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Oral and Maxillofacial Pathology

Regulation of interleukin-6 expression by arecoline in human buccal mucosal fibroblasts is related to intracellular glutathione levels

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OBJECTIVES: Cytokines play an important role in regulating fibroblast function and is likely to play a key role in regulating the initiation and progression of scarring in any fibrotic disease. Interleukin-6 (IL-6) has been implicated in the development of a variety of fibrotic diseases. The aim of this study was to compare IL-6 expression in fibroblasts cultured from normal human buccal mucosa and oral submucous fibrosis (OSF) specimens and further explore the potential mechanism that may lead to induce IL-6 expression.

METHODS: mRNA level of IL-6 in fibroblasts from OSF was compared with normal buccal mucosa. The effects of arecoline, the major areca nut alkaloid, on IL-6 expression in normal human buccal mucosa fibroblasts (BMFs) were measured *in vitro*. mRNA was quantified with AlphaImager 2000. To determine whether glutathione (GSH) levels were important in the induction of IL-6 by arecoline, we pretreated cells with 2-oxothiazolidine-4-carboxylic acid (OTZ) to boost GSH levels or with buthionine sulfoximine (BSO) to deplete GSH.

RESULTS: Fibroblasts derived from OSF exhibited higher IL-6 gene expression than BMF in mRNA levels ($P < 0.05$). The exposure of quiescent BMF to arecoline resulted in the elevation of IL-6 mRNA expression in a dose-dependent manner ($P < 0.05$). IL-6 gene regulated by arecoline correlated with intracellular GSH levels in BMF. Arecoline at a concentration of 129 μM induced about 2.7-fold IL-6 mRNA levels over the 6-h incubation period. However, BSO enhanced the IL-6 mRNA levels by 3.9-fold ($P < 0.05$). In addition, OTZ was found to marginally reduce the arecoline-induced IL-6 expression by about 1.7-fold ($P < 0.05$).

CONCLUSIONS: Taken together, these results suggest that IL-6 expression is significantly upregulated in OSF fibroblasts in areca quid chewers and arecoline may be responsible for the enhanced IL-6 expression. In addition, the regulation of IL-6 expression induced by arecoline is critically dependent on the intracellular GSH concentrations.

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Keywords: arecoline; buccal mucosal fibroblasts; oral submucous fibrosis; interleukin-6; glutathione

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Introduction

Oral submucous fibrosis (OSF) is an insidious oral disease, the principal feature of which is progressive inability to open month because of loss of elasticity. OSF involves the lamina propria and the submucosa and often extends to the underlying muscle. Although the exact pathogenesis of OSF is not yet known, there is strong epidemiological evidence to implicate chewing of areca quid as the major etiological factor (Sinor *et al*, 1990; Maher *et al*, 1994; Mutri *et al*, 1995).

Experimental studies have shown that arecoline, a major areca nut alkaloid, could stimulate human buccal mucosal fibroblast (BMF) proliferation (Harvey *et al*, 1986; Chang *et al*, 1998) and collagen synthesis *in vitro* (Harvey *et al*, 1986). Stabilization of collagen and prevention of collagenase degradation in oral mucosa (Kuo *et al*, 1995) and the attendant increase of lysyl oxidase activity (Ma *et al*, 1995; Trivedy *et al*, 1999) also contribute to abnormal deposition of collagen in OSF. In addition, our recent studies have shown that the upregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) (Chang *et al*, 2002a), vimentin (Chang *et al*, 2002b), cyclooxygenase-2 (Tsai *et al*, 2003), and plasminogen activator inhibitor-1 (Yang *et al*, 2003a) and

decreased matrix metalloproteinase-2 activity (Chang *et al*, 2002a) may also contribute to the extracellular components accumulation in OSF. Despite above evidences, pathogenesis for OSF remains unclear.

