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第一型類胰島素生長因子及其接受器在人類正常頰黏膜與口腔黏膜下纖維化症的表現

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The upregulation of insulin-like growth factor-1 in oral submucous fibrosis

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Summary Insulin-like growth factor-1 (IGF-1) is a member of a family of two interacting polypeptide hormone ligands with close homology to proinsulin. IGF-1 can influence mesenchymal cell migration, proliferation, and extracellular matrix deposition, thus implicating it in the progression of fibrotic disorders. Currently, there is limited information about the regulation of IGF-1 expression in areca quid-associated oral submucous fibrosis (OSF). The aim of this study was to compare IGF-1 expression in normal human buccal mucosa and OSF specimens and further explore the potential mechanism that may lead to induce IGF-1 expression. Twenty OSF specimens and 10 normal buccal mucosa were examined by immunohistochemistry. The activity of IGF-1 from cells cultured from OSF and normal buccal mucosa were by using reverse-transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). Furthermore, the effect of arecoline, the major areca nut alkaloid, was added to explore the potential mechanism that may lead to induce IGF-1 expression. IGF-1 expression was significantly higher in OSF specimens ($p < 0.05$) and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. OSF demonstrated significantly higher IGF-1 protein expression than normal buccal mucosa fibroblast (BMF) both in mRNA and protein levels ($p < 0.05$). In addition, arecoline was also found to elevate IGF-1 mRNA and protein expression in a dose-dependent manner ($p < 0.05$). Taken together, the data presented here demonstrated that IGF-1 expression is significantly upregulated in

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OSF from areca quid chewers and arecoline may be responsible for the enhanced IGF-1 expression in vivo.

Introduction

Oral submucous fibrosis (OSF) has been identified as a precancerous condition.[1] One of the clinical symptoms of OSF is trismus, a limitation of mouth opening. These may eventually impair the ability to eat and speak. Areca quid chewing has been recognized as one of the most important risk factors for OSF.[2,3]

The main histopathological characteristic of OSF is the deposition of collagen in the oral mucosa.[4,5] It has been found that arecoline, a major areca nut alkaloid, could stimulate human buccal mucosal fibroblasts (BMFs) proliferation[6,7] and collagen synthesis.[6] A reduced degradation of the α1(I) collagen trimer synthesized by OSF fibroblasts may induce the alteration of the ratio of α1(I):α2(I) chains.[8] The attendant increase of lysyl oxidase activity may also contribute to abnormal deposition of collagen in OSF.[9] Recently, our studies have shown that the upregulation of tissue inhibitor of metalloproteinase-1,[10] vimentin,[11] cyclooxygenase-2,[12] plasminogen activator inhibitor-1,[13] interleukin-6,[14] and keratinocyte growth factor-1[15] may contribute to the extracellular components accumulation in OSF. Despite above evidences, the pathogenesis of OSF related areca quid chewing still remains to be elucidated.

Insulin-like growth factor-1 (IGF-1) is a 70 amino acid, 7.6 kd, single-chain nonglycosylated polypeptide with structural similarity to insulin. It may act as an autocrine or paracrine growth hormone[16] and mediates most of the peripheral IGF-1 directly stimulates fibroblast proliferation and perhaps collagen synthesis.[17,18] IGF-1 is consistently and dramatically upregulated in a variety of fibrotic diseases, such as idiopathic pulmonary fibrosis,[19] systemic sclerosis [21] bleomycin-induced pulmonary fibrosis,[20] and carbon tetrachloride (CCl_4)-induced hepatic fibrosis.[22]

The biologic roles of IGF-1 induce cell proliferation and collagen synthesis, which may be important in fibroproliferative process in areca quid-associated OSF. The purpose of this study was to test whether IGF-1 expression regulated within OSF specimens and to further explore possible pathogenic mechanisms that might lead to enhanced expression of IGF-1 in vivo. More specifically, we also set out to explore where expression of IGF-1 can be triggered in human buccal mucosa fibroblasts (BMFs) stimulated by arecoline in vitro.

