ORIGINAL ARTICLE

EXPRESSION OF INTEGRIN αvβ6 (ALPHAVBETA6) IN ORAL SUBMUCOUS FIBROSIS: A POTENTIALLY MALIGNANT CONDITION OF ORAL CAVITY

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ABSTRACT

Oral submucous fibrosis (OSF) is a chronic progressive fibrosing disorder of the oral cavity. A common finding in tissue fibrosis is that stromal fibroblasts become 'activated' myofibroblasts and express α-smooth muscle actin (SMA). TGF-β1 is considered to have a central role in inducing this myofibroblastic phenotype, and its expression is increased in numerous fibrotic conditions. The epithelial-specific integrin αvβ6 is not detectable on normal oral keratinocytes but is upregulated during tissue remodelling. αvβ6 is a key activator of TGF-β1 through its interaction with the latency-associated peptide (LAP) of the cytokine.

The objective of the study was to investigate the role of αvβ6 integrin in the pathogenesis of OSF. We used immunochemistry to examine expression of αvβ6 in 41 cases of OSF compared with 14 cases of fibroepithelial hyperplasia, and found significantly higher expression in OSF (*p=0.009*). We carried out a TGF- β1 bioassay using a keratinocyte cell line genetically modified to express high levels of αvβ6 (VB6), and found that inhibition of αvβ6 significantly reduced TGF-β1 activation (*p<0.0001*).

Co-culture of HFFF2 fibroblasts with VB6 cells induced myofibroblast transdifferentiation, producing a marked increase in SMA expression. This was inhibited using anti- αvβ6 antibodies, confirming that myofibroblast generation was αvβ6-dependent.

In conclusion, these data show that αvβ6-dependent TGF-β1 activation promotes myofibroblast transdifferentiation, and may be responsible, in part, for the chronic fibrosis seen in OSF.

INTRODUCTION

Oral submucous fibrosis (OSF) is a chronic, insidious disorder of the oral cavity characterized by progressive fibrosis of oral tissues resulting in a significant impact on the quality of life of the affected individual.[1] The precise aetiology of the disease is unknown but it is strongly linked to chewing betel nut, a common habit in the regions where the disease is of high prevalence.[2] The main principal presentation of OSF is progressive limitation of mouth opening.[3] In OSF, evidence suggests that the pathological fibrosis appears to result from the disruption of equilibrium between MMP's (Matrix metalloproteinases) and TIMP's (Tissue inhibitor of metalloproteinases). Recent studies have shown that MMP-2 and MMP-9 are found in reduced expression minimal/amounts in diseased tissues.[4] Other studies have shown that arecoline down regulates MMP-2 secretion and upregulates TIMP-1 levels resulting in increased deposition of collagen in the ECM.[5,6]

The transforming growth factor–beta (TGF-β) is regarded as the principal fibrogenic cytokine; it is a potent stimulator of ECM (extracellular matrix) production and has been implicated in the pathogenesis of numerous fibrotic/scarring disorders. It causes ECM deposition by increasing synthesis of matrix proteins such as collagen, and decreasing its degradation by various inhibitory mechanisms.[7]

Overview of Integrins

Integrins are the principal ECM receptors, which are expressed by all cells.[8] They meditate signaling pathways that regulate diverse cell processes including adhesion, migration, proliferation, differentiation, apoptosis, immune response, leukocyte traffic, homeostasis.[9,10] Integrins are heterodimers composed of an α and a β subunit.[08] Both α and β subunits are type l membrane glycoproteins, containing a large extracellular

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domain (700-1100 amino acids), a transmembrane domain and, generally (except β4), a short carboxy- (C-) terminal cytoplasmic domain (30-50 amino acids).[11] The cytoplasmic domain of the β subunit of integrins associates with the actin cytoskeleton of the cell and also initiates signaling cascades.[12]

The αvβ6 (alphavbeta6) integrin

αvβ6 is an epithelial-specific integrin that is not expressed on healthy oral epithelium but is upregulated during tissue remodeling, including wound healing and carcinogenesis.[13] αvβ6 binds to its ligand via the tripeptide recognition sequence arginine-glycine-aspertic acid (RGD). Known ligands of αvβ6 are extracellular proteins such as fibronectin, tenascin-C, vitronectin, latency-associated peptide of TGF-β1 and TGF-β3.[13]

