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Regulation of extracellular matrix genes by arecoline in primary gingival fibroblasts requires epithelial factors

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Background and Objective: Oral submucous fibrosis, a disease of collagen disorder, has been attributed to arecoline present in the saliva of betel quid chewers. However, the molecular basis of the action of arecoline in the pathogenesis of oral submucous fibrosis is poorly understood. The basic aim of our study was to elucidate the mechanism underlying the action of arecoline on the expression of genes in oral fibroblasts.

Material and Methods: Human keratinocytes (HaCaT cells) and primary human gingival fibroblasts were treated with arecoline in combination with various pathway inhibitors, and the expression of transforming growth factor-beta isoform genes and of collagen isoforms was assessed using reverse transcription–polymerase chain reaction analysis.

Results: We observed the induction of transforming growth factor-beta2 by arecoline in HaCaT cells and this induction was found to be caused by activation of the M-3 muscarinic acid receptor via the induction of calcium and the protein kinase C pathway. Most importantly, we showed that transforming growth factor-beta2 was significantly overexpressed in oral submucous fibrosis tissues ($p = 0.008$), with a median of 2.13 ($n = 21$) compared with 0.75 ($n = 18$) in normal buccal mucosal tissues. Furthermore, arecoline down-regulated the expression of collagens 1A1 and 3A1 in human primary gingival fibroblasts; however these collagens were induced by arecoline in the presence of spent medium of cultured human keratinocytes. Treatment with a transforming growth factor-beta blocker, transforming growth factor-beta1 latency-associated peptide, reversed this up-regulation of collagen, suggesting a role for profibrotic cytokines, such as transforming growth factor-beta, in the induction of collagens.

Conclusion: Taken together, our data highlight the importance of arecoline-induced epithelial changes in the pathogenesis of oral submucous fibrosis.

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Oral submucous fibrosis is a chronic inflammatory condition, which is always associated with epithelial atrophy and gradual hardening of the oral cavity as a result of the enhanced production and deposition of collagen (1,2). Enhanced production of extracellular matrix proteins, including collagens, as well as inhibition of their degradation, might lead to a preferential deposition of extracellular matrix protein in the subepithelial layer of the oral mucosa (3). Inflammatory and fibrogenic cytokines have been proposed to play key roles in the

pathogenesis of oral submucous fibrosis. Transforming growth factor-beta is a profibrotic cytokine and has been shown to be important in the development of fibrosis in several organs (3). Transforming growth factor-beta has also been shown to be overexpressed in oral submucous fibrosis tissues (4) and in keratinocytes obtained from oral submucous fibrosis tissues (5), suggesting its role in oral submucous fibrosis pathogenesis. A higher incidence of oral submucous fibrosis has been reported in betel quid chewers than in those who do not chew betel quid. Arecoline, one of the major constituents of betel quid, is present in the saliva of betel quid chewers, and a causative link between arecoline and oral submucous fibrosis development has been proposed (3,6). There is increased production of collagen in oral submucous fibrosis tissues and in fibroblasts isolated from oral submucous fibrosis tissues (7,8). Arecoline has been reported to induce collagen production in buccal mucosal fibroblast cells (9). However, in a different study, the down-regulation of collagen synthesis by arecoline was shown in gingival fibroblasts and this leads to poor adhesion and migration, etc. (10). The reasons for this discrepancy are not known. Arecoline is a muscarinic acetylcholine receptor agonist and acts via M-1, M-2 and M-3 muscarinic acetylcholine receptor subtypes (11–13). Activation of muscarinic acid receptors leads to either the induction of intracellular Ca^{2+} or the down-regulation of cytosolic cAMP levels, depending on the subtype of receptors activated (14). Moreover, arecoline has been reported to induce oxidative stress in cells (15,16). Although arecoline has been shown to regulate collagen synthesis, the molecular mechanism of this is not established.

Hence, in this study, we attempted to delineate the role of arecoline in the regulation of collagen isoforms in gingival fibroblast cells *in vitro* and also studied the possible role of epithelial factors in the regulation of collagens by arecoline in fibroblasts. In addition, we tested the role of transforming growth factor-beta in this regulation. We showed that the regulation of collagen isoforms in gingival fibroblasts by arecoline is modulated by epithelial factors that are induced by arecoline and we identified one of these to be a transforming growth factor-beta isoform.

