Oral Diseases (2004) 10, 201–206

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Oral and Maxillofacial Pathology

Alterations in expression of retinoid receptor β and p53 in oral submucous fibrosis

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OBJECTIVE: Knowledge of the molecular pathogenesis of oral submucous fibrosis (OSF), a potentially malignant condition with high risk of transition to oral cancer, is meagre. Alterations in the expression of retinoic acid receptor β (RARβ) and tumor suppressor gene, p53 are early events in oral tumorigenesis. The aim of this study was to investigate the alterations in the expression of RARβ and p53 in OSF lesions and determine their association with disease pathogenesis.

METHODS: The expression of RARβ and p53 proteins was analyzed by immunohistochemistry in 50 cases of OSF and 30 histologically normal oral tissues.

RESULTS: No detectable RARβ expression was observed in 35 of 50 (70%) OSF cases. p53 protein accumulation was observed in 24 of 50 (48%) OSF cases analyzed. Thirty-six percent OSF lesions showed loss of RARβ and p53 overexpression. Interestingly, 41 of 50 (82%) of OSF lesions showed altered expression of at least one of these two proteins.

CONCLUSION: Altered expression of either RARβ or p53 in majority of OSF lesions suggests their association with disease pathogenesis and warrants follow-up to determine whether OSF lesions harboring concomitant alterations in RARβ and p53 are at a high risk of transition to malignancy.

Oral Diseases (2004) **10**, 201–206

Keywords: RARβ; p53; OSF; areca nut

Introduction

Oral submucous fibrosis (OSF) is a chronic oral mucosal disease characterized by inflammation and progressive fibrosis of lamina propria and the underlying submucosal layer of the oral cavity. The global estimates indicate that about 2.5 million people are affected (Cox and Walker, 1996a). Northwest India has an incidence of 5–8 and 2–6 per 100 000 year^{-1} for males and females, respectively; whereas in south India, the estimates are higher – 9 and 20 per 100 000 year^{-1} for males and females, respectively (Pindborg *et al*, 1980; Gupta *et al*, 1990). The risk of transition to malignancy is estimated to be 7.6% of cases over a period of 10 years (Murti *et al*, 1985). There is a rapid upsurge in the incidence of oral cancer among younger males that may be attributed to the intense promotion and widespread marketing of commercially manufactured chewing products containing areca nut and/or tobacco (Gupta *et al*, 1998). One of the most important etiological factors in the pathogenesis of OSF is the habit of chewing areca nut, either alone, or as a component of betel quid, or gutkha/pan masala (perfumed sweetened mixture of slaked lime), catechu, areca nut powder with/without tobacco (Cox and Walker, 1996a; Gupta *et al*, 1998; Lee *et al*, 2003). The areca nut extract, in particular the alkaloid arecoline, has been shown to stimulate collagen synthesis by buccal mucosal fibroblasts *in vitro*, and is proposed to be involved in the etiopathogenesis of OSF (Harvey *et al*, 1986; Van Wyk *et al*, 1994). However, molecular mechanisms implicated in the pathogenesis of OSF remain to be elucidated.

The potentially malignant oral lesions at high risk of transition to malignancy, if identified, can be effectively managed by early intervention. Chemoprevention using retinoids (RAs) (analogs of vitamin A) is a promising strategy under clinical trials for primary prevention (DiPaola *et al*, 1999; Gravis *et al*, 1999). Retinoids exert their pleiotropic effects by binding to nuclear receptors in cells and causing inhibition of cell proliferation, modulation of cell differentiation and enhancement of apoptosis (Sidell *et al*, 1991; Horn *et al*, 1996). Two families of nuclear receptors, the retinoic acid receptor isotypes (RARα, RARβ and RARγ) and the retinoid X receptor isotypes (RXRα, RXRβ and RXRγ) are implicated in the transduction of the RA signal (Leid, Kastner and Chambon, 1992; Allenby *et al*, 1993; Chambon, 1996). Alterations in the expression of specific receptors could abrogate the retinoid-signaling pathway and may be implicated in carcinogenesis.

