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半胱氨酸蛋白水解酵素與 cystatin C 在人類正常頰黏膜及口腔黏膜下纖維化的表現

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The upregulation of cystatin C in oral submucous fibrosis

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Summary Cystatin C is a 13 kDa non-glycosylated basic protein belonging to cystatin family. It is consistently and dramatically upregulated in a variety of fibrotic diseases. The aim of this study was to compare cystatin C expression in normal human buccal mucosa and oral submucous fibrosis (OSF) specimens and further explore the potential mechanism that may lead to induce cystatin C expression. Twenty-five OSF specimens and six normal buccal mucosa were examined by immunohistochemistry. The activity of cystatin C from fibroblasts cultured from OSF and normal buccal mucosa were by using reverse-transcriptase polymerase chain reaction and enzyme-linked immunosorbent assay. Furthermore, the effect of arecoline, the major areca nut alkaloid, was added to explore the potential mechanism that may lead to induce cystatin C expression. Cystatin C expression was significantly higher in OSF specimens ($p < 0.05$) and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. OSF demonstrated significantly higher cystatin C expression than normal buccal mucosa fibroblasts both in mRNA and protein levels ($p < 0.05$). In addition, arecoline was also found to elevate cystatin C mRNA and protein expression in a dose-dependent manner ($p < 0.05$). Taken together, the data presented here demonstrated that cystatin C expression is significantly upregulated in OSF from areca quid chewers and arecoline may be responsible for the enhanced cystatin C expression in vivo.

Introduction

Areca quid chewing is one of the most worldwide addictive oral habits.[1] Areca quid chewing has been recognized as one of the most important risk factors for oral submucous fibrosis

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(OSF).[2,3] OSF is regarded as a precancerous condition,[4] and is a chronic insidious disease of oral subepithelial connective tissue resulting in stiffness of the oral mucosa and inability to open the mouth.

OSF is characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria and submucosa of the oral mucosa.[4] It results from excessive deposition of extracellular matrix (ECM) due to an imbalance between degradation and synthesis. Serine proteinases, cysteine proteinases and metalloproteinases participate in the degradation of ECM. Collagenases as well as members of the matrix metalloproteinases (MMPs) are enzymes that break down collagens in oral mucosa.[5,6] Tissue inhibitors of metalloproteinases (TIMPs), specific inhibitors of MMPs, are also reported to play a role in the remodelling associated with OSF.[6,7]

The protein inhibitors of cysteine proteinases, the cystatins, comprise three families: stefins, cystatins, and kininogens.[8] Cystatins are tight- and reversible-binding inhibitors of the papain-like cysteine proteinases. Cystatin C, one member of the cystatin family, is a non-glycosylated 13 kDa basic protein that has two disulphide bonds,[9] and is a very potent inhibitor of lysosomal cysteine proteinases such as cathepsin B, H, L and S.[10] Cystatin C is consistently and dramatically upregulated in a variety of fibrotic diseases, such as multiple sclerosis sclerosis,[11] chronic hepatic fibrosis,[12] and the progression of liver fibrosis.[13]

Previously, Chiu et al.[14] have demonstrated the relationship between genotype distribution and risk for human cystatin C genotype for OSF. This suggests that cystatin C may play an important role in the pathogenesis of areca quid chewing-associated OSF. However, the authors do not take into account the cellular source of the cystatin C in OSF as well as normal buccal mucosa. On the basis of these observations, the present work was undertaken to identify the in situ localization of cystatin C expression in normal buccal mucosa and OSF specimens. More specifically, we have therefore measured the relative levels of cystatin C in OSF compared with normal buccal mucosa and the effects of arecoline, a major areca nut alkaloid, on cystatin C in normal human buccal mucosa fibroblasts (BMFs) in vitro.