Materials and methods

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of 10 normal buccal mucosa from non-areca quid chewers, and twenty 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 micron sections were stained with the monoclonal anti-IGF-1 antibody (Santa Cruz Biotechnology, CA, USA) (1:100 dilution) using a standard avidin—biotin—peroxidase complex method.[23] AEC (DAKO, Carpinteria, 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

Nine healthy individuals without areca quid chewing habits were selected from the Department of Oral Surgery (Chung Shan Medical University Hospital, Taichung, Taiwan) with the informed consent for this study. Biopsy specimens were derived from histologically normal areas of surgical third molar extraction from patients. The OSF specimens were obtained from 22 male patients with areca quid chewing habits during surgical biopsy. Clinical diagnosis was confirmed by histopathological

examination of the biopsy specimens. Fibroblasts were cultured using an explant technique as described previously.[24] The tissues were minced using sterile techniques and wash twice in phosphate buffer saline (PBS) supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml of fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

Expression of IGF-1 mRNA in OSF and BMF

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 units 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 two units of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for IGF-1 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:[25]

[(A) GAPDH]
Forward: 5′-TCCTCTGACTTCAACAGCGACACC-3′
Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′

[(B) IGF-1]
Forward: 5′-AAATCAGCAGTCTTCCAACC-3′
Reverse: 5′-CTTCTGGGTCTTGGGCATGT-3′

The PCR products were analyzed by agarose gel electrophoresis and a 395 bp band for IGF-1 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 IGF-1 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 allowed to achieve confluence. The conditioned medium samples were collected after 2 day cultured period. Levels of IGF-1 antigen were determined by enzyme-linked immunosorbent assay (ELISA) (human IGF-1, Quantikine, DG100, R&D Systems, Inc. MN, USA). 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 IGF-1 levels were determined with a calibration curve using human IGF-1 as a standard.

Effect of arecoline on IGF-1 mRNA and protein in BMF

BMF 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 IGF-1 between the BMF and OSF, the Wilcoxon rank sum test was applied. The significance of the results obtained from control and treated groups was statistically analyzed by the Student *t*-test. A *p*-value of <0.05 was considered to be statistically significant.

Results

The connective tissue from normal buccal mucosa consists of loosely woven collagen bundles in the lamina propria revealing a fine reticular pattern next to the epithelium and a coarser pattern deeper in the lamina propria. The connective tissue of normal human buccal mucosa demonstrated

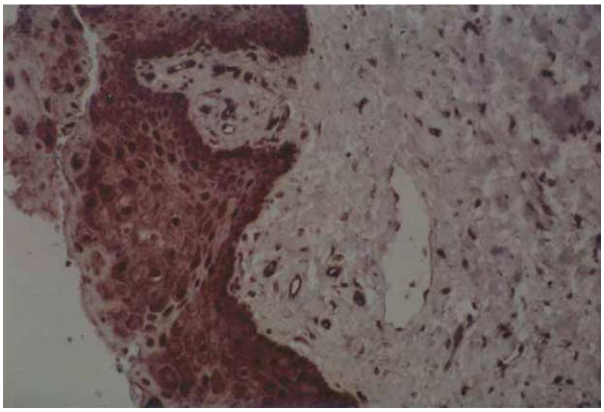

Figure 1 Very faint immunoreactivity of KGF-1 was observed in normal human buccal mucosal connective tissue and the strong signal was seen in the epithelium.