Material and methods

Cell culture and treatment

Human keratinocytes (HaCaT cells) and primary human gingival fibroblasts were maintained in Dulbecco's modified Eagle's minimal essential medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (heat inactivated for HaCaT cells), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) at 37°C in a humidified chamber with 5% CO_2. The cells were serum starved for 24 h and then treated with 0–50 μg/mL of arecoline (Sigma Aldrich) under serum-free conditions for up to 72 h. To investigate the roles of oxidative stress, muscarinic acetylcholine receptor stimulation and intracellular Ca^{2+}, the cells were pretreated for 1 h with 10 mM *N*-acetyl-L-cysteine (a glutathione precursor and reactive oxygen species scavenger), 1 mM atropine (a general muscaric acid receptor antagonist), 100 μM 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; an M-3 subtype selective antagonist), 10 nM staurosporin (a protein kinase C inhibitor), 1 mM EGTA, or 10 μM BAPTA/AM (a cell-permeable Ca^{2+} chelator), in the presence or absence of 50 μg/mL of arecoline hydrobromide (Sigma Aldrich) under serum-free conditions. To study in greater detail the influence of the transforming growth factor-beta pathway on arecoline-mediated gene regulation, the cells were treated with 5 ng/mL of recombinant human transforming growth factor-beta1 or with 2 μg/mL of transforming growth factor-beta1 latency-associated peptide (all from R & D Systems, Minneapolis, MN, USA) in the presence or absence of arecoline.

Tissue samples

Tissue biopsies from patients presenting with oral submucous fibrosis were surgically excised by the dental surgeon at the R. V. Dental College and Hospital, Bangalore. The nonoral submucous fibrosis tissues (considered as normal) were excised from patients who did not have oral submucous fibrosis and were attending the hospital for other conditions. Informed consent to participate in the study was collected from all subjects by the surgical team at the time of tissue excision. The institutional ethical committee of the R. V. Dental College and Hospital approved the protocol and the patient consent form. All the tissues were evaluated by an oral pathologist. Altogether, 21 normal and 18 oral submucous fibrosis tissue samples were used in this study.

RNA extraction, and semi- and real-time quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted from the cells and human tissues using TRI-reagent (Sigma Aldrich), according to the manufacturer's protocol. Two micrograms of total RNA was reverse transcribed using a High Capacity Archive cDNA kit (Applied Biosystems, Foster, CA, USA), and cDNA equivalent to 20 ng of total RNA was used per 20 μL of polymerase chain reaction (PCR) mix. All PCR amplifications were performed using 2× DyNAZYME Master mix (Finnzyme, Espoo, Finland) with gene-specific primers. RPL-35a (a ribosomal protein coding RNA) was used as the normalizing control. The PCR products were resolved on a 2% agarose gel containing ethidium bromide, and the band intensities were determined using the Gel Documentation System from Uvitech, Cambridge, UK.

Real-time PCR quantification was performed using cDNA, equivalent to 20 ng of total RNA, isolated from either cell lines or human tissues in a 20-μL final reaction volume using Dynamo™ SYBERgreen 2× mix (Finnzymes) in an ABI Prism 7900HT sequence detection system (Applied Biosystems) and analysed using SDS 2.1 software (Applied Biosystems). RPL-35a expression was used for normalization and the data obtained

Table 1. Primer description

Gene name	5′–3′ sequence	Description
Transforming growth factor-beta1	Sense: TCCGAGAAGCGGTACCTGAA Antisense: TGCTGTCACAGGAGCAGTGG	266 bp, 63.7°C
Transforming growth factor-beta2	Sense: AGTGCCTGAACAACGGAT Antisense: GTACAAAAGTGCAGCAGG	218 bp, 55°C
Transforming growth factor-beta3	Sense: GCGTGAGTGGCTGTTGAGA Antisense: CCAAGTTGCGGAAGCAGTA	306 bp, 52.7°C
Collagen 1A1	Sense: TCCCCAGCCACAAAGAGTCTA Antisense: TTTCCACACGTCTCGGTCA	201 bp, 58°C
Collagen 3A1	Sense: TTGACCCTAACCAAGGATGC Antisense: GGAAGTTCAGGATTGCCGTA	202 bp, 58°C
Collagen 4A2	Sense: TTGGCGGGTGTGAAGAAGTTT Antisense: CCTTGTCTCCTTTACGTCCCTG	117 bp, 50°C
Collagen 6A3	Sense: TGGTCCAGTTCAACGGAAACC Antisense: CACTACGATAACCTGAGGGACT	206 bp, 58°C
Collagen 7A1	Sense: GCCGCTGACATTGTGTTCT Antisense: GTCAGCCACATGGAGAATTG	279 bp, 52.5°C
Rpl-35a	Sense: GAACCAAAGGGAGCACACAG Antisense: CAATGGCCTTAGCAGGAAGA	236 bp, 58°C

represented fold change in expression over the expression of the control (either untreated cells or the normal tissues). The descriptions of primers employed are given in Table 1.

