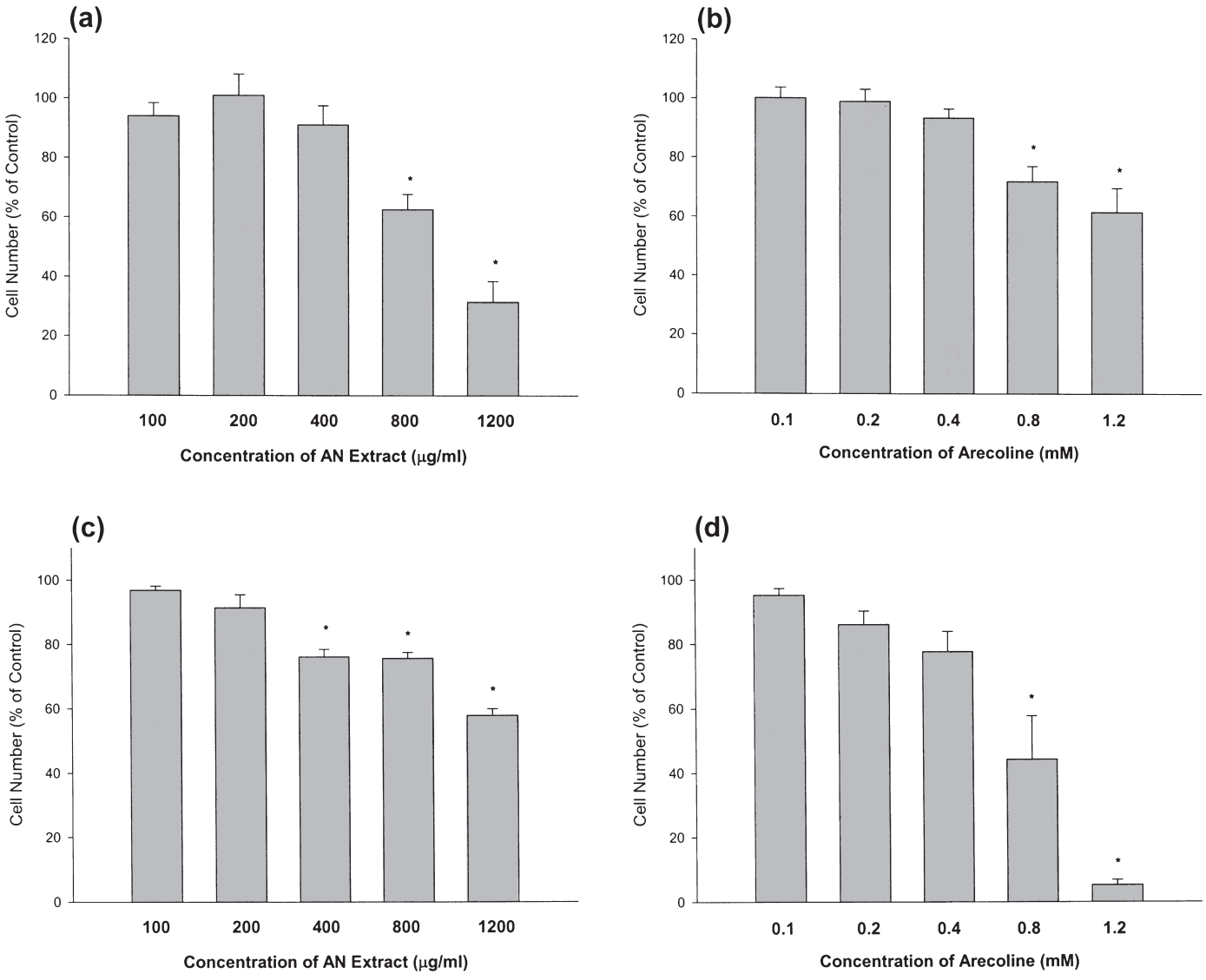

Fig. 3. Cytotoxicity of AN extract and arecoline to GK and KB cells following 48 h exposure. Cells were treated similarly to as described for the measurement of PGE_2 and IL-6. Following washing of cells with PBS, cells were incubated in fresh medium containing 0.5 mg/ml MTT for 2 h. The insoluble formazan was dissolved in 2 ml of DMSO and read against a blank at OD_{540}. Results are expressed as a percentage of control (as 100%). (**a**) GK treated with 100–1200 μg/ml AN extract for 48 h ($n = 8$); (**b**) GK treated with 0.1–1.2 mM arecoline for 48 h ($n = 7$); (**c**) KB cells treated with 100–1200 μg/ml AN extract for 48 h; (**d**) KB cells treated with 0.1–1.2 mM arecoline for 48 h. * denotes marked difference ($P < 0.05$).

generally in accord with a recent report that AN extract up-regulates COX-2 protein production (17). However, arecoline (0.1–1.2 mM) showed little effect on COX-2 production within 24 (Figure 5b) and even 48 h of exposure (data not shown).

Effects of IL-6 and IL-6 neutralizing ab on the growth of GK and KB cells

IL-6 has been shown to be mitogenic to skin keratinocytes (23–25) and esophagus cancer cells (12), which could be crucial for tissue inflammation and carcinogenesis. We need to know whether IL-6 is directly mitogenic to GK and oral KB cells. As demonstrated in Figure 6a, IL-6 slightly suppressed the proliferation of GK at concentrations ranging from 1 to 100 ng/ml, with 20–33% inhibition. On the other hand, IL-6 showed a tendency to enhance the growth of KB cells, with cell number increasing by 11–23% at concentrations ranging from 0.5–100 ng/ml. However, a statistical difference was not evident ($P > 0.05$) (Figure 6b). Although PGE_2 may induce cAMP production and calcium signaling in dental pulp cells (Jeng *et al.*, unpublished observations), PGE_2 is not mitogenic to GK (17). Similarly, PGE_2 showed little effect on the growth of KB cells at concentrations of 0.05–5 μg/ml (Figure 6c). Incubation of GK with IL-6 ab (50–1000 ng/ml) for 5 days also showed little effect on their growth (data not shown). Interestingly, IL-6 stimulated the growth of OMF by 14–26% at concentrations ranging from 1.25 to 125 ng/ml (Figure 6d), a potential contributing factor to OSF (44).

