Up-regulation of matrix metalloproteinase-8 by betel quid extract and arecoline and its role in 2D motility

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Summary Betel quid (BQ) and matrix metalloproteinase-8 (MMP-8) play roles in oral diseases. Here, we analyzed the regulation of MMP-8 by BQ and its effect on cell migration. We found that BQ extract (BQE) increased the secretion of an 85 kDa caseinolytic proteinase, specifically precipitated by an anti-MMP-8 antibody, in the culture medium of OECM-1, an oral squamous cell carcinoma (OSCC) cell line. BQE also stimulated MMP-8 secretion in an esophageal carcinoma cell line, CEB1T/VGH, in a dose-dependent manner, and MMP-8 protein was maximally expressed at 24 h after BQE treatment in OECM-1. The BQE-induced MMP-8 expression was dose-dependently inhibited by PD98059. Arecoline, the major alkaloid of areca nut, was tested to dose-dependently up-regulate MMP-8 protein level. Moreover, both arecoline- (4.7-fold) and BQE-selected (5.5-fold) CEB1T/VGH cells expressed higher MMP-8 protein level and exhibited enhanced two-dimensional (2D) motility ($p = 0.009$ in both cells) than parental cells. The enhanced motility of arecoline- ($p = 0.006$) and BQE-selected ($p = 0.002$) cells was both specifically blocked by an anti-MMP-8 antibody. We conclude that BQ may accelerate tumor migration by stimulating MMP-8 expression through MEK pathway in at least some carcinomas of the upper aerodigestive tract. Furthermore, arecoline may be one of the positive MMP-8 regulators among BQ ingredients.

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Introduction

Matrix metalloproteinases (MMPs) are required in both physiological and pathological conditions of the mouth, such as tooth eruption, caries lesions, peri-implantitis, and periodontitis.1–4 Among these conditions, matrix metalloproteinase-8 (MMP-8, collagenase-2) level in the peri-implantitis and periodontitis is positively correlated to the extent of unwanted tissue destruction at the site of inflammation.5 In addition, like other MMPs, MMP-8 plays a role in tissue remodeling and wound healing processes.6–8 This proteinase also participates in ulcerative, allergic, and inflammatory conditions, including tumor necrosis factor-induced lethal hepatitis.7,9–12

There are also many evidences suggesting a role of MMP-8 in tumors, such as adrenal tumors and Jurkat T leukemia cells.13,14 MMP-8 level is significantly correlated with tumor grade, stage, and a poor prognosis in 302 ovarian cancer cases, and loss of MMP-8 confers increased skin tumor susceptibility to male mice.15,16 MMP-8 is also commonly expressed in the cases of squamous cell carcinoma of the head and neck (SCCHN).17 Some MMPs are elevated in the serum of patients with SCCHN, and only MMP-8 exhibits as the potential tumor marker with the significant correlation with SCCHN.18

Oral cancer, strongly associated with betel quid (BQ) chewing habit, is one of the most common types of human cancer in the world. The International Agency for Research on Cancer (IARC) has recently concluded BQ as a human carcinogen.19 There are about 200–600 million BQ chewers in the world, including Taiwan. More than 2400 new cases of oral cancer are diagnosed each year in Taiwan due to the prevalent use of BQ.20,21 There are several distinct compositions of BQ, among which areca nut is the essential component, whose extract induces growth arrest and senescence-associated phenotypes in human oral keratinocytes.22 Copy number amplification of 3q26–27 oncogenes is found in the specimens of oral squamous cell carcinoma (OSCC) and oral brushed samples from areca chewers.23 In addition to oral cancer, areca chewing also induces a variety of oral diseases, including periodontitis.24 Therefore, it is reasonable to speculate that some MMPs, such as MMP-8, involved in the inflammatory and pathological conditions may be regulated by BQ ingredients.

We have previously shown that BQ chewing increased salivary MMP-2 and MMP-9 protein levels, and BQ extract (BQE) stimulated the secretion of MMP-2 in an OSCC cell line, OECM-1.25,26 In this study, we further investigated whether MMP-8 is regulated by BQE and arecoline (the major alkaloid present in areca nut ingredients) in OECM-1 and in another human esophageal carcinoma cell line, CE81T/VGH.27,28 To simulate and analyze a long-term stimulation effect of BQ, CE81T/VGH cells were further subjected to repeated BQE and arecoline challenge. Changes of the MMP-8 protein level and the two-dimensional (2D) motility of surviving cells were then measured and compared to parental cells.