Cytokines play an important role in regulating fibroblast function, such as proliferation, migration and matrix synthesis, and it is the balance of these mediators that is likely to play a key role in regulating the initiation and progression of scarring in any fibrotic disease (Koracs, 1991). Interleukin-6 (IL-6) has been implicated in the development of fibrosis. High serum levels of IL-6 are seen in systemic sclerosis patients (Hasegawa *et al*, 1998). IL-6 is also involved in platelet derived growth factor-dependent proliferation of human fibroblasts (Roth *et al*, 1995) and is also known to upregulate fibroblast α1 procollagen *in vivo* (Greenwel *et al*, 1995).

An areca quid-induced alternation in cytokine profile within oral mucosa tissue could thus cause dysregulation of connective tissue turnover with the resultant accumulation of matrix components found in OSF. The aims of this study were to investigate the relative levels of IL-6 in the fibroblasts cultured from OSF compared with normal buccal mucosa and the effects of arecoline on IL-6 expression in normal human BMFs *in vitro*. In addition, glutathione (GSH) level was found to be decreased in areca quid chewers with OSF compared with normal buccal mucosa of healthy individuals (Wong *et al*, 1994). GSH depletion might play an important role in the pathogenesis of fibrosis. It is well-known that buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamyl cysteine synthetase, inhibits GSH synthesis, is relatively non-toxic, and is quite efficient in decreasing intracellular GSH levels. 2-Oxothiazolidine-4-carboxylate (OTZ), a precursor of cysteine, metabolically promotes GSH synthesis, increasing intracellular GSH levels by as much as two to three times the control level (Williamson *et al*, 1982; Meister, 1983). Thus, we pretreated cells with the OTZ to boost GSH levels or with BSO to deplete GSH and determined whether GSH levels may be important in the regulation of IL-6 by arecoline.

Materials and methods

Cell culture

Six healthy individuals without areca quid chewing habits were selected from the Oral Medicine Center (Chung Shan Medical University Hospital, Taichung, Taiwan) with their informed consent for this study. Biopsy specimens were derived from histologically normal areas at surgical extraction of third molars from these healthy controls. The OSF specimens were obtained from 10 male patients with areca quid chewing habits by surgical biopsy. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens. Fibroblasts were cultured using an explant technique as described previously (Chang *et al*, 2000, 2001). The tissues were minced using sterile techniques and washed twice in phosphate buffered saline supplemented with antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} of fungizone). Explants were placed into 60 mm dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco Laboratories) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

Expression of IL-6 mRNA in OSF and BMF

Total RNA was prepared using TRIzol reagent (Gibco Laboratories) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco Laboratories). The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for the polymerase chain reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for IL-6 in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C. The sequences of primers used were as follows (Yang *et al*, 2003b):

(A) GAPDH Forward: 5′-TCCTCTGACTTCAACAG CGACACC-3′ Reverse: 5′-TCTCTCTTCCTCTT GTGCTCTTGG-3′
(B) IL-6 Forward: 5′-CCACTCACCTCTTCAGAA-3′ Reverse: 5′-GCGCAGAATGAGATGAGT-3′

The PCR products were analyzed by agarose gel electrophoresis and a 453 bp band for IL-6 was noted. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

Effect of arecoline on IL-6 mRNA expression in BMF

Prior to treatment, the cells were washed with serum-free DMEM and immediately thereafter exposed to arecoline (0–514 μM) for 6 h. In selected experiments, cells were pre-exposed to OTZ or BSO for 24 h before the addition of arecoline. The concentrations of OTZ (5 mM) and BSO (50 μM) were not cytotoxic to BMFs as described by Tsai *et al* (2003). This protocol was necessary to allow changes in GSH levels to occur prior to exposure to arecoline. Total RNA was isolated after 6-h incubation period for reverse transcriptase polymerase chain reaction (RT-PCR) as described above.