Effects of IL-6 neutralizing ab on AN-induced cytotoxicity

Since AN extract may stimulate IL-6 production by GK and KB cells, we subsequently tested whether stimulation of IL-6 production by AN extract is responsible for its cytotoxicity. We used IL-6 ab to neutralize AN extract-induced IL-6 in KB cells. As illustrated in Figure 7, exposure of KB cells to 800 μg/ml AN extract for 48 h induced marked cytotoxicity, with the average cell number decreasing by 54%. Inclusion of IL-6 ab (250 and 1000 ng/ml) showed no marked protection against AN-induced cytotoxicity.

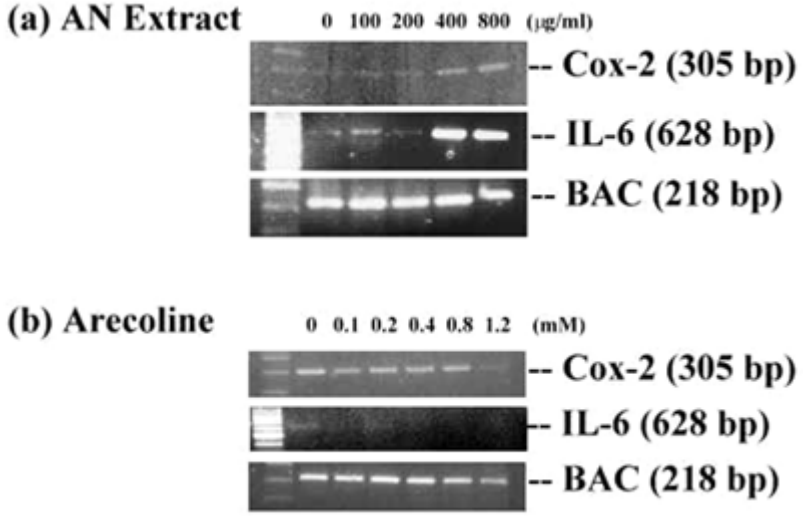

Fig. 4. Effects of AN extract and arecoline on IL-6 and Cox-2 mRNA expression in KB cells. KB cells were exposed to (**a**) AN extract or (**b**) arecoline for 24 h. IL-6 and Cox-2 mRNA expression was measured by RT–PCR.

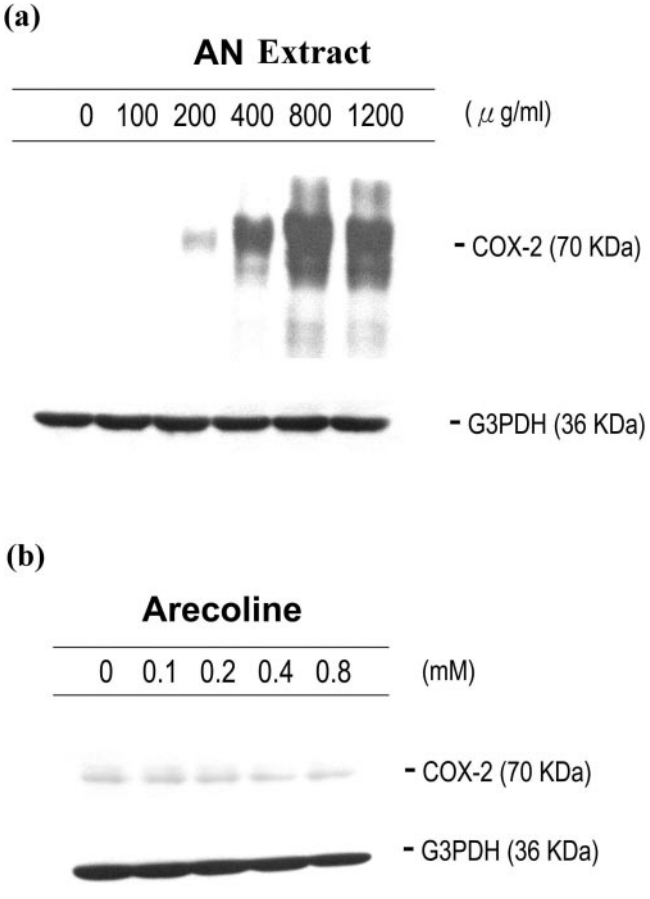

Fig. 5. Effect of AN extract and arecoline on COX-2 protein (70 kDa) production by KB cells. KB cells were exposed to (**a**) 100–1200 μg/ml AN extract or (**b**) 0.1–1.2 mM arecoline for 24 h. COX-2 protein production by KB cells was measured by western blot using COX-2 monoclonal antibody. G3PDH (36 kDa) was used as a control protein.

Effects of aspirin, IL-6 neutralizing ab and TNF-α neutralizing ab on arecoline- and AN-induced cell cycle arrest and apoptosis of KB cells

To further clarify whether PGE_2, IL-6 and TNF-α also play some role in arecoline- and AN-induced DNA changes and cell growth control, KB cells were pretreated with aspirin (25 μM), IL-6 neutralizing ab (1000 ng/ml) or TNF-α ab (500 ng/ml) for 30 min and then exposed to AN extract (400 and 800 μg/ml) or arecoline (0.8 and 1.2 mM) for 24 h. Exposure of KB cells to AN extract and arecoline under serum-free conditions for 24 h led to marked S and G_2/M phase cell cycle arrest and apoptosis (Figure 8a and b). Inclusion of anti-IL-6 ab showed little effect on AN extract- and arecoline-induced cell cycle arrest and apoptosis (Figure 8a). Similarly, addition of anti-TNF-α ab was also not effective in prevention of AN extract- and arecoline-induced cell cycle arrest and apoptosis (Figure 8b). Aspirin also lacked a preventive effect against AN extract- and arecoline-induced cell cycle arrest and apoptosis (data not shown).