Materials and methods

Preparation of BQ extract (BQE)

The preparation of BQE was described in our previous works.25 Briefly, one areca nut (Areca catechu L.) (3.6–4.0 g), a piece of inflorescence of Piper betle Linn. (0.3–0.4 g), and 0.4–0.5 g lime was ground in 5 ml phosphate-buffered saline (PBS) (150 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). The soluble fraction was used as BQE and its concentration was 24.8 mg/ml. Aliquots of 1 ml BQE were stored at −80°C for subsequent usage.

BQE or arecoline treatment

Confluent cells (7 × 10⁶) of OECM-1 or CE81T/VGH were cultured in 10-cm-plate supplemented with 10 ml Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Paisley, UK) containing 10% fetal bovine serum (FBS) (HyClone, VT, USA) at 37°C in the humid atmosphere with 5% CO2. After washing twice with 10 ml PBS, cells were cultured in 6 ml serum-free (SF) medium overnight. Again, after washing twice with PBS, cells were treated with 6 ml SFM containing 0.39 mg/ml BQE or 0, 1, 10, 40, and 100 μg/ml arecoline (Sigma Diagnostics, Inc., St. Louis, MO, USA) for 10 min and conditioned in fresh SF medium (5 ml/plate) for 24 h at 37°C. Conditioned medium (CM) and cells were separately collected for further lyase preparation and MMP-8 protein detection. Alternatively, cells were pretreated with 0, 6.25, 12.5, or 25 μM PD98059 (Sigma) for 2 h before BQE treatment.

Western blot analysis

The collected 6 ml CM was subjected to centrifugal concentration by passing through the 10,000 MWCO PES membrane (Vivascience, Hannover, Germany) as described by the manufacturer. The final concentrated volume was about 60 μl. On the other hand, scraped cells were centrifuged and lysed in 60 μl NET buffer (0.5% NP-40, 2 mM EDTA, 50 mM Tris–HCl, pH 7.4) containing onefold strength of protease inhibitor cocktail set I (Calbiochem-Novabiochem Corp., San Diego, CA, USA). The protein concentrations of the cell lysate and CM were measured with Bio-Rad protein assay kits (Bio-Rad Laboratories, Hercules, CA, USA). One-fourth of each concentrated CM (15 μl) containing about 1.5 μg protein (or 20 μg of lysate protein) was subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Bio-Rad Laboratories). Separated proteins were transferred onto nitrocellulose (NC) paper (Amersham Pharmacia Biotech, Uppsala, Sweden). The NC paper was immediately soaked in 10-fold diluted Ponceau S solution (Sigma) for 1 min and photographed. The NC paper was then extensively washed with H2O and subsequently blocked with 5% lipid-extracted milk at room temperature. After washing three times with TBS washing buffer [50 mM Tris–HCl, pH 7.35, 0.85% NaCl, 0.5% Tween 20], the NC paper was further incubated with 1 × 10⁴-fold diluted goat-anti-mouse-IgG monoclonal antibody coupled with horseradish peroxidase (Chemicon) in the same buffer as the first antibody at room temperature for 1 h. This immunoblotted
paper was then extensively washed with TBS buffer, and signals of MMP-8 protein were detected with Western Lightening Chemiluminescence Reagent (Perkin–Elmer Life Sciences, Inc., Boston, MA, USA) according to the manufacturer’s instructions.

Zymography

The 1.5 μg CM protein was subjected to SDS–PAGE analysis with 10% acrylamide gel containing 1% casein. The electrophoretic current was set at 8 mA and 15 mA when the dye front was localized within the stacking and separation gel, respectively. The entire electrophoresis was performed at 4 °C. Afterwards, the gel was soaked in 100 ml 2.5% Triton-X 100 (Amersham) at room temperature for 1 h and transferred to 100 ml reaction buffer [50 mM Tris–HCl, 200 mM NaCl, 5 mM CaCl2, 0.02% Brij-35 (Sigma), pH 7.5]. The soaked gel was held at 37 °C in the water bath for 16 h, and then rinsed with 100 ml MQ water for 5 min at room temperature followed by staining with PhastGel Blue R (Amersham) according to the manufacturer’s instructions. Finally, the gel was destained with destaining solution containing 10% acetic acid (Merck KGaA, Darmstadt, Germany) and 30% methanol (Merck) at room temperature for 1–4 h.