Materials and methods

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of six normal buccal mucosa from non-areca quid chewers, and 25 OSF specimens from areca quid chewers, were drawn from the files of the Department of Pathology, Chung Shan Medical University Hospital. Diagnosis was based on histological examination of hematoxylin- and eosin-stained sections. Five micrometer sections were stained with the polyclonal anti-cystatin C antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100 dilution) using a standard avidin-biotin-peroxidase complex method.[15] 3-Amino-9-ethylcarbazole (AEC, DAKO, Carpinteria, CA, USA) was then used as the substrate for localizing the antibody binding. Negative controls included serial sections from which either the primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy.

Cell culture

Ten healthy individuals, without areca quid chewing habits, attending the Oral Medicine Center (Chung Shan Medical University Hospital, Taichung, Taiwan) were enrolled with informed consents for this study. Biopsy specimens were derived from histologically normal oral mucosa at the time of surgical third molar extraction. The OSF specimens were obtained from 20 male patients with areca quid chewing habits during surgical biopsy. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens. Fibroblast cultures were grown and maintained using procedures described previously.[16–18] Cell cultures between the third and eighth passages were used in this study.

Expression of cystatin C mRNA in OSF and BMF

Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in 60 mm culture dish and allowed to achieve confluence. Total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) 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 U of Moloney murine leukemia virus reverse transcriptase (Gibco Laboratories, Grand Island, NY, USA). 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 U of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for cystatin C in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C, and 1 min of extension at 72 °C. The sequences of primers used were as follows[19]:

(A) GAPDH Forward: 5′-TCCTCTGACTTCAACAGCGACACC-3′.
Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′.
(B) Cystatin C Forward: 5′-GCT CTT TCC AGA TCT ACG CT-3′.
Reverse: 5′-AGG CAG CCG ATG CTA CTATT-3′.

The PCR products were analyzed by agarose gel electrophoresis and a 277 bp band for cystatin C 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).

Assessment of cystatin C activity in OSF and BMF

Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in 60 mm culture dish and al-

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lowed to achieve confluence. The conditioned medium samples were collected after 2 day cultured period. Levels of cystatin C antigen were determined by enzyme-linked immunosorbent assay (ELISA) (Biovendor Laboratory Medicine, Inc., Heidelberg, Germany). Briefly, 20 μl of conditioned media were directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer and cystatin C levels were determined with a calibration curve using human cystatin C as a standard.

Effect of arecoline on cystatin C mRNA and protein in BMF

BMF derived from three strains were seeded 1×10^5 cells per well into 10 cm culture dish and incubated for 24 h. Then the medium was changed to a medium containing 10% heated activated FCS and various concentrations of arecoline (Sigma, St. Louis, MO, USA) (0—80 μg/ml). Total RNA was isolated after 6 h incubation period for RT-PCR as described above. Condition medium were collected after 24 h incubation period for ELISA as described earlier.

Statistical analysis

Triplicate or more separate experiments were performed throughout this study. For testing of differences in the cystatin C between the BMF and OSF, the Wilcoxon—Mann—Whitney rank sum test was applied. The significance of the results obtained from control and treated groups was statistically analyzed by the Student's *t*-test. A *p*-value of <0.05 was considered to be statistically significant.

Results

The connective tissue of normal human buccal mucosa demonstrated with very faint cystatin C expression (Fig. 1). All OSF samples exhibited the features of either moderately advanced or advanced submucous fibrosis. Cystatin C expression was observed mainly in the cytoplasm of fibroblasts, endothelial cells, and inflammatory cells throughout the connective tissue (Fig. 2). However, cystatin C staining was also expressed in the epithelium both OSF and normal buccal mucosa specimens. The intensity of cystatin C within epithelium between normal buccal mucosa and OSF was not statistical significant ($p > 0.05$).

RT-PCR assay was used to compare cystatin C mRNA gene expression of the fibroblasts cultured from BMF and OSF. As shown in Figure 3, OSF specimens exhibited significantly higher cystatin C mRNA expression than BMFs. From the AlphaImager 2000, the intensity of cystatin C mRNA from OSF was elevated about 2.7-fold as compared with BMFs ($p < 0.05$).