Effects of PGE_2 on IL-6 production by GK and KB cells

Exposure of GK to PGE_2 for 48 h markedly elevated the IL-6 level in the culture medium. As shown in Table II, PGE_2 stimulated IL-6 production by GK by 2.1- to 2.6-fold at concentrations ranging from 50 ng/ml to 1.25 μg/ml. PGE_2 treatment elevated the IL-6 concentration in the culture medium from 21.3 to 44.3–56.7 pg/ml. In contrast, PGE_2 decreased IL-6 production by KB cells at concentrations >0.5 μg/ml. IL-6 levels decreased from 292 to <200 pg/ml (Table II).

Effect of TNF-α neutralizing ab on AN extract- and arecoline-induced cytotoxicity, PGE_2 and IL-6 production by KB cells

Unexpectedly, TNF-α neutralizing ab partly affected the basal level and AN extract- and arecoline-mediated PGE_2 and IL-6 production in KB cells (Tables III and IV) and GK ($n = 2$, data not shown). AN extract stimulated IL-6 and PGE_2 production by KB cells, but led to death of KB cells to some extent (Table III). Inclusion of TNF-α ab slightly enhanced the basal levels of IL-6 and PGE_2 (but $P > 0.05$) and AN extract (800 μg/ml)-stimulated IL-6 production ($P < 0.05$). However, TNF-α ab showed little effect on AN extract-induced cytotoxicity (Table III). TNF-α ab also lacked a protective effect against arecoline-induced cytotoxicity toward KB cells (Table IV). However, TNF-α ab partly prevented the arecoline-induced IL-6 reduction and arecoline-stimulated PGE_2 production by KB cells ($P < 0.05$) (Table IV).

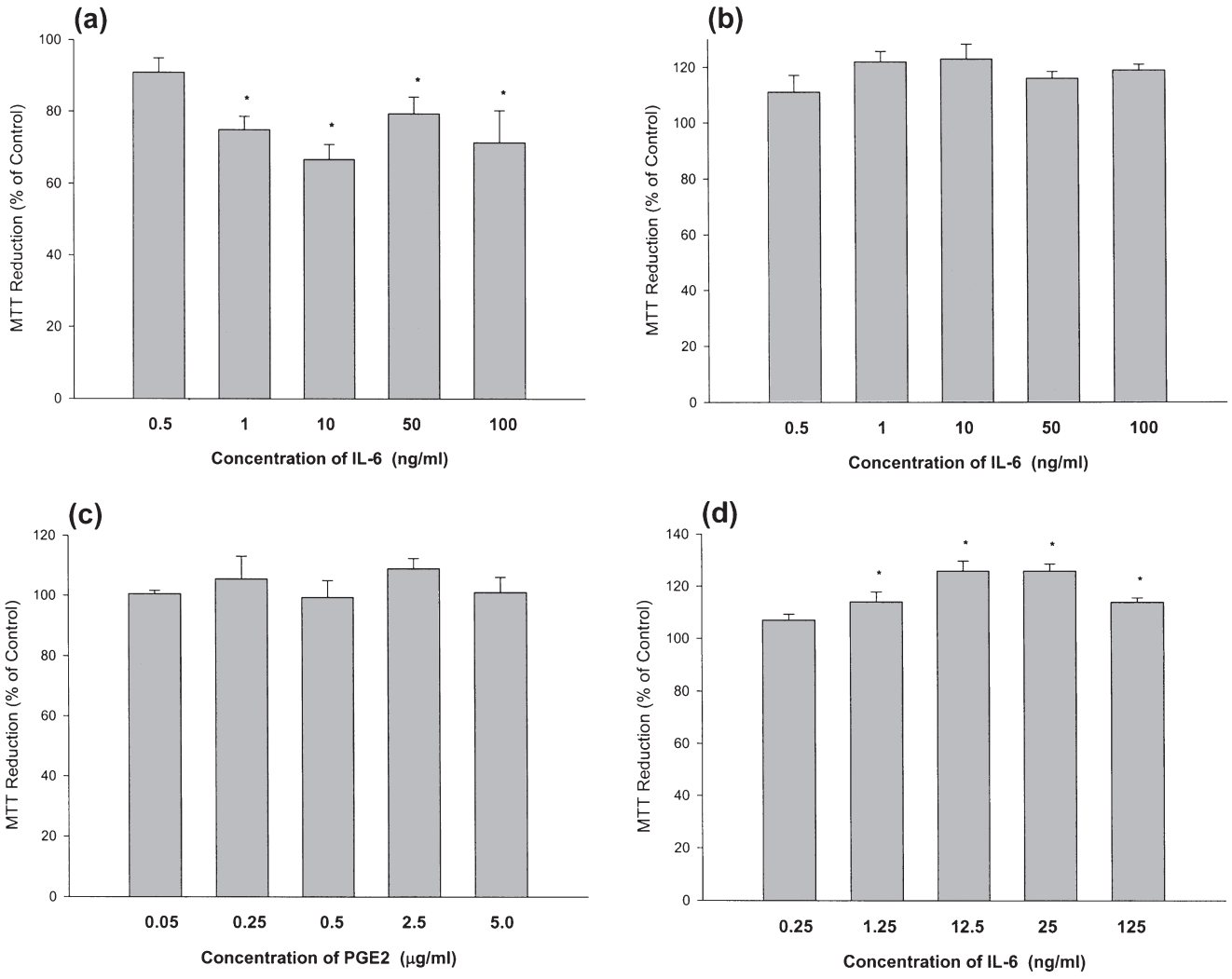

Fig. 6. Effects of IL-6 and IL-6 neutralizing ab on the growth of OMF, GK and KB cells. (**a**) Exposure of GK to IL-6 for 5 days; (**b**) exposure of KB to IL-6 for 5 days; (**c**) exposure of GK to IL-6 ab for 5 days; (**d**) exposure of OMF to IL-6 for 5 days. Two or more separate experiments were done with similar results. Data from one representative experiment in quadruplicate are shown ($n = 4$). * denotes marked difference ($P < 0.05$).