In an independent experiment, the remaining half of the BQE-treated CM (30 μl) of OECM-1 cells was equally divided into three tubes, incubated with no antibody in the first tube, with 2 μg of the same MMP-8 antibody in the second tube, and with 2 μg of an anti-MMP-28 antibody (Sigma) in the last tube at 4 °C for 1 h. Protein A-agarose beads were then added and the mixture was mixed and stood on ice for 2 h. The beads were washed three times with PBS, eluted from the agarose beads by adding the equal volume of 2X sample buffer at 37 °C for 10 min, and analyzed by casein zymography.

XTT assay

Cell viability was measured by using a XTT labeling reagent, Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA), as instructed by the manufacturer. Briefly, 1 × 104 cells/well were seeded onto a 96-well plate for 24 h. Cells were then serum starved for 24 h, followed by treatment with the indicated concentrations of BQE or arecoline for 10 min. After washing twice with PBS, cells were cultured in SF medium for another 24 h. Cells were then serum starved for 24 h, an artificial scratch wound was created by a 20-μl tip. The average width of the wound was 0.65 (mean) ± 0.01 (SD) mm by measuring the width at the top, middle, and bottom of the scratch with a microscopic ruler. Cells were then cultured in SF medium with or without anti-MMP-8 antibody (2 μg) or normal rabbit serum (1 μl) for 48 h. The scratches were photographed at 0, 24, and 48 h after scratching, and the average width was identically determined as described above. Finally, cells were subjected to XTT assay for a reference of cell numbers.

Statistical analysis

Two groups of data presented as mean ± SD were analyzed by Student’s t test. A value of p < 0.05 was regarded as statistically significant.

Results

Induction of caseinolytic proteinases by BQE

To simulate that the soluble ingredients of a BQ may dissolve into the similar volume of salivary fluid in the beginning of chewing a BQ, one BQ was ground with 5 ml PBS and the concentration of soluble BQE was 24.8 mg/ml. Before analyzing the effect of BQE on the secretion of caseinolytic proteinase, OECM-1 cells were treated with various concentrations of BQE (0.78–24.8 mg/ml) for 10 min, and the viability was determined by XTT labeling reagent after 24 h. The results showed that all the tested concentrations of BQE were non-cytotoxic (Fig. 1A).

To determine whether BQE modulates the secretion level of caseinolytic proteinases, the conditioned medium (CM) of OECM-1 treated with or without 24.8 mg/ml BQE for 10 min was assayed by casein zymography. A 70 kDa signal was strongly induced and an 85 kDa caseinolytic activity was also evidently enhanced after BQE treatment (Fig. 1B). For a loading control purpose, the same volume of the CM was subjected to the same SDS–PAGE. The separated CM proteins in the gel were then transferred onto NC paper, and stained with Ponceau S solution in a parallel experiment. The result indicated that similar amount of secreted proteins were applied in the zymographic analysis (Fig. 1B).

Identification of BQE-induced 85 kDa caseinolytic proteinase to be MMP-8

According to the molecular weight, we speculated the enhanced 85 kDa caseinolytic activity to be the full length molecule of MMP-8 protein as described by others previously. Thus, the remaining BQE-treated CM of OECM-1 was further subjected to immunoprecipitation analysis. The result showed that the 85 kDa proteinase was specifically precipitated by an anti-MMP-8 antibody rather than by Protein A/agarose beads alone or by an anti-MMP-28 antibody (Fig. 1C). The identity of the 70 kDa proteinase remains unknown.
Regulation of MMP-8 by BQE in OECM-1 and CE81T/VGH

To assess the kinetics of BQE effect on MMP-8 expression, we measured intracellular MMP-8 protein level by immunoblotting after 4, 8, and 24 h of 10-min BQE treatment. The results revealed that MMP-8 protein was moderately and maximally induced at 8 h and 24 h time points, respectively (Fig. 2A). β-Actin, in this case, served as the protein loading control. The maximally induced intracellular MMP-8 protein after 24 h of BQE treatment was further detected in the CM of OECM-1 (Fig. 2B).

Since the swallowed ingredients of BQ may stimulate the lining epithelium of esophagus as well, it is intriguing and important to investigate whether esophageal epithelial cells respond similarly to BQE. Thus, in addition to OECM-1, CE81T/VGH was subjected to various concentrations of BQE treatment (for 10 min), and MMP-8 protein was detected by immunoblotting after 24 h. The results showed that BQE increased MMP-8 protein level in the CM of both
CE81T/VGH and OECM-1 in a dose-dependent manner (Fig. 2C).