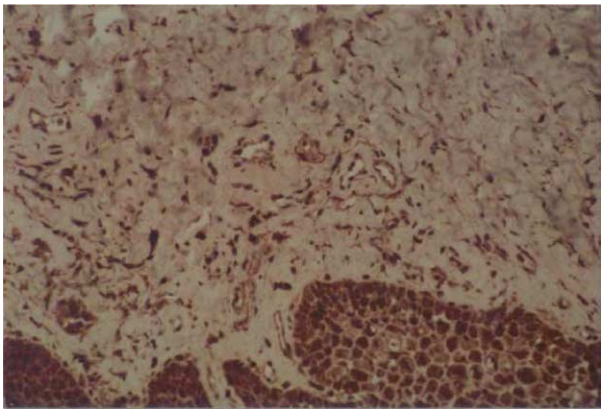

Figure 2 In OSF specimen, KGF-1 was evident as intense, diffuse brown coloring throughout the connective tissue and detected at relatively high levels in the epithelium.

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

RT-PCR assay was used to compare IGF-1 mRNA gene expression of the fibroblasts cultured from BMF and OSF. As shown in Fig. 3, OSF specimens exhibited significantly higher IGF-1 mRNA expression than BMFs. From the AlphaImager 2000, the intensity of IGF-1 mRNA from OSF was elevated about 3.4 fold as compared with BMFs ($p < 0.05$).

The individual values of IGF-1 from BMF and OSF cultures from ELISA were shown in Table 1. The amount of IGF-1 protein in BMF was about 10.96 ± 1.63 ng/10^6 cells. The amount of IGF-1 protein in OSF was about 35.44 ± 6.51 ng/10^6 cells. In addition, IGF-1 was found increased about 3.2 fold in OSF as compared with BMF ($p < 0.05$).

To examine the effect of arecoline on the IGF-1 expression, human BMFs were treated with arecoline and the levels of mRNA and protein were measured. The effects of arecoline on the IGF-1 gene expression in three different cell strains were similar, and their intracellular variations were limited.

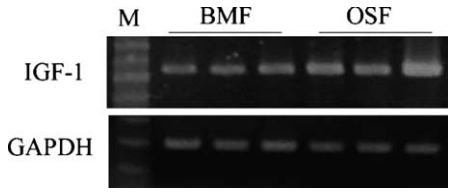

Figure 3 Comparison of the IGF-1 mRNA level from BMFs and OSFs using RT-PCR assay. GAPDH gene was performed in order to monitor equal RNA loading. Fibroblasts derived from OSF are significantly upregulated IGF-1 mRNA gene expression than BMFs.

RT-PCR were used to verify whether arecoline could affect IGF-1 mRNA gene expression by human BMFs. Fig. 4 reveals a dose-dependent change following treatment of BMF with arecoline for 6 h. Arecoline was found to elevate IGF-1 mRNA gene expression in a dose-dependent manner ($p < 0.05$). From the AlphaImager 2000, the amount of IGF-1 was elevated about 2.1, 2.3, 3.2, and 3.4 fold at

Table 1 Summary of IGF-1 protein levels from BMF and OSF by using ELISA

Subjects	BMF ($n = 9$)		OSF ($n = 22$)	
	Media	Range	Media	Range
IGF-1	11.69 (ng/10^6 cells)	7.84—14.6 (ng/10^6 cells)	29.52* (ng/10^6 cells)	13.5—83.78 (ng/10^6 cells)
Mean ± SD	10.96 ± 1.63 (ng/10^6 cells)		35.44 ± 6.51* (ng/10^6 cells)	

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

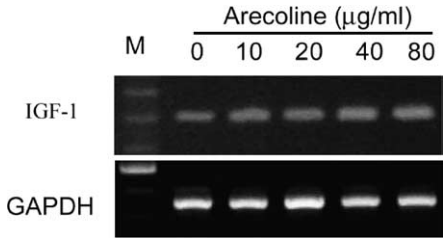

Figure 4 Expression of IGF-1 mRNA gene in arecoline-treated human BMFs by RT-PCR assays. Cells were exposed for 6 h containing arecoline concentrations as indicated. *M* = DNA molecular size marker. GAPDH gene was performed in order to monitor equal RNA loading.