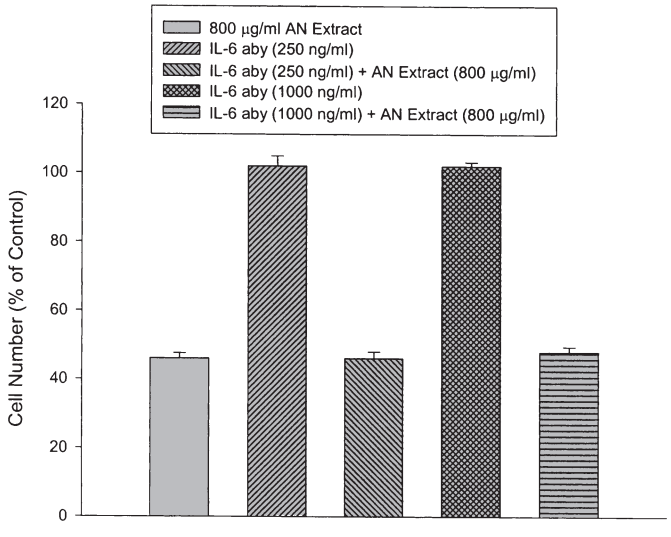

Fig. 7. Effect of IL-6 ab on AN cytotoxicity to KB cells. KB cells were exposed to IL-6 ab (250 and 1000 ng/ml) 30 min prior to the addition of AN extract (400 or 800 μg/ml). Cells were further incubated for 48 h and cytotoxicity was measured by the MTT assay. Results are expressed as a percentage of control (100%) cell number ($n = 3$).

Effect of conditioned cell culture supernatants on $CD4^+$ and $CD8^+$ cell activation

Conditioned medium from GK and KB cells treated with AN extract and arecoline was unable to induce CD69 activation in both $CD4^+$ and $CD8^+$ T cells (data not shown). On the contrary, arecoline obviously inhibited PHA-induced $CD4^+$ and $CD8^+$ T cell activation. As shown in Figure 9a–c, PHA (4 μg/ml) stimulated $CD4^+$ and $CD8^+$ T cell activation as revealed by the increase in cells expressing $CD69^+$ to 41.4 and 52%, respectively (upper right region, Figure 9a). In the presence of arecoline (0.8 and 1.2 mM)-conditioned cell culture supernatant, the expression of CD69 on $CD4^+$ and $CD8^+$ T cells was markedly suppressed (Figure 9b and c). Exposure of isolated PBMC to AN extract-conditioned medium also inhibited the activation of $CD4^+$ and $CD8^+$ T cells, as revealed by a decrease in the percentage of cells expressing CD69 (Figure 9d and e). Similar results were observed when AN extract- and arecoline-conditioned medium isolated from GK was tested (data not shown).