MMP-1 (collagenase-1) is up-regulated by different stimuli through MEK/ERK pathway.\(^{30,31}\) In contrast, cytokine-induced MMP-13 (collagenase-3) expression is ERK-independent.\(^{31}\) Thus, MEK/ERK differentially regulate the collagenase subfamily of MMP. To analyze whether BQE-mediated up-regulation of MMP-8 relies on MEK/ERK pathway, a MEK specific inhibitor, PD98059, was used to pretreat cells before BQE treatment. The results indicated that BQE-induced MMP-8 expression was dose-dependently inhibited by PD98059 (Fig. 2D).

**Induction of MMP-8 by a BQ ingredient, arecoline**

The inhibition of BQE-induced MMP-8 expression by PD98059 implied that BQE may up-regulate MMP-8 expression through MEK pathway. Among BQ ingredients, arecoline was shown to stimulate ERK1/2 phosphorylation.\(^{32}\) Taken together, arecoline was thought to be one of the possible MMP-8 inducers in BQ ingredients. Before testing this possibility, the cytotoxicity of arecoline was firstly tested. Figure 3A showed that after 24 h of 10-min arecoline treatment, none of the arecoline concentrations used was cytotoxic. Figure 3B further shows that arecoline dose-dependently increased protein level of MMP-8 below the concentrations of 40 \(\mug/ml\). Higher concentration of arecoline (100 \(\mug/ml\)) lost the MMP-8 inducing effect.

**Elevation of MMP-8 protein level and accelerated 2D motility after BQE or arecoline selection**

To simulate a constant stimulation and analyze the effect of BQ on carcinoma cells, especially from the upper aerodigestive tract origin, we repeatedly challenged CE81T/VGH cells with cytotoxic concentrations of BQE and arecoline for 24 h (detailed in "Materials and methods"), and measured the changes of expression level of MMP-8 protein and of 2D motility of the survived cells. The results showed that BQE- and arecoline-selected CE81T/VGH cells expressed 5.5- and 4.7-fold of MMP-8 protein than that of parental cells, respectively (Fig. 4A). The arecoline- and BQE-selected cells also exhibited an increased 2D healing motility than parental cells in scratching test (\(p = 0.009\) in both cases, Fig. 4B, a vs. b and a vs. e; and Fig. 4C, *1 and *3). This enhanced motility of arecoline- and BQE-selected cells was specifically and significantly blocked by an anti-MMP-8 neutralizing antibody (\(p = 0.0006\) and 0.002, respectively, Fig. 4B, b vs. c and e vs. f; Fig. 4C, *2 and *4). Addition of normal rabbit serum did not affect healing mobility in both arecoline- and BQE-selected cells (Fig. 4B, b vs. d and e vs. g).

To rule out that the accelerated motility in the two selected cell types was due to the increased growth rate, each of the selected cells after 48 h of scratching test were subjected to XTT assay. The results showed that cell proliferation was not affected by any of the selection or treatment (Fig. 4D).

**Induction of MMP-8 secretion by both BQE and arecoline in another OSCC cell line**

To verify whether the inducing effect of BQE and arecoline on MMP-8 expression can be applied to other OSCC cells, HCC-15 cells were subjected to BQE (24.8 mg/ml) and arecoline (10 \(\mug/ml\)) treatment, and MMP-8 protein in the conditioned medium was detected by immunoblotting. The results showed that MMP-8 secretion was induced by both BQE and arecoline treatment in HCC-15 cells (Fig. 5).

**Discussion**

We have previously described the elevation of MMP-2 and MMP-9 proteins in the saliva after BQ chewing, as well as the increase of MMP-2 protein in the CM of primarily cultured oral fibroblasts and OECM-1 after BQE treatment.\(^{25,26}\) In this study, we have further demonstrated the induction of MMP-8 expression and secretion by BQE treatment in both oral and esophageal carcinoma cell lines. Accordingly, BQ consumption may have the potential to up-regulate MMP-8 in the line-up of epithelial cells along the upper aerodigestive tract, including oral cavity and esophagus. It has also been illustrated that this induction is MEK-dependent (Fig. 2D), and one of the BQE ingredients, arecoline, is a positive MMP-8 regulator (Fig. 3). Furthermore, cells survived from BQE or arecoline selection process expressed higher MMP-8 protein level (Fig. 4A), and exhibited an MMP-8-dependent acceleration of 2D motility (Fig. 4B–D). It is suggested that BQE may contribute to oral diseases partly through the induction of disease-associated MMPs like MMP-8. Induction of MMP-8 may thus be one of BQE-mediated physiological consequences resulting in the promotion of tumor invasion/migration.