Role of αvβ6 in wound healing

The correlation of αvβ6 with TN-C expression has led to the suggestion that the principle functions of αvβ6 in wound healing are modulated through this matrix protein.[14,15] TN-C is also a ligand for α9β1, which is upregulated in the early stage of wound healing,[14] but down regulated in the later stages, coinciding with induced expression of αvβ6.[13]

Role of αvβ6 expression in carcinomas

αvβ6 integrin promotes tumour progression by modulating several fuctions including cell proliferation, invasion, apoptosis, and MMP expression.[13]

Role of αvβ6 expression in fibrotic disease

αvβ6 has been shown modulate fibrosis through its ability to activate TGF-β1 in several different organs. Munger and colleagues originally showed that αvβ6-dependent TGF-β activation promoted lung fibrosis.[16]

Aims and objectives of the study

Several studies have implicated αvβ6 in modulating tissue fibrosis[16,17,18] These data raise the possibility that a similar mechanism occur in OSF. The aim of this study is to investigate expression and function of αvβ6 integrin in submucous fibrosis.

Specific objectives include

1 To examine the expression of αvβ6 in normal oral mucosa and submucous fibrosis using immunochemistry.

2. To determine the role of αvβ6 in oral keratinocyte cell line genetically manipulated to express high levels of αvβ6.

3. To investigate the mechanism by which oral keratinocytes induce myofibroblastic transdifferentiation in co-culture assays with fibroblasts.

4. To examine relative expression of collagen mRNA in fibroblasts and myofibroblasts.

5. To determine whether myofibroblasts are present in the lamina propria of submucous fibrosis specimen using immunochemistry.

METHODOLOGY

Immunohistochemistry

Antibodies used were anti- αvβ6 (6.2G2, 0.5 µg/mL; Biogen Idec) or anti-SMA (IA4, 1;100; Sigma). Antigen retrieval varied according to primary antibody: 0.1% α- chymotrypsin and 0.1% calcium chloride (pH 7.8) for 20 min at 37ºC (IA4) or Digest-All 3 Pepsin Solution (Zymed Laboratories) for 5 min at 37ºC (6.2G2), and microwaving for 30 min in 0.1 mol/L citrate buffer. Endogenous peroxidase was neutralized with 0.45% hydrogen peroxidase in methanol for 15 min and primary antibodies were applied in TBS (pH 7.6) for 1 h. Antimouse IgG biotinylated secondary antibody (Vectastain Elite ABC Reagent, Vector Laboratories) was applied for 30 min followed by peroxidase-labeled streptavidin (Vectastain Elite ABC Reagent, Vector Laboratories) for 30 min. Peroxidase was visualised using DAB+ (Dako) for 7 min and counterstained in Mayer's hematoxylin (Sigma). Forty-one OSF and 14 cases of fibroepithelial hyperplasia were selected from archival material and stained for αvβ6 and SMA, then scored. Appropriate ethical approval was obtained. The staining intensity of αvβ6 was scored on a scale of 1 to 3 (1, weak; 2, moderate; and 3, strong), and the proportion of cells staining positively was scored on a scale of 1 to 4 (1, focal basal; 2, linear basal; 3, basal and suprabasal; and 4, full epithelium thickness). The score for intensity was added to the score for proportion to give a score in the range of 0 to 7 and grouped as score = 0 (-), score = 1–4 (low), score = 5-7 (high).

Cell culture

Human foetal foreskin fibroblasts (HFFF^2, American Type Culture Collection) were maintained in fibroblast growth medium (FGM) consisting of DMEM supplemented with 10% fetal calf serum (FCS) and glutamine. Keratinocytes genetically modified to express high levels of αvβ6 (VB6 cells) were cultured in standard keratinocyte growth medium (KGM) consisting of α-MEM supplemented with 10% FCS, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 1.8×10^{-4} M adenine,

5μg ml^{-1} insulin, 0.5 μg ml^{-1} hydrocortisone, 10 ng ml^{-1} epidermal growth factor and 1×10^{-10} M cholera toxin. Cells were maintained in a humidified atmosphere of 5% CO2 at 37ºC and passaged routinely using trypsin–EDTA. All chemicals were purchased from Sigma (Poole, Dorset, UK) unless stated otherwise. Cells were tested routinely for mycoplasma.