Detection of intracellular (Ca^{2+})

HaCaT cells were treated with 50 μg/mL of arecoline under serum-free conditions for 30 min. To test whether arecoline activated muscarinic acetylcholine receptors in HaCaT cells, the cells were pretreated for 1 h with 10 μM atropine and then incubated for 30 min in the presence or absence of 50 μg/mL of arecoline. The cells were trypsinized, washed with ice-cold 17 mM potassium phosphate buffer (pH 6.4) and then $1–2 \times 10^5$ cells were incubated with 2 μM Indo-1/AM dye (Molecular Probes Inc., Eugene, OR, USA) in the presence of 0.2% Pluronic acid F-127 detergent for 30 min at 37°C and 5% CO_2 in the dark with frequent tapping. The cells were then washed three times with ice-cold potassium phosphate buffer. The cells were resuspended in potassium phosphate buffer and then mounted on a glass slide with a drop of water on the cover slip. The cells loaded with Indo-1AM dye were excited with ultraviolet light of 355 nm, and the fluorescence of Ca^{2+}-bound Indo-1AM was observed at 405 nm using an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 60× objective.

Statistical analysis

Statistical significance was evaluated using the Student's *t*-test. The results were considered significant at a *p*-value of ≤ 0.05.

Results

Regulation of transforming growth factor-beta2 expression by arecoline in normal keratinocytes (HaCaT cells)

Epithelial changes could be crucial for the development of oral submucous fibrosis. Arecoline, a major risk factor of oral submucous fibrosis development, primarily targets oral epithelial cells and fibroblast cells. One well-established profibrotic cytokine is transforming growth factor-beta and it has been reported to be involved in the fibrosis of a variety of tissues (17). A previous study showed that transforming growth factor-beta was over-expressed in the epithelium of oral submucous fibrosis tissues (4). Thus, it is possible that arecoline might influence the development of oral submucous fibrosis via the induction of transforming growth factor-beta expression in epithelial cells. In order to test this, we investigated whether arecoline regulates any of the transforming growth factor-beta isoform genes in HaCaT cells. Figure 1A shows the results of reverse transcription (RT) -PCR analysis, which demonstrates the induction of transforming growth factor-beta2 mRNA after treatment with arecoline. The highest level of transforming growth factor-beta2 was induced by 50 μg/mL of arecoline and was about 2.58-fold greater than that of the untreated control. Expression of the other transforming growth factor-beta genes, transforming growth factor-beta1 and 3, were not affected by arecoline (data not shown) in HaCaT cells. Furthermore, it was observed that the induction of transforming growth factor-beta2 mRNA in HaCaT cells was independent of the autoregulatory action of transforming growth factor-beta, as transforming growth factor-beta1 latency-associated peptide, a transforming growth factor-beta blocker (18), could not block this induction (Fig. 1B). The addition of 10 mM *N*-acetyl-L-cysteine (an antioxidant) elevated the basal expression level of transforming growth factor-beta2 mRNA, which was not further induced by arecoline. In addition, 1 mM atropine, a muscarinic acid receptor antagonist, reduced the induction of transforming growth factor-beta2 by arecoline by nearly 50% (Fig. 1C). It has been reported that only the M-3 muscarinic acetylcholine receptor subtype is expressed in HaCaT cells (19). This suggests that arecoline might regulate the transforming growth factor-beta2 isoform in HaCaT cells via the muscarinic acid receptor, M-3. To establish in greater detail the role of the M-3 receptor in the regulation of transforming growth factor-beta2 mRNA by arecoline, an M-3 muscarinic acetylcholine receptor subtype selective antagonist, 4-DAMP, was added, along with arecoline, to HaCaT cells. Arecoline alone induced transforming growth factor-beta2 mRNA expression by four-fold, whereas the addition of 0.1 mM 4-DAMP blocked the induction completely. Interestingly, the presence of 4-DAMP increased the basal expression level by two-fold (Fig. 1D). This confirms the role of the M-3