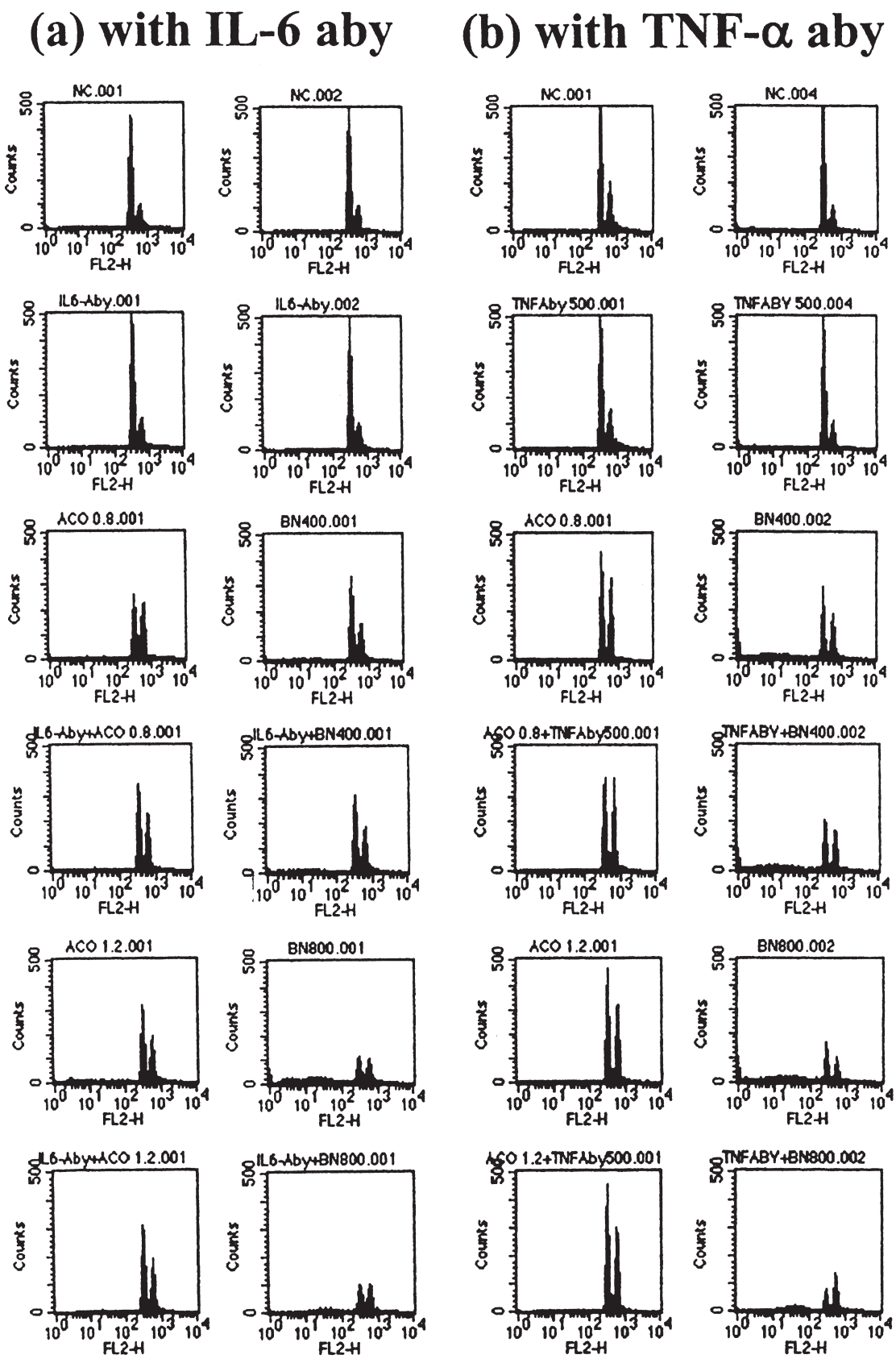

Fig. 8. Effect of IL-6 and TNF-α neutralizing ab on AN extract- and arecoline-induced cell cycle arrest and apoptosis. Cells were treated with (**a**) IL-6 ab (1000 ng/ml) or (**b**) TNF-α ab (500 ng/ml) and then exposed to arecoline or AN extract for 24 h. Cells were harvested, stained with propidium iodide and then analyzed by flow cytometry. DNA content is represented on the *x*-axis and cell count on the *y*-axis. Cell cycle arrest and apoptosis of KB cells were noted following exposure to AN extract and arecoline under serum-free conditions. The results of one representative histogram are shown here with more than three similar results. The concentrations of arecoline, AN extract and antibodies are depicted in the upper part of each figure. NC, untreated control; ACO 0.8, 0.8 mM arecoline; BN400, 400 μg/ml AN extract.

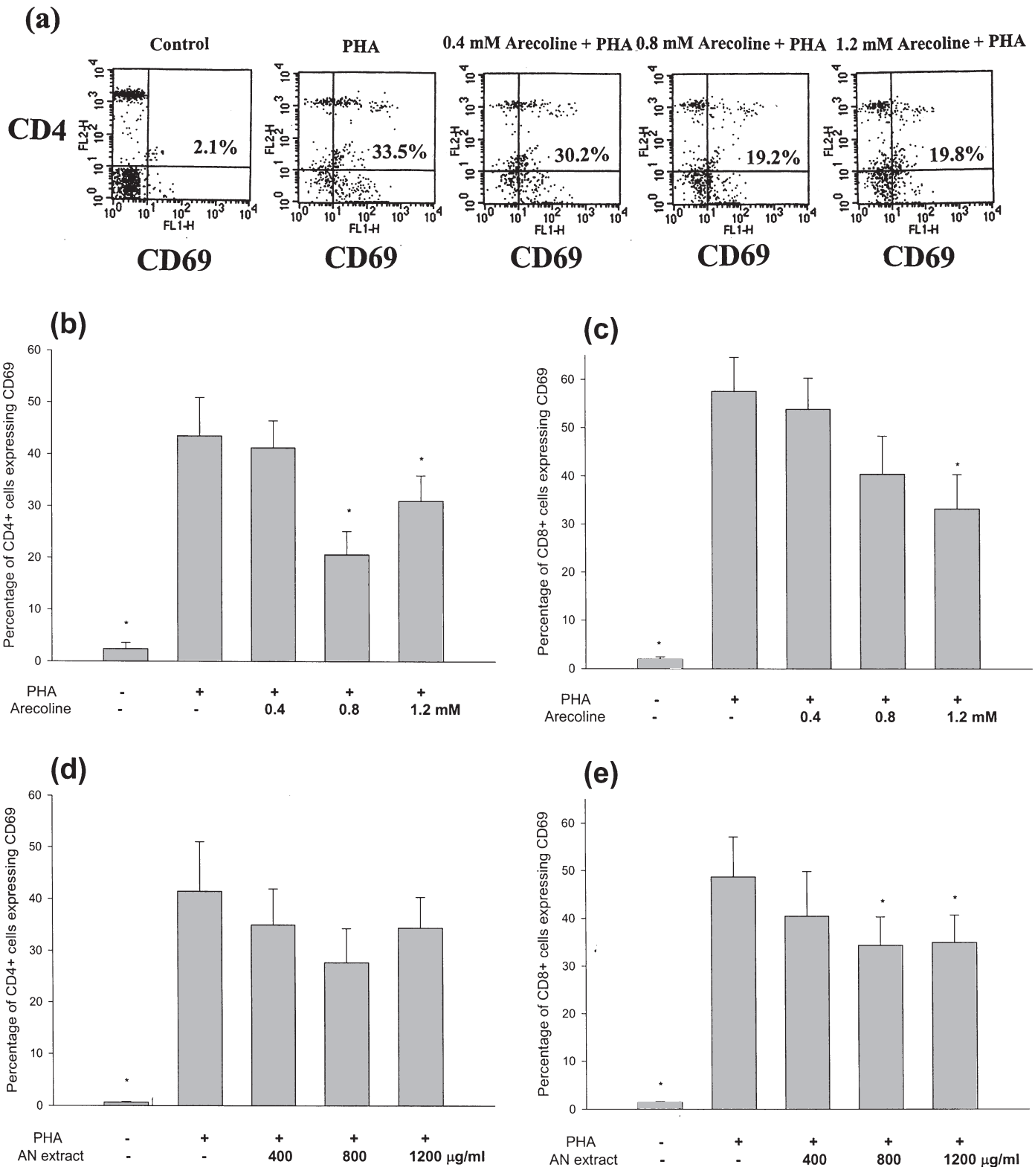

Fig. 9. Effect of AN extract- and arecoline-conditioned medium on PHA (4 μg/ml)-induced T cell activation. (**a**) Isolated PBMC were exposed to RPMI 1640/conditioned medium (1:1 v/v) with or without PHA for 24 h. Cells were collected and subjected to flow cytometric analysis for $CD4^+/CD69^+$ and $CD8^+/CD69^+$ T cell populations. Activation of T cells by PHA was evident as increasing numbers of $CD69^+$ T cells (upper right region). The results of one representative experiment are shown. Quantitatively the percentages of (**b**) $CD4^+$ T cells expressing the $CD69^+$ surface marker and (**c**) $CD8^+$ T cells expressing the $CD69^+$ surface marker after exposure to PHA with/without arecoline (0.4, 0.8 and 1.2 mM)-conditioned medium for 24 h are shown ($n = 5$). The percentages of (**d**) $CD4^+$ T cells expressing the $CD69^+$ surface marker ($n = 5$) and (**e**) $CD8^+$ T cells expressing the $CD69^+$ surface marker after exposure to PHA with/without AN (400, 800 and 1200 μg/ml)-conditioned medium for 24 h ($n = 5$) are also shown. * denotes marked difference when compared with PHA-treated group.