Flow cytometry

Flow cytometry was performed using anti-αvβ6 antibody (10D5; Chemicon) and Alexafluor 488-conjugated secondary antibody (Molecular Probes). Negative controls used secondary antibody only. Labelled cells were scanned on a FACS Calibur cytometer (BD Biosciences) and analyzed using CellQuest software, acquiring 1×10^4 events.

TGF-β bioassay

Mink lung epithelial reporter cells stably expressing a TGF-β-responsive luciferase reporter construct were plated overnight in 96-well plates in DMEM and 10% FCS (5×10^4 cells/well). The medium was changed to serum-free α-MEM, and VB6 cells (2.5×10^4 cells/well) were added to each well in serum-free α-MEM containing anti- αvβ6 antibody (10 μg/mL, 63G9, Biogen Idec) or control antibody (10 μg/mL, mouse monoclonal anti-α4 integrin, mAb7.2; produced in-house). The cells were cocultured overnight, washed once in PBS, and lysed in reporter lysis buffer (Promega). Luciferase assay buffer (Promega) was added to the supernatant and the luminescence measured using a Wallac plate reader.

Coculture experiments

HFFF2 cells and VB6 cells were plated in six-well dishes (2.5×10^5 cells of each cell type). Cells were seeded in DMEM, 10% FCS ± antibodies (as above), or TGF-β1 (2 ng/mL; R&D Systems) and left to attach. The medium was then changed to serum-free DMEM containing antibodies or TGF-β and cultured for an additional 48 h. The cells were either lysed for analysis by Western blotting or fixed and processed for immunofluorescence.

Western blot analysis

Cells were lysed in NP40 buffer (Biosource). Samples containing equal amounts of protein were electrophoresed under reducing conditions in 10% SDS-PAGE gels. Protein was electroblotted to nitrocellulose membranes (Amersham Biosciences). Blots were probed with antibodies against αvβ6 (made in-house) or α-SMA (Dako). Horseradish peroxidase-conjugated anti-goat or anti-rat (Dako) was used as secondary antibodies. Bound antibodies were detected with the enhanced chemiluminescence Western blotting detection kit system (Amersham). Blots were probed for HSC70 (Santa Cruz Biotechnology) as a loading control. Exposures of blots in the linear range were quantified by densitometry software (Scion Corp).

Confocal microscopy

Cells were fixed for 10 min in 4% formaldehyde in cytoskeletal buffer (10 mmol/L MES, 3 mmol/L MgCl2, 138 mmol/L KCl, 2 mmol/L EGTA; pH 6.1) with 0.32 mol/L of sucrose. Cells were permeabilised for 5 min with 0.2% Triton X-100 and incubated for 30 min in DMEM, 0.1% bovine serum albumin, and 0.1% sodium azide. Nonspecific staining was blocked by incubation in 5% normal goat serum. Cells were then incubated with anti-SMA (IA4; Dako) or anti-αvβ6 antibodies (53a.2; in-house) for 1 hour, and binding detected by incubation with secondary antibodies conjugated with Alexafluor 488 (Molecular Probes) or Cy3 (Jackson ImmunoResearch) for 45 minutes. Nuclei were visualized using 4',6-diamidino-2-phenylindole (Invitrogen). Images were recorded and processed with a confocal laser scanning microscope (Zeiss LSM510).

Preparation and use of medium conditioned by fibroblasts and myofibroblasts

Fibroblasts (5×10^5) were plated in fibroblast growth medium in 60mm culture dishes for 24 hours. To induce a myofibroblastic phenotype, cells were incubated for 24h in medium containing recombinant TGF-β1 (2 ng/mL; R&D Systems), which was acid-activated prior to use (4 mmol/L HCl/0.1% bovine serum albumin). Control cells were cultured in medium alone. After 24 hours, the cells were cultured for a further 48 h in serum-free medium ± TGF-β1. Cells were then either lysed for Western blot analysis or RNA was extracted for RT-PCR analysis.