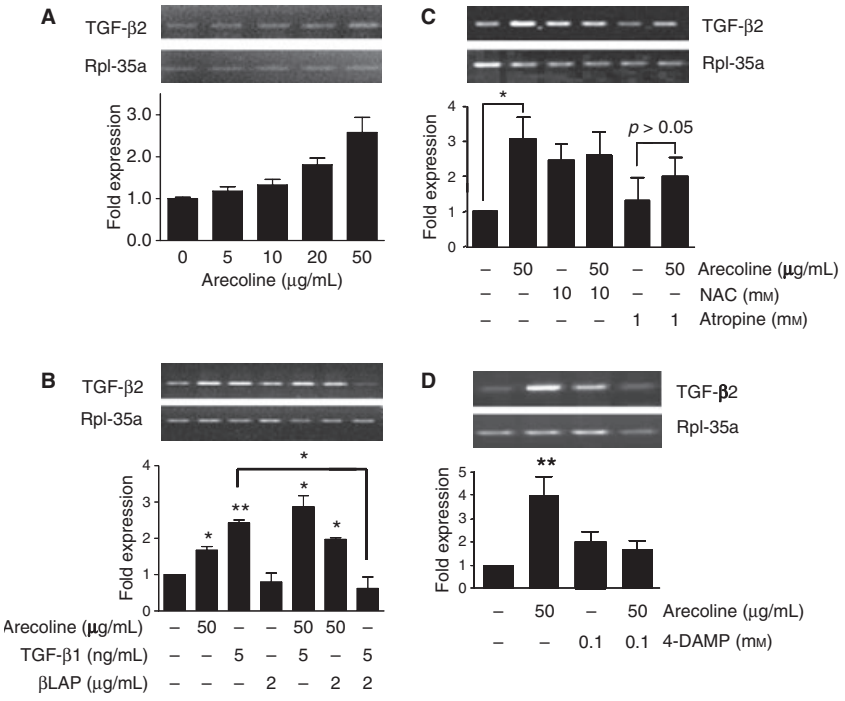

Fig. 1. (A) Regulation of transforming growth factor-beta2 mRNA expression by arecoline in HaCaT cells. HaCaT cells were treated with 0–50 μg/mL of arecoline for 48 h without serum and the expression of transforming growth factor-beta2 mRNA was evaluated by reverse transcription–polymerase chain reaction (RT-PCR) analysis. The top panel shows the ethidium bromide-stained agarose gel and the bottom panel shows the densitometric intensities of DNA bands. The data represent the mean fold changes in the mRNA expression levels (with respect to the control) of three independent experiments and bars show ± standard deviation. (B) Effect of transforming growth factor-beta1 latency-associated peptide (a transforming growth factor-beta blocker) on the induction of transforming growth factor-beta2 expression by arecoline. HaCaT cells were treated with 50 μg/mL of arecoline in the presence or absence of 5 ng/mL of transforming growth factor-beta1or 2 μg/mL of transforming growth factor-beta1 latency-associated peptide for 48 h and the expression of transforming growth factor-beta2 mRNA was assessed using RT-PCR analysis. The top panel represents the ethidium bromide-stained gel and the lower panel represents the band intensities expressed as fold change in transforming growth factor-beta2 mRNA expression with respect to the untreated control. The data represent the mean of two independent experiments and the bars show ± standard error. *$p < 0.05$; **$p < 0.005$. (C) Effect of *N*-acetyl-L-cysteine and atropine on the induction of transforming growth factor-beta2 expression by arecoline. HaCaT cells were treated with 50 μg/mL of arecoline in the presence or absence of 10 μM *N*-acetyl-L-cysteine or 1 mM atropine for 48 h under serum-free conditions and the expression of transforming growth factor-beta2 mRNA was assessed using RT-PCR. The top panel represents the ethidium bromide-stained agarose-gel and the lower panel represents the band intensities expressed as fold change in transforming growth factor-beta2 mRNA expression with respect to the untreated control. The data represent the mean of three independent experiments and the bars show ± standard deviation. *$p < 0.05$. (D) Effect of the M-3 selective antagonist, 4-DAMP, on the induction of transforming growth factor-beta2 expression by arecoline. HaCaT cells were treated with 50 μg/mL of arecoline for 48 h in the presence or absence of 100 μM 4-DAMP and the expression of transforming growth factor-beta2 was evaluated using RT-PCR. The top panel shows the ethidium bromide-stained agarose gel, and the bottom panel represents the band intensities expressed as fold changein transforming growth factor-beta2 mRNA expression with respect to the untreated control. The data represent the mean of three independent experiments and the bars show ± standard deviation. **$p < 0.005$. 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; βLAP, transforming growth factor-beta1 latency-associated peptide; NAC, *N*-acetyl-L-cysteine; TGF-β2, transforming growth factor-beta2.

muscarinic acid receptor subtype in the induction of transforming growth factor-beta2 mRNA expression by arecoline in human keratinocytes. Activation of the M-3 receptor has been shown to increase cytosolic Ca^{2+} levels via the activation of phospholipase C-gamma (14). The activation of muscarinic receptor by arecoline was tested using fluorescence microscopy analyses of Indo 1/AM (fluorescent Ca^{2+} probe)-stained HaCaT cells. Figure 2A shows an increased level of cytosolic Ca^{2+} following treatment with arecoline, which was blocked by atropine (a general muscarinic acid receptor antagonist), suggesting that arecoline is capable of Ca^{2+} mobilization via muscarinic acid receptors in HaCaT cells. An increased level of cytosolic Ca^{2+} can activate several signaling molecules, such as protein kinase C and other Ca^{2+}-dependent kinases (20). In order to study the role of Ca^{2+} and protein kinase C in the arecoline-mediated induction of transforming growth factor-beta2 expression, HaCaT cells were treated with Ca^{2+} chelators (EGTA and BAPTA/AM) or staurosporin (a protein kinase C inhibitor) in the presence or absence of 50 μg/mL of arecoline. As shown in Fig. 2B, the induction of transforming growth factor-beta2 mRNA by arecoline is dependent on Ca^{2+} influx and protein kinase C activation because EGTA and staurosporin reversed the induction. However, BAPTA/AM resulted in only partial inhibition of the induction of transforming growth factor-beta2. All data presented above suggest that arecoline is capable of inducing transforming growth factor-beta2 mRNA expression through the M-3 muscarinic acid receptor and protein kinase C activation in human keratinocytes.