Table II. Effect of PGE_2 on IL-6 production by GK ($n = 3$) and KB cells ($n = 11$)

Chemicals	IL-6 concentration (pg/ml)	
	GK cells	KB cells
Control	21.3 ± 4.6	292 ± 25
PGE_2 50 ng/ml	51.0 ± 10.7[a]	261 ± 15
PGE_2 250 ng/ml	46.0 ± 4.4[a]	250 ± 15
PGE_2 500 ng/ml	56.7 ± 10.9[a]	200 ± 17[a]
PGE_2 1250 ng/ml	44.3 ± 6.4[a]	163 ± 13[a]
PGE_2 2500 ng/ml	45.6 ± 9.4[a]	115 ± 6[a]

Data for IL-6 are presented as means ± SE (pg/ml).
[a]Marked difference when compared with untreated control group ($P < 0.05$).

Table III. Effect of TNF-α ab on AN extract-induced cytotoxicity, IL-6 and PGE_2 production by KB cells ($n = 4$)

Chemicals	PGE_2 (pg/ml)	IL-6 (pg/ml)	MTT reduction (% of control)
Control	163 ± 13	309 ± 17	100
AN extract (400 μg/ml)	113 996 ± 7002	14 073 ± 1560	75 ± 8.6
TNF-α ab (500 ng/ml)	625 ± 236	337 ± 28	134 ± 27
TNF-α ab + AN extract (400 μg/ml)	128 634 ± 16 586	15 087 ± 2330	63 ± 2.6
AN extract (800 μg/ml)	254 522 ± 15 085	4924 ± 717	55 ± 6.6
TNF-α ab + AN extract (800 μg/ml)	264 733 ± 18 359	6103 ± 876[a]	54 ± 6.7

Data for IL-6 and PGE_2 are presented as means ± SE (pg/ml). Cell numbers are presented as percent MTT reduction of control (100%) as assayed at OD_{540}.
[a]Difference ($P < 0.05$) when compared with AN-treated (800 μg/ml) group.

Table IV. Effect of TNF-α ab on arecoline-induced cytotoxicity, IL-6 and PGE_2 production by KB cells ($n = 4$)

Chemicals	PGE_2 (pg/ml)	IL-6 (pg/ml)	MTT reduction (% of control)
Control	134 ± 2.6	308 ± 20	100
Arecoline (0.4 mM)	244 ± 7	172 ± 4	69 ± 5.9
TNF-α ab (500 ng/ml)	265 ± 7[a]	317 ± 19	103 ± 6.4
TNF-α ab + Arecoline (0.4 mM)	230 ± 3.2	176 ± 5.6	70 ± 3.5
Arecoline (0.8 mM)	366 ± 13	129 ± 2.8	32 ± 2.7
TNF-α ab + Arecoline (0.8 mM)	330 ± 10[b]	144 ± 3.3[b]	36 ± 2.9

Data for IL-6 and PGE_2 are presented as means ± SE (pg/ml). Cell numbers are presented as percent MTT reduction of control (100%) as assayed at OD_{540}.
[a]Difference ($P < 0.05$) when compared with control.
[b]Difference when compared with AN-treated (800 μg/ml) group.

Discussion

In the present study, AN components, which are associated with oral cancer and OSF were found to induce keratinocyte inflammation by stimulating the production of PGE_2, TNF-α and IL-6 in primary cultured GK and KB oral carcinoma cells. We have demonstrated the induction by AN extract of PGE_2 production in KB oral carcinoma cells at concentrations (100–200 μg/ml) that showed little cytotoxicity. Consistently, AN extract also stimulates PGE_2 and prostacyclin production by GK (17). Release of PGs by AN components can induce vasodilation, changes in vascular permeability and inflammatory cell infiltration (9,45). BQ chewing therefore plays a role in inducing mucosal inflammation and promotes tumor progression by stimulating PG production by GK and KB carcinoma cells. Consistently, marked inflammatory cell infiltration is observed in the connective tissues of BQ chewers (1,4,7). Although the role of PGs in tumor promotion is not fully clear, an *in vivo* animal model supports the hypothesis that AN extract may promote 7,12-dimethylbenzanthracene-induced tumor formation in hamster cheek pouch (46). This is also supported by recent animal experiments that showed that topical application of BQ components exerted a potential tumor promoting effect by inducing epidermal hyperplasia, tissue inflammation and myeloperoxidase activity in CD-1 mouse skin (47). However, whether this event is associated with IL-6, TNF-α and PG production should be further confirmed. Interestingly, we found that arecoline stimulated PGE_2 production by KB cells, indicating that arecoline was one of the causative factors leading to mucosal inflammation in BQ chewers. However, additional factors may be present, because the extent of stimulatory effect of arecoline on PGE_2 production by KB cells was far less than that of AN extract and arecoline inhibited PGE_2 production by GK (unpublished observations). In addition, our data show that arecoline has little effect on Cox-2 mRNA and protein expression in KB cells. Some tumor cells, endothelial cells and smooth muscle cells have been shown to produce PGs in response to muscarinic receptor activation (48–50). Arecoline is a muscarinic receptor agonist, which raises the possibility of direct activation of COX in KB cells via receptor activation. Whether KB cells and GK express different isoforms of muscarinic receptor should be further elucidated. Since arecoline was unable to induce Cox-2 mRNA and protein expression, stimulation of PGE_2 production in KB cells by higher concentrations (>0.4 mM) of arecoline may also be partly due to cytotoxicity. Inhibition of arecoline-induced PGE_2 production in KB cells by aspirin (Table I) further suggests the possible direct activation of COX-1 by arecoline.