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Received 20 March 2003; revised 26 September 2003; accepted 17 November 2003

Defects in retinoid receptor structure, expression and function have been reported in various types of cancer cells *in vitro* and *in vivo* (de The *et al*, 1990a; Gebert *et al*, 1991; Xu *et al*, 1997a,b). Downregulation of RARβ mRNA levels has been reported in several malignant tumors including carcinoma of head and neck, lung, thyroid and ovaries (Rochette-Egly *et al*, 1992; Caliaro *et al*, 1994; Xu *et al*, 1994; Kawchak *et al*, 1999). The clinical response to retinoids is correlated with the levels of RARβ in cancer patients (de The *et al*, 1990b).

Alterations in tumor suppressor gene *p53* are early events in betel and tobacco-related oral cancer in the Indian population (Kaur, Srivastava and Ralhan, 1994, 1998). Murti *et al* (1998) assessed the potential of p53 protein expression as a marker for determining which oral precancerous lesions may transform to malignancy with time and proposed that p53 overexpression peaks close to the time of transition from precancer to cancer, rather than early in the history of oral precancer (Murti *et al*, 1998). Interestingly, p53 aberrations – both non-functional protein as well as gene mutations – have been observed in OSF lesions in south-east Asian (Nepalese and Pakistani) population (Cox and Walker, 1996b; Trivedy *et al*, 1998). Nevertheless, the status of RARβ and p53 in OSF lesions in the Indian population remains to be investigated. In this context, the cellular expression of RARβ and p53 in OSF lesions was analyzed by immunohistochemistry and their association with clinicopathological parameters was determined.

Material and methods

Tissue samples

Biopsy specimens from 50 untreated primary OSF lesions were obtained from the Department of Surgical Disciplines, All India Institute of Medical Sciences, New Delhi, India. Twenty of the normal cases were smears obtained from oral mucosa of the healthy volunteers working in the institute (with their prior consent) and 10 normal mucosa specimens were taken from a site contralateral to the lesion of OSF patients that had histologically confirmed normal epithelium. The tissues were fixed in 10% neutral-buffered formalin for 24–48 h and embedded in paraffin for immunohistochemical analysis. The smears were spread on glass slides and fixed with acetone for immunohistochemical analysis. Hematoxylin and eosin-stained sections were examined by a pathologist to confirm the clinical diagnosis.

Clinicopathological characteristics of patients

The OSF patients included in this study comprised of 37 males and 13 females, aged 16–60 years. The mean age of the patients is 33 ± 12.06 years. The chewing habit of tobacco, gutkha, pan masala, betel quid or areca nut was recorded in a predesigned questionnaire. Twenty-seven of 50 OSF patients included in this study were habitual consumers of betel quid with tobacco and/or panmasala with tobacco (gutkha). The consumption habits of the patients were divided into four categories. Areca consumers (12) include patients who were consumers of areca nut or panmasala or pan without tobacco. Tobacco consumers (1) include the patients who were consumers of the chewing form of tobacco alone. Areca with tobacco (30) group includes areca nut chewers with tobacco.

The mean age of the control group is 36 ± 16.3 years. Twenty-six controls were non-consumers of any form of tobacco or alcohol; four controls were consumers of bidi (tobacco rolled in temburni leaf).

Antibodies

The monoclonal antibody DO-1 used to detect p53 and polyclonal antibody, C-19 used to detect RARβ (Hayashi *et al*, 2000), were obtained from Oncogene Science Inc. (Cambridge, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Immunohistochemistry