Over-expression of transforming growth factor-beta2 mRNA in oral submucous fibrosis tissues

The identity of transforming growth factor-beta isoforms involved in the oral submucous fibrosis disease process has not been established. We performed a real-time quantitative analysis of human tissues to study the

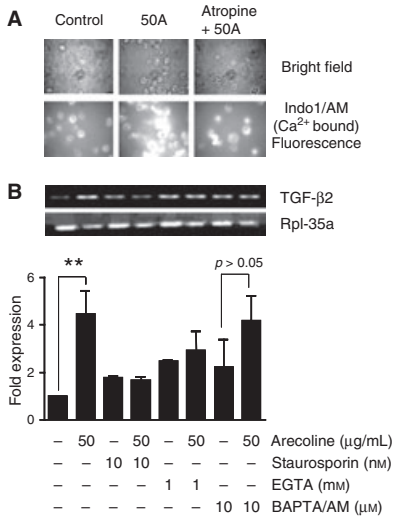

Fig. 2. (A) Induction of intracellular Ca^{2+} by arecoline in HaCaT cells. HaCaT cells were treated with 50 μg/mL of arecoline for 30 min in the presence or absence of 10 μM atropine (a nonselective muscarinic acetylcholine receptor antagonist). The cells were then loaded with the fluoresencent Ca^{2+} probe, Indo-1/AM. The fluorescence at 408 nm was observed using a Leica fluorescence microscope. (B) Effect of staurosporin, EGTA and BAPTA on the induction of transforming growth factor-beta2 expression by arecoline. HaCaT cells were treated with 50 μg/mL of arecoline for 48 h in the presence or absence of 10 nM staurosporin (a protein kinase C inhibitor), 1 mM EGTA (a Ca^{2+} chelator) or 10 μM BAPTA (a cell-permeable Ca^{2+} chelator), and the expression of transforming growth factor-beta2 was evaluated using reverse transcription–polymerase chain reaction. The top panel shows the ethidium bromide-stained agarose gel and the bottom panel represents the band intensities expressed as fold change in transforming growth factor-beta2 mRNA expression over the untreated control. The data represent the mean of three independent experiments and the bars show ± standard deviation. $^{**}p < 0.005$. 50A, 50 μg/mL of arecoline; BAPTA, EGTA, Indo-1/AM; TGF-β2, transforming growth factor-beta2.

expression of transforming growth factor-beta isoform(s) and found that transforming growth factor-beta2 mRNA was overexpressed in the majority of oral submucous fibrosis tissues; 83.3% (15/18) of the samples showed expression > 1.5-fold higher than the mean expression level of the

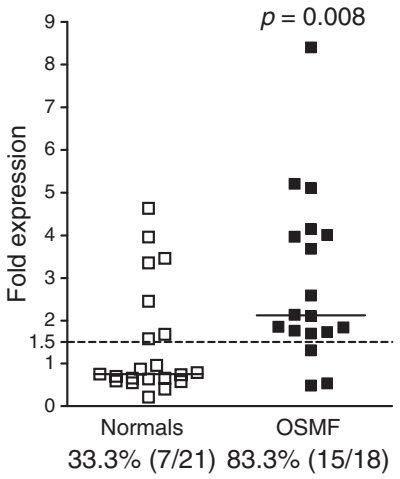

Fig. 3. Expression of transforming growth factor-beta2 mRNA in oral submucous fibrosis tissues. Real-time quantitative reverse transcription–polymerase chain reaction analysis results are shown of transforming growth factor-beta2 mRNA in oral submucous fibrosis and normal human tissues. The respective samples are indicated below the groups. Each square represents one tissue sample. The normalized values are plotted as fold change over the mean expression calculated for the normals. The horizontal solid bars indicate the corresponding medians. *P*-values of ≤ 0.05 were considered significant. OSMF, oral submucous fibrosis.

normal tissues compared with 33.3% (7/21) in normal tissues. Most importantly, the median expression level in oral submucous fibrosis tissues (2.13, $n = 21$) was significantly higher ($p = 0.008$) than the median expression level in normal tissues (0.75, $n = 18$) (Fig. 3). This suggests a possible role for transforming growth factor-beta2 in oral submucous fibrosis pathogenesis.