The individual values of cystatin C from BMF and OSF cultures from ELISA were shown in Table 1. The amount of cystatin C protein in BMF was about 728 ± 37.24 ng/10^6 cells. The amount of cystatin C protein in OSF was about 1169 ± 56.96 ng/10^6 cells. In addition, cystatin C was found increased about 1.6-fold in OSF as compared with BMF ($p < 0.05$).

To examine the effect of arecoline on the cystatin C expression, human BMFs were treated with arecoline and the levels of mRNA and protein were measured. RT-PCR were used to verify whether arecoline could affect cystatin C mRNA gene expression by human BMFs. Figure 4 reveals a dose-dependent change following treatment of BMF with

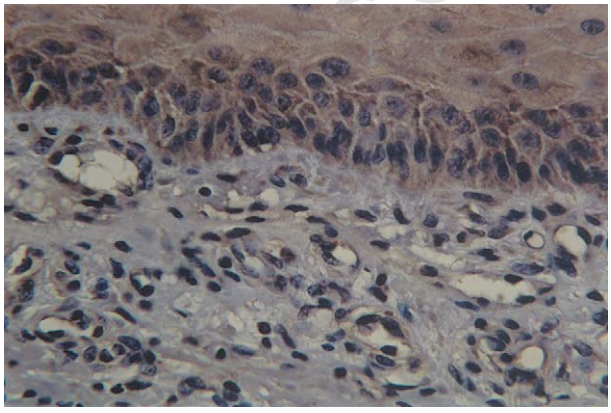

Figure 1 Very faint immunoreactivity of cystatin C was observed in normal human buccal mucosal connective tissue and the strong signal was seen in the epithelium (400×).

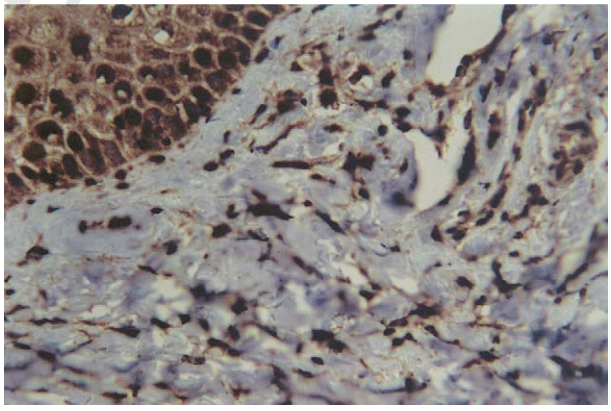

Figure 2 In OSF specimen, cystatin C was evident as intense, diffuse brown coloring throughout the connective tissue and detected at relatively high levels in the epithelium (400×).

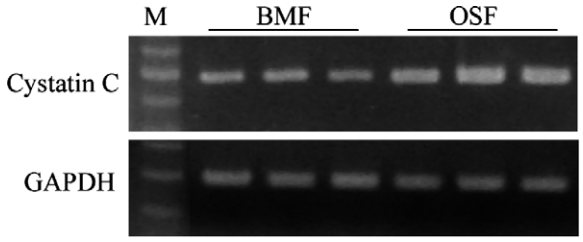

Figure 3 Comparison of the cystatin C mRNA level from BMFs and OSFs using RT-PCR assay. GAPDH gene was performed in order to monitor equal RNA loading. Fibroblasts derived from OSF are significantly upregulated cystatin C mRNA gene expression than BMFs.

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Table 1 Summary of cystatin C protein levels from BMF and OSF by using ELISA

Subjects	BMF (n = 10)		OSF (n = 20)	
	Media (ng/10^6 cells)	Range (ng/10^6 cells)	Media (ng/10^6 cells)	Range (ng/10^6 cells)
Cystatin C	693.24	417.94—953.2	1032.77*	837.46—1543.96
Mean ± SD	728 ± 37.24		1169 ± 56.96*	

* Statistically significant between BMF and OSF, $p < 0.05$.