Paraffin-embedded tissue sections (5 μm thickness) were deparaffinized in xylene, hydrated and incubated with 0.5% v/v H_2O_2 in methanol for 20 min to inactivate the endogenous peroxidase. Slides were washed with Tris-buffered saline (TBS), antigen was retrieved by microwave heating for 15 min at 100°C in 10 mM citrate buffer (pH 6.0) and bench cooling to 37°C followed by rinsing in TBS. The oral smears were re-hydrated with TBS and endogenous peroxidase activity was blocked with 0.5% v/v H_2O_2 in methanol for 20 min. Smears or tissue sections were then incubated with either anti-RARβ polyclonal antibody (dilution 1:200) or with DO1 (1:200 dilution), at 4°C overnight in the humidified chamber. After extensive rinsing with TBS, sections were incubated with biotinylated anti-mouse antiserum and subsequently after TBS washings, tissues were incubated with horseradish peroxidase streptavidin conjugate (Dako Labs, Copenhagen, Denmark). Sections were rinsed and colour was developed with the chromogen 3,3′-diaminobenzidine hydrochloride (DAB, 0.05%). Finally, the sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted for evaluation. p53- and RARβ-immunoreactivities were classified into four categories, defined as follows: number of immunostained cells $<10\%$, negative (−); 10–30%, (+1); 30–50%, (+2); and $>50\%$, (+3). The slides were examined by three of us independently (J.K., N.C., M.M.) and, where the observations were discordant, consensus was reached by discussion.

For statistical analysis, the slides with +1 to +3 grading were grouped together and taken as positive. The immunohistochemical data were subjected to statistical analyses using the SPSS software (Chicago, IL, USA). Relationships between RARβ and p53 expression and patient variables were tested by the chi-square test.

Results

Immunohistochemical analysis of RARβ in OSF lesions

Of the 50 OSF tissues analyzed for RARβ protein expression, 35 (70%) cases did not show detectable expression of the protein in the epithelial cells in OSF lesions (Figure 1a). However, 15 of 50, 30% of the OSF specimens showed RARβ-immunopositivity in the epithelial cells, although this immunoreactivity was