Regulation of expression of collagen isoforms by arecoline in human gingival fibroblasts

Oral submucous fibrosis is basically a disease of collagen disorder and associates with the dysregulation of collagen expression in oral fibroblast cells. In order to elucidate the molecular basis of the action of arecoline on oral fibroblasts in the development of oral submucous fibrosis, the regulation of collagen gene expression by arecoline in primary human gingival fibroblast cells was evaluated by RT-PCR analysis after treatment with varying concentrations of arecoline for 72 h. Surprisingly, the mRNA expression of interstitial collagens 1A1 and 3A1 was reduced by approximately 65% and 57%, respectively, at 50 μg/mL of arecoline in human gingival fibroblasts (Fig. 4A). However, arecoline did not affect the mRNA expression of collagens 4A2, 6A3 and 7A1 (data not shown). This observation contradicts the expected role of arecoline as an inducer of fibrosis. In order to explain this paradox, we hypothesized that perhaps factors produced by epithelial cells in response to arecoline might be involved in the regulation of collagen expression in the oral fibroblast cells, thus invoking a crucial role of oral epithelium during oral submucous fibrosis progression. To test this hypothesis, human gingival fibroblasts were cultured in HaCaT-conditioned medium collected after 48 h of treatment with or without arecoline, and the expression of Col 1A1 and 3A1 was assessed. It was observed that the mRNA expression of collagens 1A1 and 3A1 was induced approximately 6- and 1.9-fold, respectively, by 50 μg/mL of arecoline in human gingival fibroblasts cultured for 72 h in control HaCaT-conditioned medium. Interestingly, conditioned medium from HaCaT cells treated with arecoline had similar effects on the regulation of mRNA expression of collagens 1A1 and 3A1 (Fig. 4B). This suggests a cooperative effect of arecoline and a factor secreted by HaCaT cells for the induction of collagen isoforms 1A1 and 3A1 in human gingival fibroblasts.

As demonstrated in Fig. 1, one of the factors produced by epithelial cells in response to arecoline is transforming growth factor-beta2. Transforming growth factor-beta is also a well-known stimulator of matrix genes in fibroblasts (17). Hence, we tested the effect of transforming growth factor-beta1 latency-associated peptide (that binds to active transforming growth factor-beta and prevents its action) on the regulation (by arecoline) of collagen isoforms in human gingival fibroblasts in HaCaT-conditioned medium.

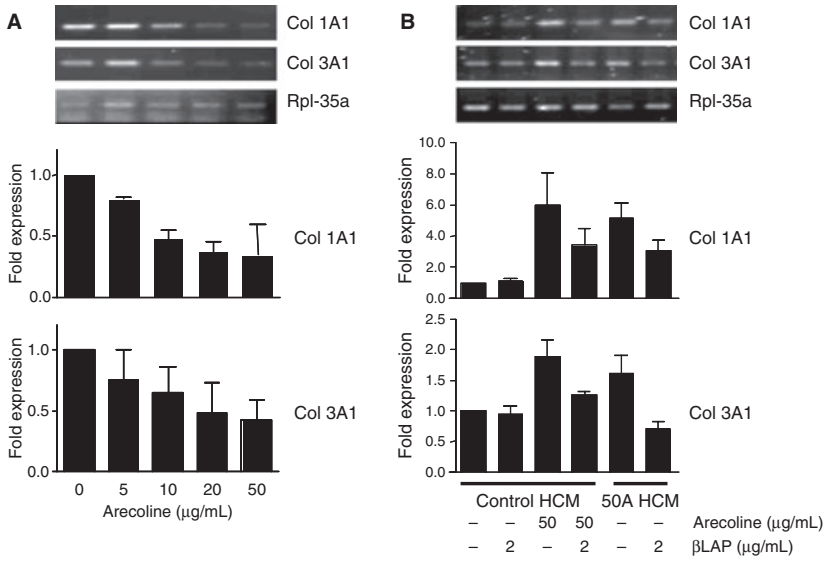

Fig. 4. (A) Regulation of collagen expression by arecoline in human gingival fibroblasts. Human gingival fibroblasts were treated with 0–50 μg/mL of arecoline for 72 h under serum-free conditions and the expression of collagens 1A1, 3A1, 4A2, 6A3 and 7A1 was assessed using reverse transcription–polymerase chain reaction. The top panel shows a representative ethidium bromide-stained agarose gel of the polymerase chain reaction products. The bar diagrams show the fold change in expression of Col 1A1 and 3A1 mRNA following arecoline treatment, as determined by densitometric scanning of the polymerase chain reaction products. The data represent the results of four independent experiments. (B) Induction, by arecoline, of the expression of collagens 1A1 and 3A1 in human gingival fibroblasts cultured in HaCaT-conditioned medium. Human gingival fibroblasts were cultured for 72 h in serum-free control HaCaT-conditioned medium or in HaCaT conditioned medium treated with 50 μg/mL of arecoline collected after 48 h either in the presence or absence of 50 μg/mL of arecoline or transforming growth factor-beta1 latency-associated peptide (2 μg/mL). The expressions of collagen 1A1 and 3A1 were then assessed using reverse transcription–polymerase chain reaction. The top panel shows the ethidium bromide-stained agarose-gel picture and the bottom panel represents the band intensities as fold changes in the mRNA expression with respect to the untreated control. The data represent the mean of two independent experiments, and bars show ± standard error. 50A, 50 μg/mL of arecoline; βLAP, transforming growth factor-beta1 latency-associated peptide; HCM, HaCaT-conditioned medium.