Statistical analysis

Experiments were performed in triplicate throughout this study. The significance of the results obtained from control and treated groups was statistically analyzed by

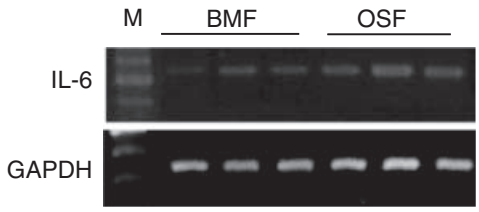

Figure 1 Comparison of the interleukin-6 (IL-6) mRNA level from buccal mucosa fibroblasts (BMFs) and the fibroblasts cultured from oral submucous fibrosis (OSF) using reverse transcriptase polymerase chain reaction assay. GAPDH gene was performed in order to monitor equal RNA loading. Fibroblasts derived from OSF specimens are significantly upregulated IL-6 mRNA expression than BMFs

the Student *t*-test. A *P*-value of < 0.05 was considered to be statistically significant.

Results

The RT-PCR for IL-6 mRNA gene was used to compare the cells cultured from BMF and OSF. As shown in Figure 1, 10 fibroblast strains derived from OSF specimens exhibited significantly higher IL-6 mRNA expression than BMFs. From the AlphaImager 2000, the intensity of IL-6 mRNA from the fibroblasts cultured from OSF was elevated 2.1-fold as compared with BMFs ($P < 0.05$).

To examine the effect of arecoline on the IL-6 expression, human BMFs were treated with arecoline and the levels of mRNA and protein were measured. The effects of arecoline on the IL-6 gene expression in three different cell strains were similar, and their intracellular variations were limited.

Reverse transcriptase polymerase chain reactions were used to verify whether arecoline could affect IL-6 mRNA expression in human BMFs. Arecoline upregulated IL-6 expression in BMFs at concentrations (0–514 μM) tested (Figure 2a). The quantitative measurement was made by the AlphaImager 2000 (Figure 2b). The levels of the IL-6 mRNAs increased about 2.5- and 1.3-fold after exposure to 64.3 and 257 μM arecoline for 6 h ($P < 0.05$), respectively. The decrease of detectable IL-6 mRNA at arecoline concentrations exceeding 514 μM may result from increasing cytotoxicity. Moreover, the peak of IL-6 mRNA levels induced by arecoline was 129 μM (Figure 2a).

To determine whether GSH levels modulated IL-6 gene expression in human BMFs by arecoline, monolayers were pretreated with OTZ or BSO. As shown in Figure 3a, addition of OTZ or BSO alone did not alter IL-6 mRNA levels in comparison with untreated control. However, pretreatment with OTZ led to decrease in induction of IL-6 mRNA by arecoline. In parallel studies, cells were pretreated with BSO. BSO was found to increase arecoline-induced IL-6 mRNA levels. From the AlphaImager 2000 (Figure 3b), arecoline at a concentration of 129 μM induced about 2.7-fold IL-6 mRNA levels over the 6-h incubation period. However, BSO enhanced the IL-6 mRNA levels up to 3.9-fold ($P < 0.05$). In addition, OTZ was found to marginally