RT-PCR analysis of collagen 1

Total RNA from fibroblasts was isolated using the RNeasy™ Mini kit (Qiagen, Hilden, Germany). The reverse transcription polymerase chain reactions (RT-PCR) were performed in an automated thermal cycler (Eppendorf) using an RT-PCR kit (Strategene) with pairs of primers amplifying the mRNAs encoding human a_1I collagen. The primer sequences were:

Forward 5'-CCCACCAATCACCTGCGTACAGA-3'
Reverse 5'-TTCTTGGTCGGTGGGTGACTCTGA-3'

The reverse transcription step (70° C for 15 minutes) was followed by a 2-minute incubation at 94°C and then by amplification for 23 cycles at 94°C for 15 seconds, 66°C for 20 seconds and 72°C for 10 seconds. The PCR products were run on a 2% agarose gel and detected using ethidium bromide. Expression levels were normalised to a known housekeeping gene (GAPDH).

RESULTS

1 αvβ6 expression in OSF cases

Results of the present study showed that strong expression of αvβ6 was present in over 50% of OSF cases examined, but was not detected in normal oral epithelium (Table 1; Figure 1 A-E).

TABLE 1: αvβ6 EXPRESSION IN OSF PATIENTS

	αvβ6 Expression				
	Cases	**Nil**	**Low**	**High**	**% High**
Normal Oral Mucosa	14	3	11	0	0%
Submucous fibrosis	41	9	10	22	53.6%

2 αvβ6 activates TGF-β1

When MLEC cells were cocultured with VB6 cells, high luciferase activity was observed indicating TGF-β activation. Inhibition of αvβ6 using antibody (63G9) significantly reduced TGFβ activation confirming that the activation of TGF-β was αvβ6-specific. This assay demonstrated that TGF-β activation by VB6 cells is αvβ6-dependent (Figure 2).

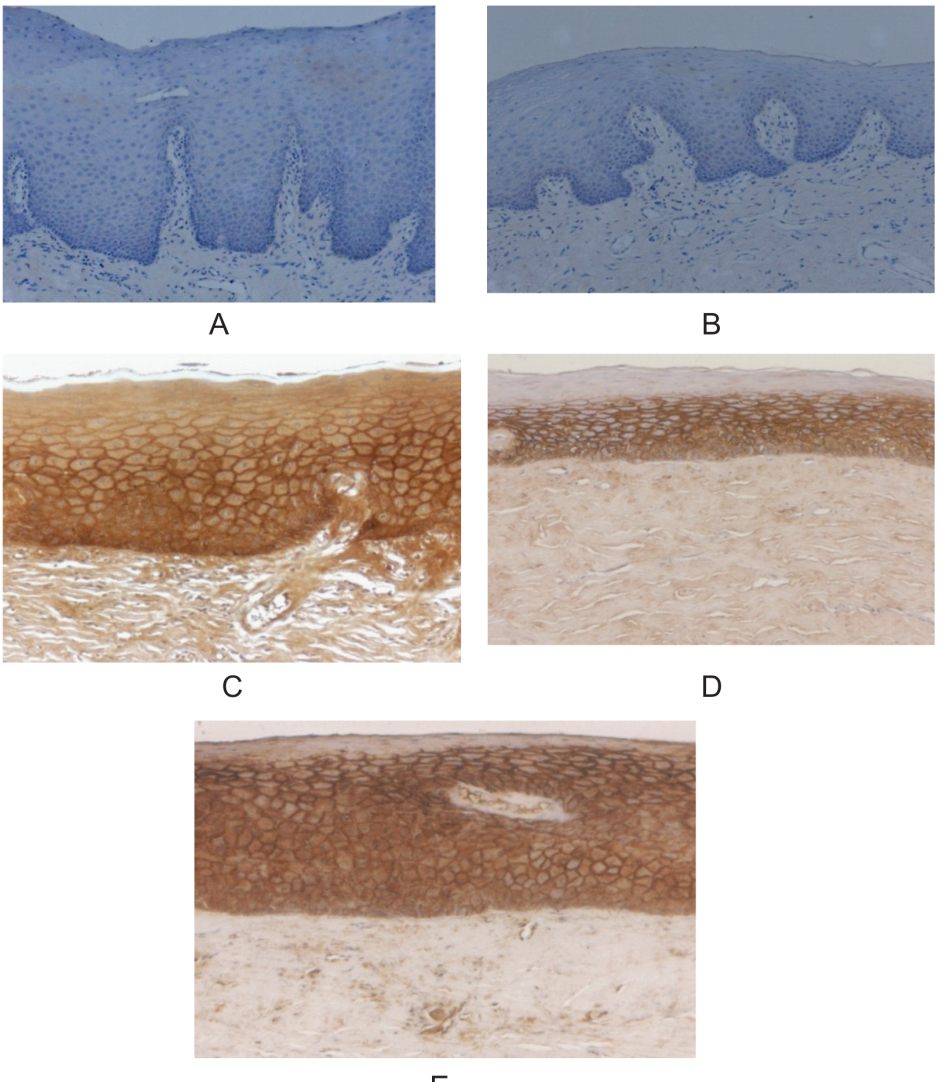


Fig 1: Immunohistochemistry of αvβ6 expression in normal (A and B) and OSF clinical samples (C, D and E)