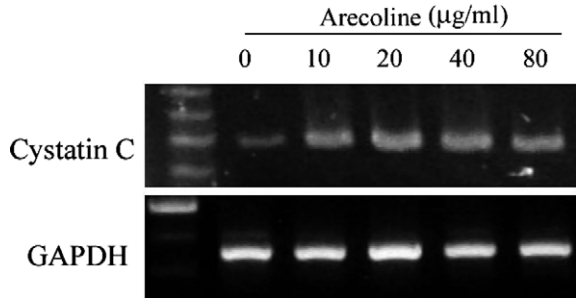

Figure 4 Expression of cystatin C mRNA gene in arecoline-treated human BMFs by RT-PCR assays. Cells were exposed for 6 h containing arecoline concentrations as indicated. M = DNA molecular size marker. GAPDH gene was performed in order to monitor equal RNA loading.

arecoline for 6 h. Arecoline was found to elevate cystatin C mRNA gene expression in a dose-dependent manner ($p < 0.05$). From the AlphaImager 2000, the amount of cystatin C was elevated about 2.5-, 3.7-, 3.0-, and 2.6-fold at concentrations of 10, 20, 40, and 80 μg/ml, respectively, as compared with control (Fig. 5).

As shown in Figure 6, arecoline was found to upregulate cystatin C activity in BMF cultures. The effect of arecoline on cystatin C protein in BMF during 24 h incubation period, arecoline was found to elevate cystatin C expression in a dose-dependent manner ($p < 0.05$).

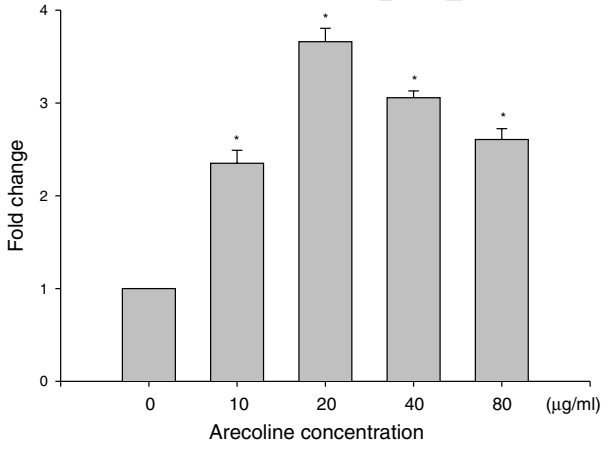

Figure 5 Densitometric analysis of the cystatin C bands was calculated from their mRNA activity. Optical density values represent the means of three different BMF ± standard deviations.

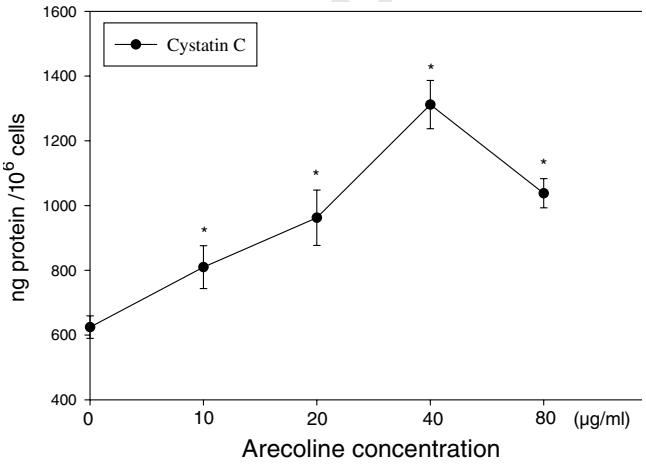

Figure 6 Expression the protein level of cystatin C in arecoline-treated human BMFs by using ELISA. '*' Significant differences from control values with $p < 0.05$.