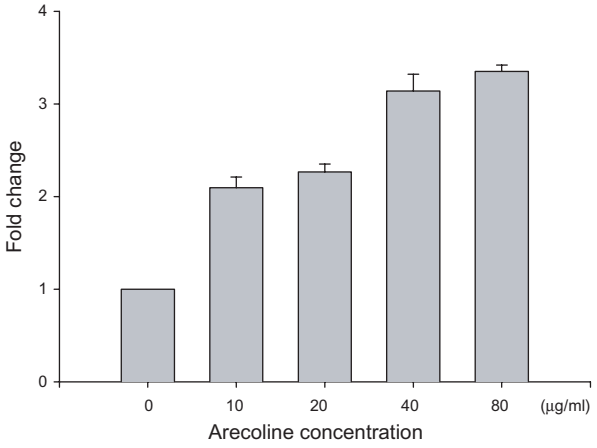

Figure 5 Densitometric analysis of the IGF-1 bands was calculated from their mRNA activity. Optical density values represent the means of three different BMF ± standard deviations.

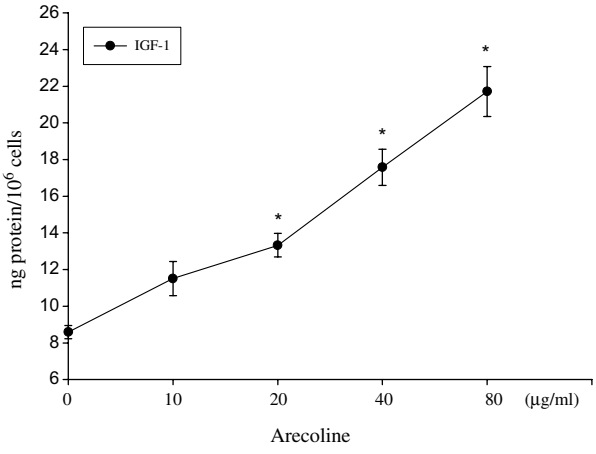

Figure 6 Expression the protein level of IGF-1 in arecoline-treated human BMFs by using ELISA. *Significant differences from control values with $p < 0.05$.

concentrations of 10, 20, 40, and 80 μg/ml, respectively, as compared with control (Fig. 5).

As shown in Fig. 6, arecoline was found to upregulate IGF-1 activity in BMF cultures. The effect of arecoline on IGF-1 protein in BMF during 24 h incubation period is shown in Fig. 6. Arecoline was found to elevate IGF-1 expression in a dose-dependent manner ($p < 0.05$).

Discussion

The relationship between areca quid chewing and OSF is well established from epidemiological studies,[2,3] although the underlying biochemical mechanisms are not completely understood. It has been described that areca quid as well as its component affect fibroblasts proliferation [6,7] increase collagen synthesis.[6] IGF-1 is a profibrogenic growth factor, promoting fibroblast proliferation and extracellular matrix deposition, where increased expression may be critical to the pathogenic of OSF. From the results of immunohistochemistry, our findings demonstrated, for the first time, that positive staining for IGF-1 in connective tissue was found to be upregulated in OSF specimens compared to normal buccal mucosa. Strong immunostaining for IGF-1 was detected throughout the connective tissues, mainly fibroblasts, endothelial cells and inflammatory cells.

Fibroblasts are the principal cell type residing in connective tissue and are responsible for the formation and turnover of extracellular 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 fibroblasts derived from OSF demonstrated significantly higher IGF-1 expression than BMF. Similar results were found other fibrotic pathogenesis like lung fibrosis,[19,20] systemic sclerosis,[21] and hepatic fibrosis.[22] This result may be the reason why there is an excessive increase of collagen in OSF. This phenomena promoted us to elucidate that OSF may be due to increased synthesis and deposition of extracellular matrix.