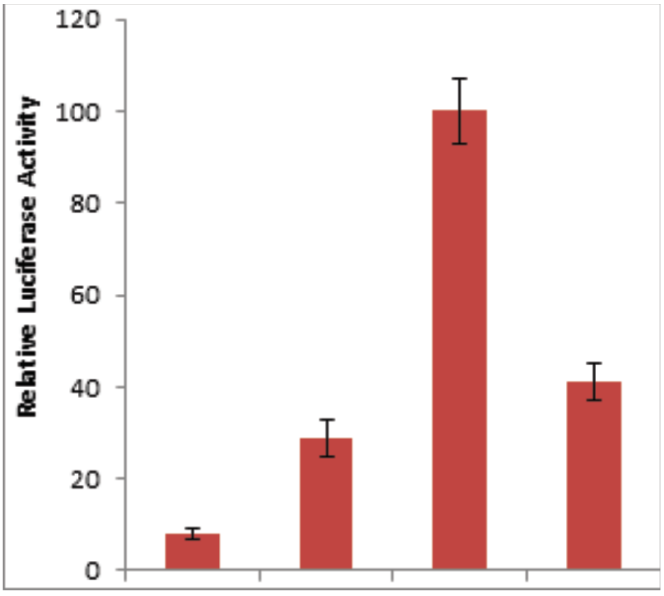


Fig 2: TGF-β activation assay Co-culture of VB6 with reporter MLEC cells resulted in increased luciferase activity indicating TGF-β activation. This was inhibited by anti-αvβ6 antibody.

3 TGF-β induces myofibroblasts transdifferentiation

Confocal microscopy was used to assess induction of SMA in HFFF2 cells that had been treated with TGF-β for 48 hours. Our results demonstrated that TGF-β-treated HFFF2 cells strongly expressed SMA. In contrast, HFFF2 cells that had not been treated with TGF-β expressed very low levels of SMA (Figure 3).

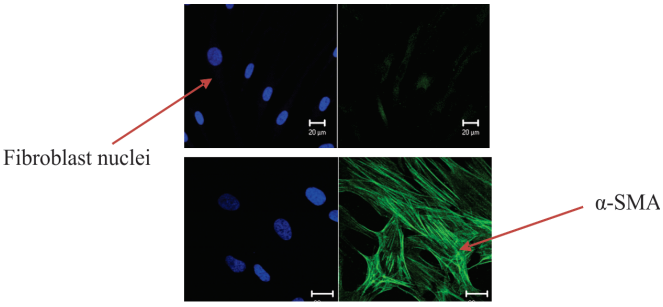


Fig 3: TGF-β1 induces SMA expression in fibroblasts. Confocal micrographs showing DAPI-stained HFFF2 nuclei (blue) and smooth muscle actin (SMA; green). HFFF2 cells in culture express low levels of SMA (A). Treatment of HFFF2 cells with TGF-β1 causes upregulation of SMA (B) indicating myofibroblasic differentiation.

4 Inhibition of αvβ6 prevents myofibroblast transdifferentiation in co-culture

To determine whether VB6 cells could induce myofibroblastic differentiation, we carried out coculture experiments with HFFF2 cells. Coculture of HFF2 with VB6 cells induced myofibroblastic transdifferentition, producing significant increase in SMA expression (Figure 4 A). To show that myofibroblasts generation was αvβ6 dependent, we repeated this culture assay in the presence of an αvβ6 inhibitory antibody. When the αvβ6 antibody was added, SMA expression was inhibited significantly. This was confirmed by Western blotting. Figure 4B shows the low level of SMA expression by cultured HFFF2 (lane 1) which increases when