In addition to PGs, IL-6 is also a major inflammatory mediator responsible for oral mucosal inflammation (12,51) and may influence the oral mucosa immune response in a quantitative and qualitative manner (52). Interestingly, we clearly indicated a stimulatory effect of AN extract on IL-6 production by both primary GK and KB oral carcinoma cells. In support of this, Haque *et al*. (18) found that OSF tissues have higher expression of IL-6 when compared with healthy tissues. Haque *et al*. (19) further found elevated IL-6 production by PBMC isolated from OSF patients, relative to normal healthy subjects. Thus, induction of IL-6 production by AN components may act synergistically with PGs in eliciting the systemic and oral mucosal immune response as observed in BQ chewers (1,4,5,7) and animal experiments (47). AN-stimulated IL-6 may subsequently modulate a variety of biological processes, including the immune response, inflammation and carcinogenesis, by regulating the growth, survival and differentiation of target cells (16,21,22). Nevertheless, PGs and IL-6 have been shown to be mitogenic to several types of epithelial cells (12,15,23–25). In the present study, PGE_2 showed little effect on the growth of KB cells, similar to what we reported for GK (17). In addition, primary GK and KB carcinoma cells showed differential growth responses to exogenous IL-6. IL-6 slightly inhibited the growth of GK, whereas it showed a tendency to stimulate the growth of OMF and KB carcinoma cells. These

different responses of epithelial cells may be due to differences in the cell culture conditions (such as culture medium, growth factor supplementation, etc.), IL-6 receptor (IL-6R or gp130) expression or subsequent signaling pathways. IL-6 inhibits the growth of breast and lung carcinoma cell lines (53,54). In contrast, IL-6 serves as an autocrine growth factor in the proliferation of colon cancer cells (55) and a choriocarcinoma cell line (JET-3) (56). Hirano suggested that if STAT levels are suppressed, exogenous IL-6 stimulates proliferation, whereas IL-6 induces cell cycle arrest and differentiation when STAT levels are high (57). Similar diverse responses of primary buccal keratinocytes and a carcinoma cell line to terminal differentiation by AN components have also been reported by Sundqvist and Grafstrom (58). This may explain why GK and KB cells showed differential PGE_2 production in response to arecoline. Whether, similarly, IL-6 shows disparities on the differentiation of GK and KB carcinoma cells should be further addressed. Since AN components are genotoxic to oral keratinocytes (35,59), the lack of a mitogenic effect of IL-6 on GK may perhaps be a cellular response to a genotoxic agent to prevent fixation of the DNA damage in cells. Further study found that exposure to IL-6 neutralizing ab showed little effect on the growth of GK and KB cells. This revealed that endogenous IL-6 production was not crucial for the growth of primary GK and KB oral carcinoma cells. However, inducing the growth of KB oral carcinoma cells by exogenous IL-6 indicates that BQ chewing may potentially promote tumor progression by stimulating IL-6 production. Arecoline was not the agent responsible for AN-induced IL-6 production in oral epithelial cells, because arecoline markedly decreased cellular production of IL-6. In accord with this, no marked stimulatory effects of arecoline and arecaidine on IL-6 production by gingival fibroblasts, OMF and OSF fibroblasts have recently been reported (60). Thus, from the present results and those published previously (35,59), AN-induced PGE_2 and IL-6 production may be mediated by arecoline-independent and -dependent pathways. Various cytokines have been found to diffuse through the basement membrane (61) and regulate the biological functions of connective cells (e.g. fibroblasts). Since IL-6 is a well-known inflammatory mediator and fibrogenic cytokine (44,62,63), IL-6 induced by AN components may penetrate the basement membrane and stimulate the growth of OMF, contributing to the pathogenesis of OSF. However, more in depth *in vivo* studies are needed to further confirm this hypothesis.

AN components are able to induce DNA breaks, DNA–protein cross-links and unscheduled DNA synthesis in oral keratinocyte (35,37,59), leading to transformation of epithelial cells. Since TNF-α has been shown to provide a selective growth advantage to transformed cells (27), induction of TNF-α production in GK by AN extract may possibly contribute to the clonal expansion of transformed oral epithelial cells. The induction of TNF-α production by AN extract was also not due to its arecoline content, because an effect of arecoline on TNF-α production by GK was not obvious in this experiment. In addition, arecoline inhibits the secretion of TNF-α by PBMC (64). Since, TNF-α mediates the arsenic trioxide-induced death of myeloid leukemia cells (65) and an elevation of TNF-α expression in oral squamous cell carcinoma has been noted (26), we further investigated whether TNF-α is crucial in mediating IL-6 and PGE_2 production induced by AN extract and arecoline in GK and KB cells. Interestingly, we noted that TNF-α is not the major factor responsible for induction by AN extract or arecoline of PGE_2 and IL-6 production in GK and KB cells. Although neutralization of TNF-α by antibodies inhibits the generation of IL-6 in synovial mononuclear cells and rheumatoid synovial membrane culture (66,67), IL-6 production may also be induced in a TNF-α-independent fashion (68,69). In addition, neutralization with anti-TNF-α ab slightly increased basal- and AN-induced levels of PGE_2 and IL-6 production. This reveals the presence of complex cross-talk between cytokine networks and a compensatory response of cells to exogenous stimuli. Possibly the induction of production of TNF-α by AN extract is a concomitant event comparable with PGE_2 and IL-6 production.