Areca quid chewing-related oral mucosal lesions are potential hazards to a large population worldwide. Many of the undesirable effects of areca nut have been attributed to arecoline. Data from our in vitro experiment show that arecoline is capable of stimulating IGF-1 expression in human BMFs. The data presented here may partly explain why arecoline could increase oral fibroblasts proliferation[6,7,26] and collagen synthesis.[6,26] During areca quid chewing, arecoline could stimulate synthesis

and deposition of extracellular matrix by elevating IGF-1 levels.

As far as we known, this is the first systematic attempt to evaluate the role of IGF-1 expression in areca quid associated-OSF in human at both in vivo and in vitro levels. We have demonstrated for the first time that IGF-1 expression is upregulated in OSF than normal buccal mucosa. Data from our in vitro experiments showed that arecoline was capable of stimulating IGF-1 mRNA and protein expression in human BMFs. This suggests that one of the pathogenic mechanisms of OSF in vivo may be the synthesis of IGF-1 by resident cells in response to areca nut challenge. However, the genetic and environmental determinants of IGF-1 expression are still incompletely understood. Further research is required, however, including detection of IGF-1 gene transcripts, specifically whether OSF evolves solely as a result of increased/altered de novo synthesis and deposition of IGF-1 by areca nut constitutes.

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References

1. Pindborg JJ, Murt PR, Bhomsle RB, Gupta PC, Daftary DK, Mehta FS. Oral submucous fibrosis as a precancerous condition. *Scand J Dent Res* 1984;**92**:224—9.
2. Murt PR, Bhonsle RB, Gupta PS, Daftary DK, Pindborg JJ, Mehta FS. Etiology of oral submucus fibrosis with special reference to the rate of areca nut chewing. *J Oral Pathol Med* 1995;**24**:145—52.
3. Warnakulasuriya KAAS, Trivedy C, Maher R, Johnson NW. Aetiology of oral submucous fibrosis. *Oral Dis* 1997;**3**:286—7.
4. Caniff JP, Harvey W, Harvis M. Oral submucous fibrosis: its pathologensis and management. *Brit Dent J* 1986;**160**: 429—34.
5. Van Wyk CW, Seedat HA, Phillips VM. Collagen in submucous fibrosis: and electron microscopic study. *J Oral Pathol Med* 1990;**19**:182—7.
6. Harvey W, Scutt A, Meghji S, Canniff JP. Stimulation of human buccal mucosa fibroblasts in vitro by betel-nut alkaloids. *Arch Oral Biol* 1986;**31**:45—9.
7. Chang YC, Tai KW, Cheng MH, Chou LSS, Chou MY. Cytotoxic and non-genotoxic effects of arecoline on human buccal fibroblasts in vitro. *J Oral Pathol Med* 1998;**27**:68—71.
8. Kuo MYP, Chen HM, Hahn LJ, Hsieh CC, Chiang CP. Collagen biosynthesis in human oral submucous fibrosis fibroblast cultures. *J Dent Res* 1995;**74**:1783—8.
9. Trivedy C, Warnakulasuriya KAAS, Hazarey VK, Tavassoli M, Sommer P, Johnson NW. The upregulation of lysyl oxidase in oral submucous fibrosis and squamous cell carcinoma. *J Oral Pathol Med* 1999;**28**:246—51.
10. Chang YC, Yang SF, Tai KW, Chou MY, Hsieh YS. Increased tissue inhibitor of metalloproteinase-1 expression and inhibition of gelatinase A activity in buccal mucosal fibroblasts by arecoline as possible mechanisms for oral submucous fibrosis. *Oral Oncol* 2002;**38**:195—200.
11. Chang YC, Tsai CH, Tai KW, Yang SH, Chou MY, Lii CK. Elevated vimentin expression in buccal mucosal fibroblasts by arecoline in vitro as a possible pathogenesis for oral submucous fibrosis. *Oral Oncol* 2002;**38**:425—30.