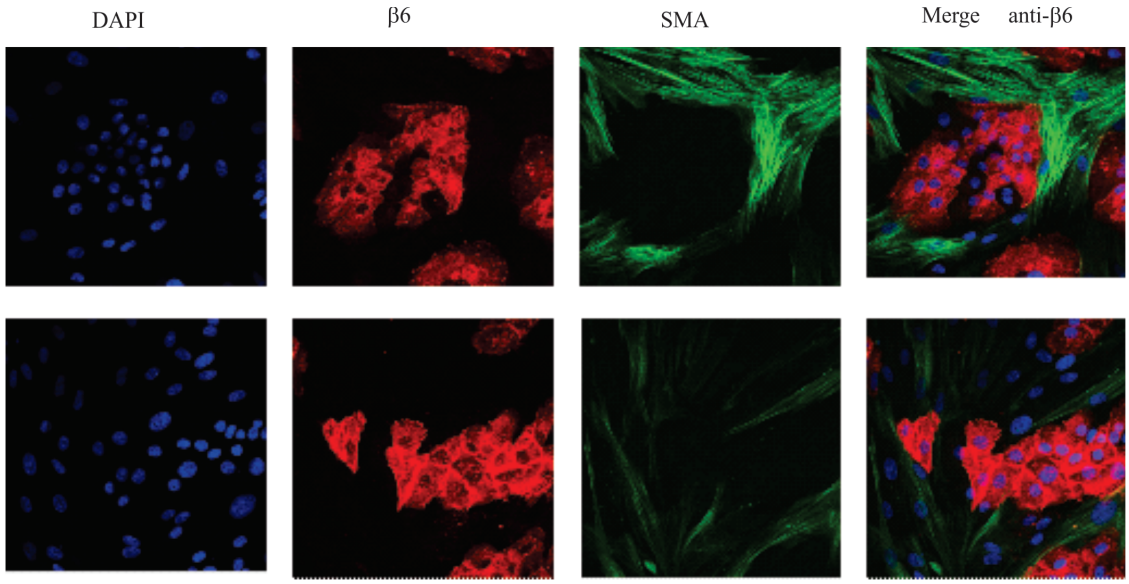


Fig 4-A: Confocal micrographs of VB6 and HFFF2 co-culture experiments. Nuclei are shown in blue, αvβ6-positive VB6 cells in red, SMA-positive myofibroblasts in green. Co-culture of VB6 and HFFF2 induced SMA expression which was inhibited using an anti- αvβ6 antibody.

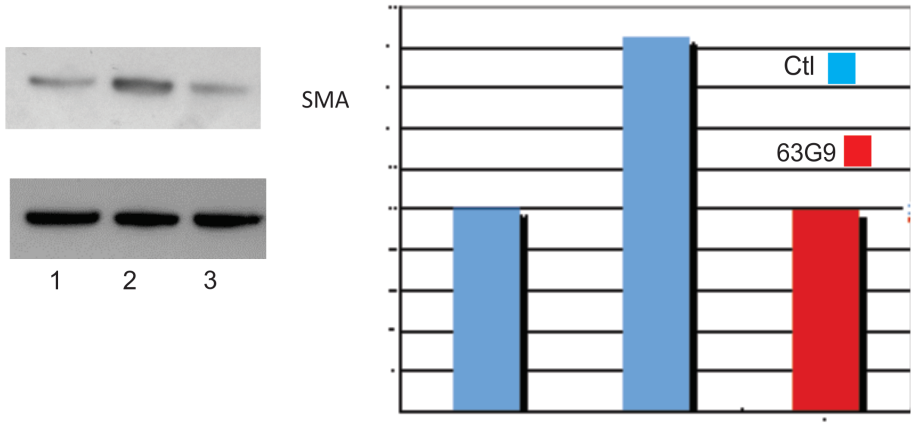


Fig 4B: Western blotting for SMA in VB6 and HFFF2 co-cultures HFFF2 cells express low levels of SMA in culture (lane 1). SMA is induced on co-culture with VB6 cells (lane 2) and this is inhibited by an αvβ6-blocking antibody (lane 3).