IL-6 and PGE_2 have been reported to be crucial mediators of the cellular response to injury (70) and oxidative stress (71,72). An epidemiological study has suggested a correlation between reactive oxygen species (ROS) production and BQ-induced cancer in Papua New Guinea (2). BQ components are able to generate ROS such as H_2O_2 and superoxide radicals during auto-oxidation of BQ phenolics (73,74). *In vitro* cell culture models also reveal that AN components may induce ROS production by KB cells (36). Antioxidants such as thiols and vitamin C prevent AN-induced unscheduled DNA synthesis in GK (37). ROS is also reported to induce IL-6 and COX-2 via activation of mitogen-activated protein kinase (MAPK) pathways (71,72). Consistent with this, a recent study indicated that AN induced platelet aggregation and thromboxane B_2 production. AN-induced thromboxane B_2 production can be inhibited by genistein (a tyrosine kinase inhibitor), catalase and superoxide dismutase (38). Because a western blot study has found stimulation of MAPK phosphorylation in KB cells by AN extract (unpublished observations), induction of IL-6 and PGE_2 production by AN extract may possibly be via two mechanisms, one involving ROS production, MAPK activation and then induction of Cox-2 and IL-6 gene expression, and the other involving induction of the protective cellular response to injury. In the present work, AN extract induced PGE_2 and IL-6 production by KB and GK cells at non-cytotoxic (100–400 μg/ml) and cytotoxic concentrations (800 and 1200 μg/ml), indicating that the cellular response of IL-6 mRNA expression to injury and ROS induction perhaps occur concomitantly. In support of this, we clearly noted the induction of Cox-2 and IL-6 mRNA expression in KB cells by 100–800 μg/ml AN extract. This implies the regulation of Cox-2 and IL-6 by an AN component at the transcriptional level. In addition, COX-2 protein production by KB cells was also increased following exposure to AN extract for 24 h. These results may partly explain why HNSCC tissues show a higher level of IL-6 and COX-2 protein and mRNA expression than comparable healthy oral tissues (12,28). Modulation of Cox-2 and IL-6 gene expression is therefore crucial in the pathogenesis of BQ chewing-related oral mucosal diseases.

The mechanisms responsible for AN toxicity are still not fully understood. Glutathione depletion, ROS production and mitochondrial dysfunction are possible contributing factors (36). IL-6 and PGE_2 production by GK and KB cells were induced by AN extract; an intriguing question was whether AN cytotoxicity was mediated by induction of these mediators. Indomethacin, a COX inhibitor, showed little protective effect against AN-induced cytotoxicity to GK (17). In this study, AN extract and arecoline induced G_2/M cell cycle arrest and apoptosis of KB cells under serum-free conditions. This indicates that serum contains some factor(s) which may prevent or delay

the AN extract- and arecoline-induced toxic event (36). Moreover, inclusion of aspirin, TNF-α and IL-6 neutralizing ab was also unable to prevent arecoline- and AN-induced cell death, cell cycle arrest and apoptosis. These results indicate that AN cytotoxicity was not directly via activation of COX-2 and induction of TNF-α and IL-6. Whether IL-6 and PGE_2 production showed cross-talk is not known. However, activation of COX has been shown to be important in regulating the synthesis of IL-6 in MDA-MB-231 and Hs578T breast cancer cells (42). We also noted that PGE_2 stimulated IL-6 production in primary cultured GK, whereas PGE_2 (>0.5 g/ml) inhibited IL-6 production in KB oral carcinoma cells. PGE_2 has been found to increase or decrease IL-6 production via EP1, EP2, EP3 and EP4 receptors, which depends on the type of cell, such as human gingival fibroblasts, airway epithelial cells, human astroglioma cells, primary rat astrocytes, rat synovial cells and murine mast cells (75–80). Since KB oral carcinoma cells express mainly the EP2 PGE receptor isoform (unpublished observations), further studies are needed to evaluate the expression of other isoforms of EP receptors and their downstream cellular effects in GK and KB cells. Differences between GK and KB cells in response to PGE_2 could be due to differential activation of EP1/EP3 or EP2/EP4 PG receptors. These disparities between GK and KB carcinoma cell responses to PGE_2 may also suggest that changes in oral epithelial cells in response to IL-6 and PGE_2 are a crucial factor in the carcinogenic process.

The ability of epithelial cells to produce and respond to IL-6 without neutralization by the ab indicates production independent of ligand–receptor binding. We therefore evaluated the interplay between epithelial cells and immune effecter activity by isolation of culture supernatant containing PGE_2, IL-6 and TNF-α from the epithelial cells and incubation with isolated human PBMC. Interestingly, the cytokine-rich supernatant was not able to induce $CD4^+$ and $CD8^+$ T cell activation, as revealed by the lack of $CD69^+$ early surface activation marker expression. On the contrary, the AN extract- and arecoline-treated epithelial cell supernatant inhibited PHA-induced $CD69^+$ expression on $CD4^+$ and $CD8^+$ T cells, indicating suppression of T cell activation. Since functions of T helper cells and T suppressor cells are crucial for killing of malignant cells and inhibition of carcinogenesis (81,82), impairment of $CD4^+$ and $CD8^+$ cell activation by cell culture supernatant indicates that exposure to AN extract and arecoline may play a role in oral carcinogenesis by affecting T cell-mediated immunity. Why cytokine-rich supernatants lack a stimulatory effect on T cell activation is not clear. Antagonism by interactions with other unknown immunosuppressive cytokines in the conditioned medium is one possible reason. Whether this is due to direct inhibition by arecoline and AN extract should be further addressed, because s.c. injection of arecoline into male mice suppresses delay type hypersensitivity and antibody production in response to sheep red blood cells (83,84).

In summary, our studies indicate that IL-6, TNF-α and PGE_2 production by KB cells and GK is induced by AN extract by stimulation of COX-2 and IL-6 mRNA and protein expression. This stimulatory effect of AN is not solely due to its content of arecoline. Other AN components are also crucial for this event. IL-6, TNF-α and PGE_2 produced by primary oral keratinocytes and carcinoma cells may induce oral mucosal inflammation, as observed in BQ chewers. These mediators may also regulate the biological activity of epithelial cells by an autocrine mechanism, penetrate the basement membrane to regulate the growth of OMF by a paracrine mechanism or elevate the level of IL-6 in the serum of betel chewers, contributing to the pathogenesis of oral cancer and OSF. In addition, disturbance of T cell-mediated immunity may be another contributing factor to BQ chewing-related oral mucosal diseases. Future development of antioxidants and anti-inflammatory agents such as vitamin C, thiol and immune milk (33,37,38) can be potentially used as chemopreventive agents against BQ chewing-related oral mucosal diseases.

Acknowledgements

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