12. Tsai CH, Chou MY, Chang YC. The upregulation of cyclooxygenase-2 expression in human buccal mucosal fibroblasts by arecoline: a possible role in the pathogenesis of oral submucous fibrosis. *J Oral Path Med* 2003;**32**:146—53.
13. Yang SF, Hsieh YS, Tsai CH, Chou MY, Chang YC. The upregulation of type I plasminogen activator inhibitor in oral submucous fibrosis. *Oral Oncol* 2003;**39**:367—72.
14. Tsai CH, Yang SF, Chen YJ, Chu SC, Hsieh YH, Chang YC. Regulation of interleukin-6 expression by arecoline in human buccal mucosal fibroblasts is related to intracellular glutathione levels. *Oral Dis* 2004;**10**:360—4.
15. Tsai CH, Yang SF, Chen YJ, Chou MY, Chang YC. Raised keratinocyte growth factor-1 expression in oral submucous fibrosis in vivo and upregulated by arecoline in human buccal mucosal fibroblasts in vitro. *J Oral Pathol Med* 2005;**34**:100—5.
16. Aston C, Jagordar J, Lee TC, Hur T, Hintz RL, Rom WN. Enhanced insulin-like growth factor molecules in idiopathic pulmonary fibrosis. *Am J Resp Crit Med* 1995;**151**: 1563—97.
17. Olbruck H, Seemayer NH, Voss B, Wilhelm M. Supernatants from quartz dust treated human macrophages stimulate all proliferation of different human lung cells as well as collagen-synthesis of human diploid lung fibroblasts in vitro. *Toxicol Lett* 1998;**96**:85—95.
18. Goldstein RH, Poliks CF, Pilch PF, Smith BD, Fine A. Stimulation of collagen formation by insulin and insulin-like growth factor 1 n cultures of human lung fibroblasts. *Endocrinology* 1989;**124**:964—70.
19. Uh ST, Inoue Y, King Jr TE, Chan ED, Newman LS, Richls DW. Morphometric analysis of insulin-like growth factor-1 localization in lung tissues of patients with idiopathic pulmonary fibrosis. *Am J Resp Crit Care Med* 1998;**158**: 1626—35.
20. Maeda A, Hiyama K, Yamakido H, Ishioka S, Yamakido M. Increased expression of plate-derived growth factor A and insulin-like growth factor-1 in BAL cells during the development of bleomycin-induced pulmonary fibrosis in mice. *Chest* 1996;**129**:780—6.
21. Harrison NK, Cambrey AD, Myers AR, Southcott AM, Black RM, DuBois RM, et al. Insulin-like growth factor-1 is partially responsible for fibroblast proliferation induced by bronchoalveolar lavage fluid from patients with systemic sclerosis. *Clin Sci* 1994;**86**:141—8.
22. Castilla-Cortazar I, Garcia M, Muguerza B, Quiroga J, Perez S, Sartidrian S, et al. Hepatoprotective effects of insulin-like growth factor-1 in rats with carbon tetrachloride-induced cirrhosis. *Gastroenterology* 1997;**113**:1682—91.
23. Tsai CH, Weng SF, Yang LC, Huang FM, Chen YR, Chang YC. Immunohistochemical localization of tissue type plasminogen activator and type I plasminogen activator inhibitor in radicular cysts. *J Oral Pathol Med* 2004;**33**:156—61.
24. Chang YC, Hu CC, Tseng TH, Tai KW, Lii CK, Chou MY. Synergistic effects of nicotine on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts. *J Oral Pathol Med* 2001;**30**:458—64.
25. Nemoto E, Shimonishi M, Nitta Y, Shimauchi H. The involvement of platelet-derived growth factor receptors

and insulin-like growth factor-I receptors signaling during mineralized nodule formation by human periodontal ligament cells. *J Periodontal Res* 2004;**39**:388—97.

26. Chang YC, Tai KW, Lii CK, Chou LSS, Chou MY. Cytopathologic effects of arecoline on human gingival fibroblasts in vitro. *Clin Oral Invest* 1999;**3**:25—9.