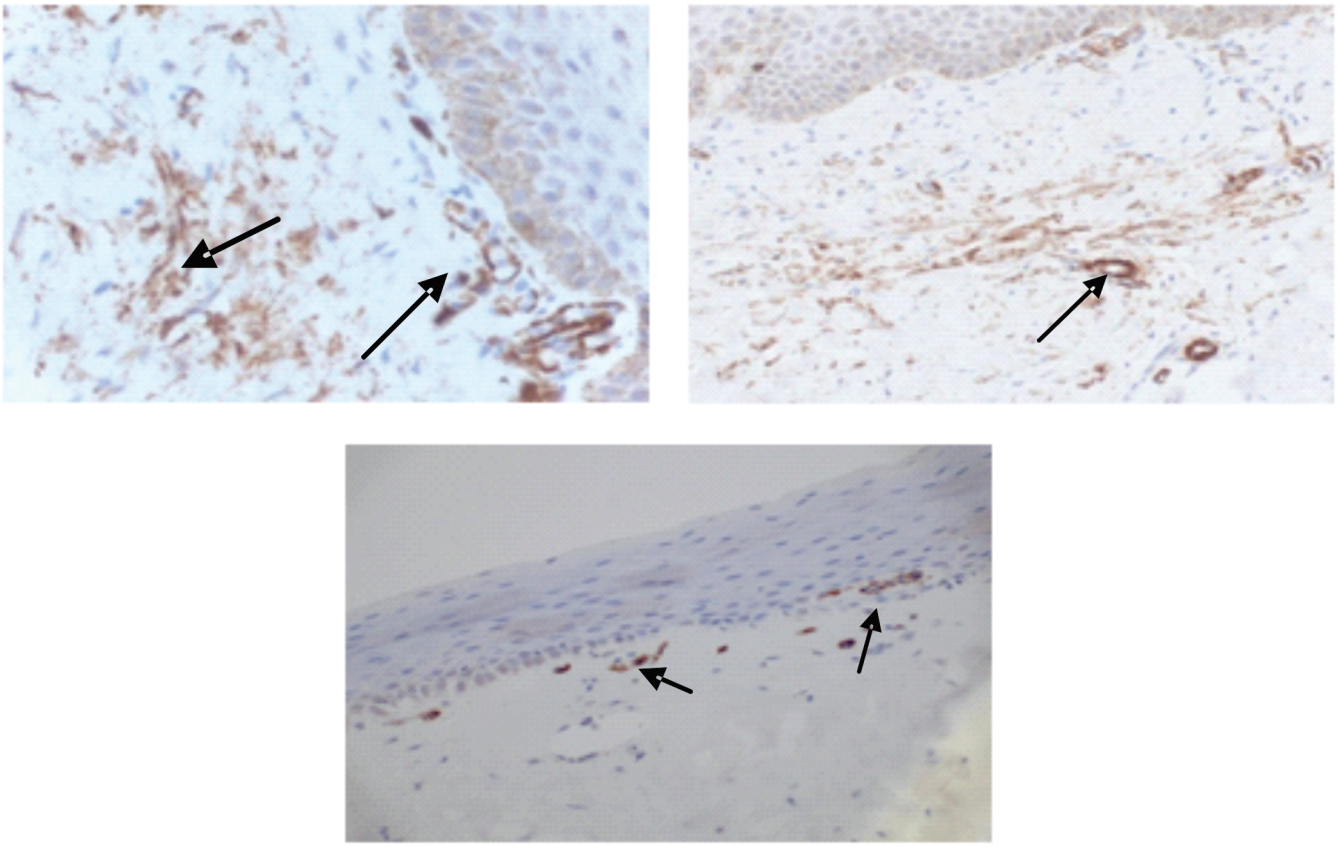


Fig 5: Immunochemistry showing SMA staining in OSF specimens (SMA) positive myofibroblasts were identified in the corium of OSF clinical samples.

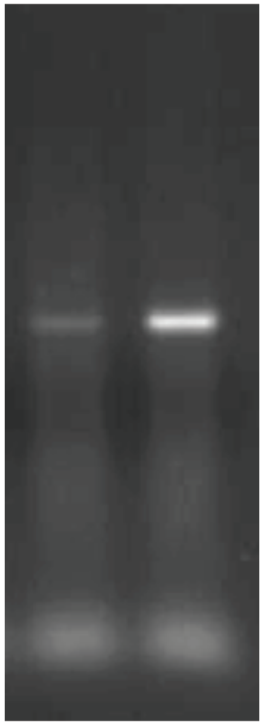
co-cultured with VB6 (lane 2), and which is inhibited by an anti-αvβ6 blocking antibody (lane 3). Blots were probed for HSC70 (Santa Cruz Biotechnology) as a loading control.