Discussion

The biochemical events involved in the development of the OSF are not well understood at present. Proteinase/antiproteinase imbalance may be caused by predominance of proteinases, resulting in severe tissue damage or abundance of proteinase inhibitors, leading to a shift in the balance of synthesis and degradation of ECM proteins and accumulation of these matrix components. Cysteine proteases have been linked with matrix turnover by degrading existing ECM.[20] Cystatin C, a member of the cystatin superfamily, has inhibitory activity toward cysteine proteinases. It has been suggested that cystatin C plays a regulatory and defensive role against exogenous cysteine proteinases present in body fluids.[10] It is reasonable to speculate that cystatin C may directly relate to the pathogenesis of OSF.

Cystatin C is a 13 kDa non-glycosylated basic protein belonging to cystatin family.[9] It is consistently and dramatically upregulated in a variety of fibrotic diseases.[11–13] To the best of our knowledge, we first found that cystatin C expression is upregulated in OSF specimens compared to normal buccal mucosa. Strong immunostaining for cystatin C was detected in fibroblasts, endothelial cells and inflammatory cells. In addition, OSF has significantly higher cystatin C expression than BMF derived from normal buccal mucosa both in mRNA and protein levels. cystatin C deposition is associated with OSF, suggesting that it could be play an important role in the ECM turnover. This phenomena promoted us to elucidate that OSF may be due to increased

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synthesis and deposition of ECM proteins, altered degradation or both.

The etiology of OSF is still not quite clear, it is known that an association exists the chewing of areca quid. In this study, we first report upregulation of cystatin C mRNA and protein expression in normal human BMFs stimulated by arecoline. This suggests that one of the pathogenetic mechanisms of OSF may be the synthesis of cystatin C expression by resident cells in response to areca nut challenge. The accumulation of ECM components in oral mucosal connective tissue may be caused by a simultaneous effect on cystatin C.

The other main extracellular proteolytic systems have been recognized as: (1) the MMP-dependent pathway and (2) the plasminogen-dependent pathway.[21] Our previous studies have demonstrated that arecoline reduces the secretion of gelatinolytic activity of MMP-2 from BMF in cell culture experiments[6] and TIMP-1[6] and type I plasminogen activator inhibitor (PAI-1)[22] were found to be upregulated in OSF. In a previous study, it has been shown that transforming growth factor beta could simultaneously increase the activities of MMP-2 and cystatin C in the differentiated podocytes.[23] Recently, one report has suggested that members of the cystatin family may protect the MMPs family from autolytic degradation without interfering with its gelatinolytic activities. In the absence of cystatins, MMPs were completely degraded within 4 h.[24] Indeed, the plasmin-dependent pathway is understood to be a significant alternative pathway for the initiation of ECM degradation by MMPs.[25] Thus, the regulatory mechanisms may be through several different pathways. The interaction between MMPs, PAI-1, and cystatin C is worthy of further investigation.

As far as we known, this is the first systematic attempt to evaluate the role of cystatin C expression in areca quid associated-OSF in human at both in vivo and in vitro levels. We have demonstrated for the first time that cystatin C is elevated in OSF than normal buccal mucosa. Data from our in vitro experiments showed that arecoline was capable of stimulating cystatin C mRNA and protein expression in human BMFs. This suggests that one of the pathogenic mechanisms of OSF in vivo may be the synthesis of cystatin C by resident cells in response to areca nut challenge. Cysteine proteases secreted or synthesized are inhibited by cystatin on a basis of ratio of 1:1. ECM remodeling is a result of the balance between synthesis and degradation. More detailed studies should be undertaken to clarify the roles of cysteine proteases in areca quid associated-OSF in human at both in vivo and in vitro levels.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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