Collagenase is thought to play a role in cellular migration. For examples, MMP-1 (collagenase-1) was shown to be expressed in the migration front of human corneal epithelial cells by a colony dispersion assay in response to hepatocyte...
Figure 4 Increased ProMMP-8 protein level and enhanced 2D motility after BQE or arecoline selection. (A) ProMMP-8 protein in the lysates of non-selected, BQE-selected (BQE-s), and arecoline-selected (ARE-s) CE81T/VGH cells was analyzed by immunoblotting. (B) CE81T/VGH (CE81T) cells, arecoline-selected CE81T/VGH (ARE-s), and BQE-selected CE81T/VGH (BQE-s) cells were subjected to scratching test with or without anti-MMP-8 antibody (MMP-8 Ab) or normal rabbit serum (NRS) (a–g). Cells were photographed after 0, 24, and 48 h after scratching. Bar = 0.2 mm. (C) Average width of each scratched wounds was measured and presented as mean ± SD. Asterisk represents the statistically significant difference ($p < 0.05$). *1: $p = 0.009$; *2: $p = 0.0006$; *3: $p = 0.009$; *4: $p = 0.002$. (D) Cell viability after 48 h of scratching was assessed by XTT assay. This scratching test has been repeated for five times with similar results.
growth factor. The migration of these cells was blocked by MMP-1 neutralizing antibody. A recent study showed that the stromal fibroblasts-produced MMP-1 facilitated the migration of breast cancer cells by cleaving protease-activated receptor 1. On the other hand, MMP-13 (collagenase-3) was shown to mediate nitric oxide-induced endothelial cell migration, which was delayed when the expression of MMP-13 was specifically blocked by RNA interference. Although MMP-8 is detected in the site of wound healing, as to our knowledge, there is no direct evidence verifying the involvement of MMP-8 (collagenase-2) in cell migration. Therefore, our present study may have provided the first in vitro evidence for the requirement of MMP-8 in cell migration.

Expression of MMP-8 has been demonstrated to be up-regulated by several cytokines, such as interleukin-1β, in distinct cell types, including human gingival fibroblasts. However, the responsible pathway in regulating MMP-8 expression remains undefined. Two pieces of independent evidences have suggested MEK as the MMP-8 regulator: (i) MMP-8 expression is CCAAT enhancer binding protein (C/EBP)-dependent during myeloid development; (ii) MEK is an upstream kinase of C/EBP in growth factor-regulated cortical neurogenesis. To our knowledge, inhibition of BQE-induced MMP-8 expression by PD98059 may be the first evidence showing MEK as the up-regulator of MMP-8 (Fig. 2D).

About 70% oral tumor patients keep BQ-chewing habit upon their hospitalization at Chi Mei Medical Center in southern Taiwan (unpublished records). Their tumor cells may thus encounter a constant and long-term selection force by BQ. According to our in vitro study, 10-min treatment of 24.8 mg/ml BQE or 200 μg/ml arecoline is not cytotoxic to both OECM-1 (Fig. 1A and Fig. 3A) and CE81T/VGH (data not shown). This may reflect the short-term stimulatory effect of BQ chewing on oral tumor cells, and one of the responses of these cells is to express more MMP-8. When the treatment is prolonged to 24 h, less than 2 mg/ml BQE or 100 μg/ml arecoline becomes cytotoxic to both OECM-1 and CE81T/VGH (data not shown). This, in turn, may reflect the repetitive BQ stimulation in heavy BQ users, which brings about a lethal impact on their oral tumor cells. Our in vitro selection model suggests that cells survived from this impact may acquire an accelerated migration potential through up-regulation of MMP-8.

OECM-1 has also been subjected to BQE or arecoline selection process. Both selected cells were more resistant to cisplatin, whereas their 2D motility remained similar to parental cells (data not shown). In contrast, BQE- or arecoline-selected CE81T/VGH cells showed enhanced 2D motility (Fig. 5), whereas their sensitivity to cisplatin was distinguishable from that of parental cells (data not shown). Therefore, selection by BQE or arecoline does not necessarily accelerate cell migration. Nevertheless, enhanced motility or drug resistance increases the malignancy of tumor cells. It is thus reasonable to speculate that cells survived from long-term BQ challenge may result in different phenotypic changes, which favors the survival (growth) or migration (invasion/metastasis). In conclusion, BQ is not only a carcinogen, but also a promoter in the process of tumor progression.

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