5 The stroma of OSF contains myofibroblasts

To determined whether the connective tissue in OSF contained myofibroblasts, we immunostained the OSF specimen for SMA expression. SMA–positive myofibroblasts were detected in the corium of OSF samples. No myofibroblasts were identified in the corium of normal oral mucosa (Figure 5).

6 TGF-β upregulates type I collagen on an mRNA level

Semiquntitative PCR was used to examine the



HFF2 TGF-β1+HFF2

Fig 6: RT-PCR analysis of collagen 1 in TGF-β-treated fibroblasts. Myofibroblastic differentiation results in an increase of collagen I mRNA.

expression of type 1 collagen in fibroblasts and myofibroblasts. Fibroblasts were treated with TGF-β for 48 hours, lysed and RNA extracted. Myofibroblastic differentiation resulted in significantly upregulated collagen I mRNA (Figure 6).

DISCUSSION

Previous research has confirmed that the constituents of areca nut include the alkaloids arecoline, arecaidine, guacine that can cause interferences in collagen deposition and its degradation. Disruption in the balance between matrix metallproteinases (MMPs) and tissue inhibitor metallo-proteinases (TIMPS) contributes in the pathogenesis of OSF.[6] Expression of αvβ6 has also been reported in numerous carcinomas.[13] Ma *et al* demonstrated αvβ60dependent activation of TGF-β1 in a mouse model of pulmonary and renal fibrosis, suggesting that this mechanism may be of general importance in fibrosis in multiple epithelial organs.[19] Thus, our rationale was to investigate whether αvβ6 has a role in the pathogenesis of OSF. To our knowledge, this is the first study examining the possible role of αvβ6 in this context. In the first instance, our immunohistochemistry results from clinical samples demonstrated that around 50% of OSF cases expressed high levels of αvβ6 (compared with 0% of fibroepithelial hyperplasias), indicating a possible role for the integrin in the disease.

One of the main functions of αvβ6 is to activate the cytokine TGF-β1 by binding to its latency-associated peptide (LAP). This is potentially a key factor for the role of αvβ6 in the pathogenesis of OSF, since TGF-β1 is a molecule of central importance in fibrotic diseases since it promotes the transdifferentiation of fibroblasts into myofibroblasts.[20] These 'activated' fibroblasts characteristically express high levels of α-SMA. We carried out TGF-β1 activation assays which confirmed that VB6 cells (transfected with αvβ6; Thomas et al, 2001) activated TGF-β1, and that this activation was inhibited by a β6-function-blocking antibody, confirming that this phenomenon was αvβ6-dependent. We also demonstrated the upregulation of SMA at a protein level in HFFF2 fibroblasts that had been cocultured with VB6 cells, and, again, blocking αvβ6 resulted in a down regulation of SMA. These results indicate that the induction of TGF-β-dependent myofibroblasts transdifferentiation was modulated through αvβ6.

To examine the downstream effect of TGF-β on collagen synthesis by myofibroblasts, we carried out semiquantitative PCR which showed an upregulation of type 1 collagen in fibroblasts that had been treated with TGF-β. Furthermore, in the co-culture setting we noted a reduction in type 1 collagen expression when αvβ6 was inhibited, suggesting that αvβ6-dependent activation of TGF-β may be responsible for the upregulation of collagen that is a histological hallmark of OSF.

Finally, and to correlate our *in vitro* findings with clinical samples, immunohistochemistry from our OSF samples revealed SMA-positive myofibroblasts in the collagen-rich connective tissue, but the pattern of expression was varied in tissue specimens.

Hypothetical model of αvβ6 role in OSF pathogenesis

Alkaloids implicated in OSF

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Genetic predisposition

↓

αvβ6

↓

Activation of TGF-β1

↓

Tansdifferentiation of myofibroblasts

↓

Upregulation of collagen 1

↓

Pathological fibrosis

Future Work

This work represents a 'pilot' study for the role of αvβ6 in the pathogenesis of OSF. The first major modification of the study would be to repeat our experiments using cells which are more physiologically relevant to the disease i.e. normal oral keratinocytes and fibroblasts (rather than VB6 OSCC cells and HFFF2 (foreskin) fibroblasts.

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