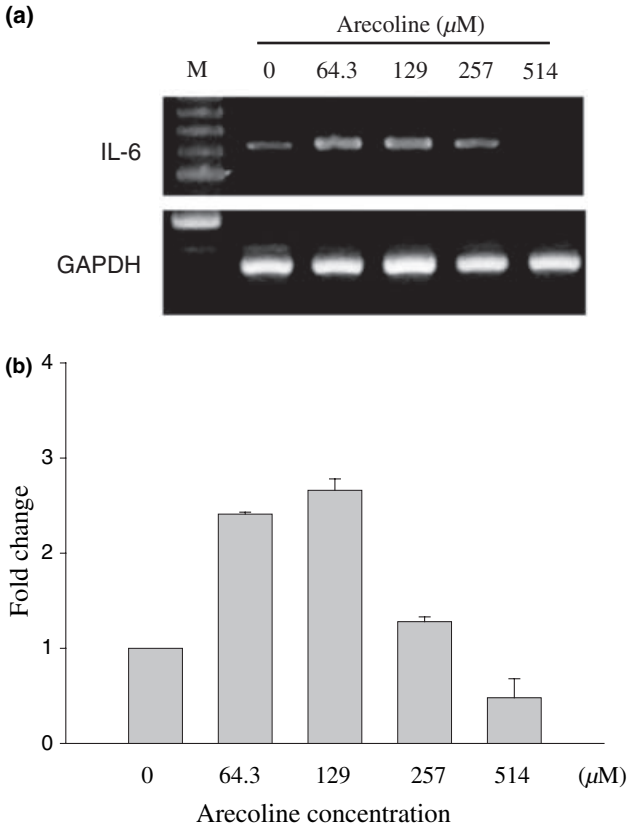

Figure 2 (**a**) Expression of interleukin-6 (IL-6) mRNA gene in arecoline-treated human buccal mucosa fibroblasts (BMFs) by reverse transcriptase polymerase chain reaction assays. Cells were exposed for 6 h containing arecoline concentrations as indicated. M = DNA molecular size marker. (**b**) Densitometric analysis of the IL-6 bands was calculated from their mRNA activity. Optical density values represent the means of three different BMF ± standard deviations

reduce the arecoline-induced IL-6 expression by about 1.7-fold ($P < 0.05$).

Discussion

Fibroblasts are the principal cell type residing in connective tissue and are responsible for the formation and turnover of the extracelluler matrix. Fibroblast function is, in turn, regulated by bioactive molecules acting in the local tissue environment. To the best of our knowledge, we first found that the fibroblasts cultured from OSF demonstrated significantly higher IL-6 expression than BMFs derived from normal buccal mucosa in their mRNA level. Consistently, Haque *et al* (1998) have shown that IL-6 expression is upregulated in OSF specimens compared with normal buccal mucosa by immunohistochemistry. These findings suggest that IL-6 may play an important role in increased synthesis and deposition of extracellular matrix components in OSF.

Furthermore, we treated BMFs with arecoline to examine its influence on IL-6 to search for the possible pathogenesis of areca quid-associated OSF *in vivo*. Data from our *in vitro* experiments also show that arecoline is