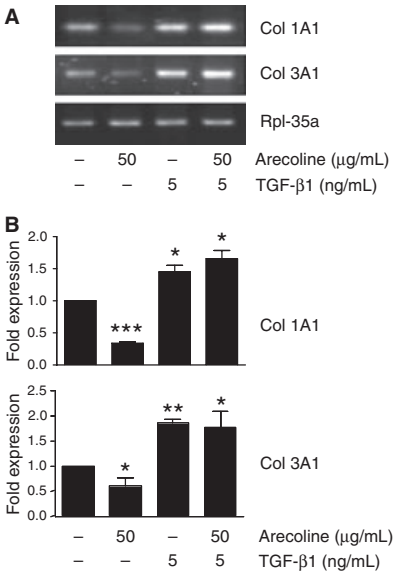

Fig. 5. Regulation of expression of collagens 1A1 and 3A1 by transforming growth factor-beta in human gingival fibroblasts. Human gingival fibroblasts were treated with 5 ng/mL of human recombinant transforming growth factor-beta1 for 72 h under serum-free conditions in the presence or absence of 50 μg/mL of arecoline and the expression of collagen 1A1 and 3A1 was assessed using reverse transcription–polymerase chain reaction. (A) Ethidium bromide-stained agarose gel and (B) quantification of band intensities expressed as fold change in mRNA expression with respect to the untreated control. The data represent the mean of two independent experiments, and bars show ± standard error. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. TGF-β1, transforming growth factor-beta1.

As shown in Fig. 4B, the addition of transforming growth factor-beta1 latency-associated peptide reduced the induction of expression of Col 1A1 and 3A1 mRNA by approximately 40–60% in human gingival fibroblasts cultured in HaCaT-conditioned medium, irrespective of the supplementation or prior presence of arecoline, confirming that transforming growth factor-beta isoforms are involved in the regulation of collagen isoforms. Furthermore, in order to test whether transforming growth factor-beta is capable of inducing collagens in human gingival fibroblasts in the presence of arecoline, human gingival fibroblasts were treated with recombinant human transforming growth factor-beta1 in the presence or absence of 50 μg/mL of arecoline and the expression of Col 1A1 and 3A1 mRNA was assessed by RT-PCR. As shown in Fig. 5, transforming growth factor-beta1 induced the expression of Col 1A1 and 3A1 mRNA in human gingival fibroblasts, even in the presence of arecoline. Our data suggest that the effects of arecoline on human gingival fibroblasts (e.g. the down-regulation of collagen isoforms) could be reversed by transforming growth factor-beta while transforming growth factor-beta is one of the factors induced by arecoline in epithelial cells. Hence, we propose a role for epithelial cell–stromal cell interactions during oral submucous fibrosis development, perhaps involving transforming growth factor-beta.

Discussion

The progression of oral submucous fibrosis has been attributed to the betel quid chewing habit amongst the people of South East Asian countries. Arecoline, an alkaloid present in the quid, is the ingredient most commonly studied to date, and several reports suggest a causative role for arecoline in the progression of oral submucous fibrosis (3). Arecoline-induced epithelial changes might have a bearing on the pathophysiology of the underlying oral

fibroblast cells, leading to the abnormal production and deposition of extracellular matrix proteins during the development of oral submucous fibrosis. A recent report suggests the regulation of alfa-actin in oral fibroblasts in the presence of keratinocyte factors in co-culture experiments, suggesting the importance of epithelial factors in arecoline-induced differentiation of oral fibroblast cells (21). Arecoline might elicit the expression of certain critical factor(s) in epithelial cells that, in turn, may modulate the activity of oral fibroblasts. In order to understand the mechanisms involved in the regulation of gene expression by arecoline in epithelial and fibroblast cell types, HaCaT cells and primary human gingival fibroblast cells were employed in our studies. Both of these cell lines were treated with arecoline, and the expression of transforming growth factor-beta genes and collagens was investigated in the presence or absence of pathway-specific inhibitors.