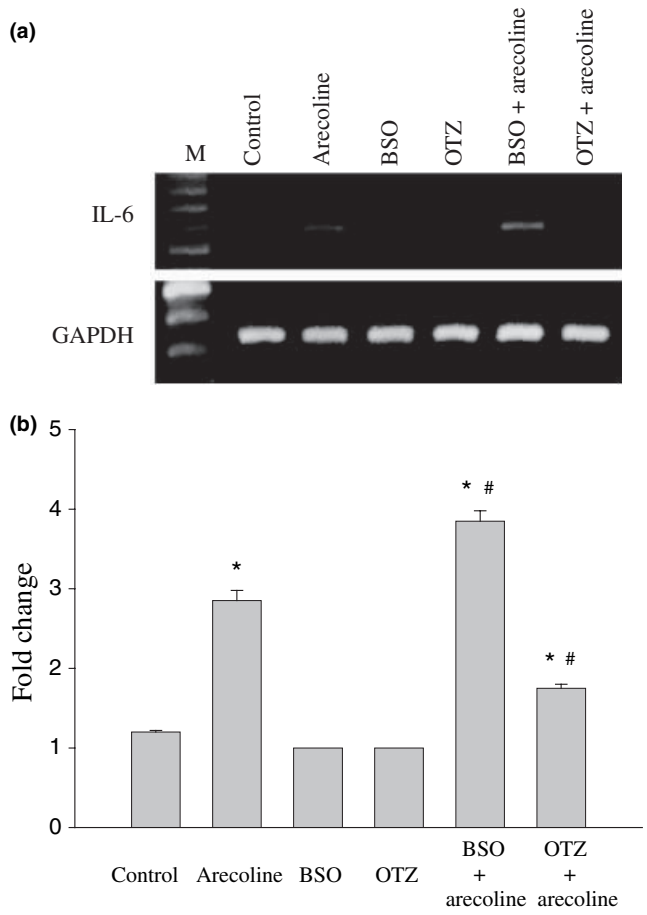

Figure 3 (**a**) Effects of 2-oxothiazolidine-4-carboxylic acid (OTZ) and buthionine sulfoximine (BSO) on arecoline-induced Interleukin-6 (IL-6) expression in human buccal mucosa fibroblasts (BMFs). Cells were pre-exposure with OTZ or BSO for 24 h then were treated for 6 h in the presence of 129 μM arecoline. M = DNA molecular size marker. (**b**) Levels of IL-6 gene in BMFs after exposure to BSO or OTZ alone and to 129 μM. Quantitation was achieved by AlphaImager 2000. *Significant difference from control values with $P < 0.05$. #Statistically significant between arecoline alone and arecoline with BSO or OTZ; $P < 0.05$

capable of stimulating IL-6 mRNA expression in human BMFs. Our data were in agreement with previous studies that arecoline can stimulate IL-6 secretion from human peripheral blood mononuclear cells (Haque *et al*, 2000), and oral fibroblasts (Chen *et al*, 1995), but not in human gingival keratinocytes (Jeng *et al*, 2003) *in vitro*. This suggests that one of the pathogentic mechanisms of OSF may be increased synthesis and expression of IL-6 by resident cells in response to areca nut challenge.

The possible mechanism why arecoline-induced IL-6 expression may play a pivotal role in the pathogenesis of OSF is not fully understood. In terms of connective tissue turnover, IL-6 was found to reduce tissue breakdown through increased TIMP expression (Sato *et al*, 1990; Lotz and Guerne, 1991). A tissue turnover imbalance in the oral mucosa could be of particular importance in this disorder. The relative overexpression of IL-6 could result in the accumulation of extracellular matrix which characterizes OSF. In addition, IL-6 has been shown to stimulate the proliferation of human keratinocyte cultures (Grossman *et al*, 1989), an effect which could account for the epithelial hyperplasia observed in overgrowth of gingival tissues (Belazi *et al*, 1993). Recently, our study has shown TIMP-1 level is upregulated in OSF than normal buccal mucosa and arecoline can elevate TIMP-1 in BMFs (Chang *et al*, 2002a). Thus, the interaction between IL-6 and TIMP-1 is worthy of further investigation.

Glutathione is the most abundant intracellular thiol that acts as a major cellular antioxidant (Meister and Anderson, 1983). Previous studies have shown GSH depletion may be one of the mechanisms underlying arecoline-induced cytotoxicity (Deb and Chatterjee, 1998; Chatterjee and Deb, 1999; Jeng *et al*, 1999; Chang *et al*, 2000, 2001). To determine whether GSH levels modulated IL-6 gene expression in human BMFs by arecoline, confluent monolayers were pretreated with OTZ or BSO. In the present study, addition of OTZ or BSO alone did not alter IL-6 mRNA levels in comparison with untreated control. However, pretreatment with OTZ led to decrease in induction of IL-6 mRNA by arecoline. In addition, BSO alone failed to induce IL-6 mRNA at 6 h or at earlier time periods. These experiments suggest that GSH depletion *per se* is not causally related to IL-6 gene induction at the transcriptional level. However, pre-exposure to BSO caused an increase in IL-6 mRNA level by arecoline. Our results are in agreement with Mantovani *et al* (2003), who reported that GSH treatment reduces serum levels of IL-6. Taken together, GSH may act as an intracellular buffer against arecoline-mediated induction of IL-6 gene.

This study represents to the best of our knowledge, the first attempt to evaluate the role of IL-6 mRNA gene expression in areca quid associated-OSF. Levels of IL-6 were found to be elevated in the fibroblasts cultured from OSF specimens. Arecoline was also capable of stimulating IL-6 mRNA expression in human BMFs. Upregulation of IL-6 may be an important mechanism is areca quid-associated OSF. In addition, IL-6 regulated by arecoline is critically dependent on the intracellular GSH concentration. Based on these findings, we propose that use of GSH-rich foods or GSH-like antioxidants may provide a valuable tool in reduction of OSF progression associated with areca quid chewing.

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