Surprisingly, the expression of Col 1A1 and 3A1 mRNA was down-regulated by arecoline in human gingival fibroblasts, which is in contrast to the proposed role of arecoline in the development of oral submucous fibrosis. Also, collagen types 1 and 3 have been reported to be overexpressed in oral submucous fibrosis tissues and in fibroblasts isolated from oral submucous fibrosis tissues (7,22). Hence, one of the possible explanations for this paradox is to invoke the role of other cell types within the tissue environment. Although oral submucous fibrosis is classified as a fibroblast disease, one important observation in oral submucous fibrosis pathogenesis is the involvement of progressive epithelial changes finally leading to atrophy of the epithelium (1,2). So far, there has been no demonstrable involvement of epithelial factors in the manifestation of oral submucous fibrosis. In order to explain the inability of arecoline to induce collagens in our experiments, we resorted to looking for an answer in epithelial cells. Our data suggest the involvement of transforming growth factor-beta, secreted by epithelial cells, in the regulation of collagens in fibroblasts. We demonstrated the induction of transforming growth factor-beta2 isoform mRNA by arecoline in keratinocytes, in line with the reported secretion of transforming growth factor-beta by human keratinocytes (23). The reported enhanced secretion of the transforming growth factor-beta1 isoform by keratinocytes isolated from oral submucous fibrosis tissue cannot be explained by our data, as we did not observe its regulation by arecoline in HaCaT cells. One possible reason for this could be the inherent differences between oral keratinocytes and HaCaT cells, which are derived from the dermal tissues (24). Hence, the effects of arecoline on epithelial cells might have an influence on fibroblasts in the oral submucous fibrosis pathogenesis. Strong staining for transforming growth factor-beta has been observed in oral submucous fibrosis tissues, predominantly in the epithelium but not in normal epithelium (4). Also, a previous report suggested that cultured keratinocytes isolated from oral submucous fibrosis tissues secrete a high level of transforming growth factor-beta1 (4661.641 $\pm$ 783.893 pg/mL) in the culture medium (5), indicating that the physiological level of transforming growth factor-beta in oral submucous fibrosis tissues is perhaps sufficiently high to influence the effect of arecoline, whose concentrations in the saliva of betel quid chewers could be as high as approximately 140 μg/mL in some cases (6). This supports our *in vitro* data that regulation of transforming growth factor-beta mRNA in epithelial cells might be an important event in the development of oral submucous fibrosis.

The role of arecoline in the regulation of transforming growth factor-beta2 is also exemplified by other findings, such as the reversal of arecoline-induced transforming growth factor-beta2 mRNA expression by the M-3 muscarinic acetylcholine receptor antagonist, 4-DAMP, or staurosporin (a protein kinase C inhibitor). Furthermore, the addition of Ca^{2+} chelators, EGTA and BAPTA/AM (cell permeable), abolished the process to different extents, suggesting the importance of Ca^{2+} influx in this process. Arecoline has been reported to function via M-1, M-2 and M-3 muscarinic acetylcholine receptor subtypes (11–13). Muscarinic acetylcholine receptors have been shown to be expressed in a variety of cell types other than neuronal and smooth muscle cells (18,25–28); however, their physiological significance has not been well characterized. Very recently, the cholinergic potential of the alkaloid has been proposed in the modulation of immune functions in mice (29). So far, muscarinic acetylcholine receptors have not been implicated in the development of oral submucous fibrosis. Our data, perhaps for the first time, provided circumstantial evidence that activation, by arecoline, of muscarinic acetylcholine receptor subtypes expressed on oral epithelial cells might be important for the development of oral submucous fibrosis in betel quid chewers. Arecoline also induced transglutaminase-2, a matrix cross-linking enzyme, via activation of the M-2 muscarinic acetylcholine receptor in primary human gingival fibroblast cells (G. S. Thangjam, P. Kondaiah, unpublished data). Our study thus warrants identification of the muscarinic acetylcholine receptor expressed in oral tissues. Taken together, the causal effect of arecoline during the development of oral submucous fibrosis might involve the activation of muscarinic acetylcholine receptors expressed in the oral tissues.

The increased production of transforming growth factor-beta2 mRNA by arecoline in keratinocytes perhaps contributes to the induction of fibrosis in the buccal mucosa. Transforming growth factor-beta is secreted as an inactive form, which has to be activated in order to become biologically active (30). Although it has yet to be established how transforming growth factor-beta is activated from its latent form by arecoline in oral fibroblasts, we speculate that arecoline may contribute to the activation process by inducing (activating) proteases in fibroblasts. In summary, our findings provide a basis for the role of epithelial changes mainly mediated by transforming growth factor-beta2 during oral submucous fibrosis development in betel quid chewers and also a role for muscarinic acid receptor activation

as one of the important mechanisms of action of arecoline in epithelial cells.

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