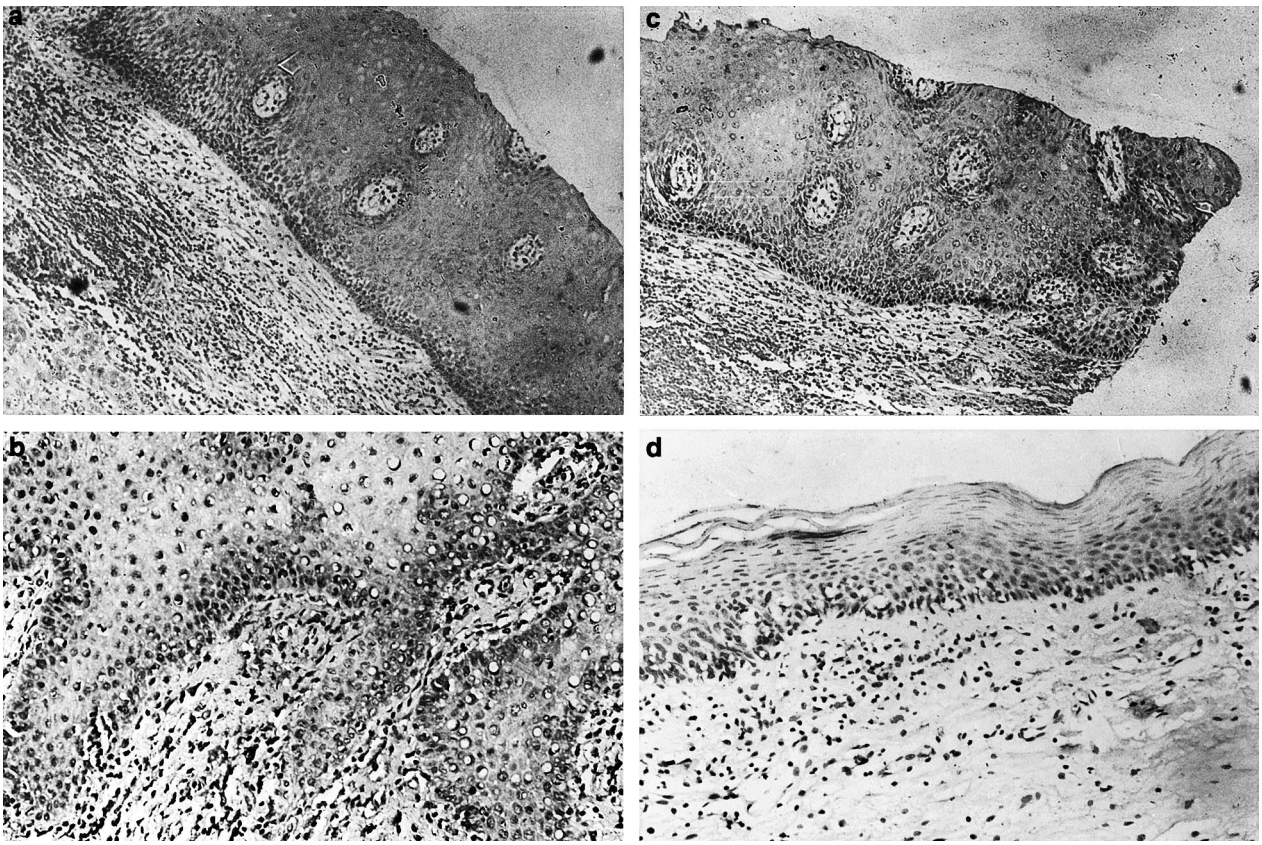

Figure 1 Immunohistochemical analysis of RARβ and p53 proteins in oral lesions using anti-RARβ polyclonal antibody C-19, and p53 monoclonal antibody DO-1, respectively. (a) Epithelial cells in OSF lesion showing decreased expression of RARβ; (b) mucosal epithelium in OSF lesion showing RARβ immunopositivity; (c) oral mucosal epithelium in OSF lesion showing nuclear staining for p53; (d) normal oral mucosal epithelium showing no staining for p53. Original magnification (a, b, c and d) 200×. All sections were counterstained with hematoxylin

Table 1 Expression of RARβ and p53 proteins in normal oral mucosa and oral submucous fibrosis lesions

	Tissue type		
Protein expression	*Normal [n = 30; n (%)]*	*OSF [n = 50; n (%)]*	*Level of significance* (P-value)*
RARβ			
Positive (+1 to +3)	17 (57)	15 (30)	$P = 0.018$
Negative (−)	13 (43)	35 (70)	(OR = 3.054)
p53			
Positive (+1 to +3)	3 (10)	24 (48)	$P = 0.001$
Negative (−)	27 (90)	26 (52)	(OR = 8.307)

*Chi-square analysis.

localized in the differentiated layer of the mucosal epithelium (Figure 1b). A significant decrease in RARβ immunopositivity was observed in OSF lesions compared with normal oral tissues [$P = 0.018$, odd's ratio (OR) = 3.054; Table 1].

Of the 20 normal controls (smear) which were taken from healthy volunteers, 13 were positive for RARβ. Four of the 10 histologically normal tissue taken from the contralateral site of OSF lesion showed RARβ immunopositivity. However, six of 10 did not show RARβ immunopositivity.

Immunohistochemical analysis of p53 in OSF lesions

p53 immunostaining was detected in 24 of 50 (48%) of the OSF cases analyzed. Increased expression of p53 in the nuclei of epithelial cells was predominantly limited to the basal layer, which is the proliferative invasive layer of the epithelia (Figure 1c). The staining was mainly nuclear, while in some cases both nuclear and cytoplasmic immunoreactivity was observed. However, no detectable p53 immunopositivity neither nuclear nor cytoplasmic, was detected in 27 of 30 (90%) normal oral mucosa analyzed (Figure 1d). A significant increase in p53 protein expression was observed in OSF lesions in comparison with normal oral mucosal specimens ($P = 0.001$, OR = 8.307; Table 1).

Three of the 10 histologically normal tissues taken from the contralateral site of OSF lesions showed p53 immunopositivity. However, 20 normal controls (smear) which were taken from healthy volunteers did not show p53 immunopositivity.

Relationship between RARβ and p53 expression in OSF lesions

The relationship between expression of RARβ and p53 proteins in serial tissue sections of OSF lesions was determined by immunohistochemical analysis. Eighteen of 50 (36%) OSF lesions demonstrated accumulation of p53 protein and did not show detectable RARβ immu-

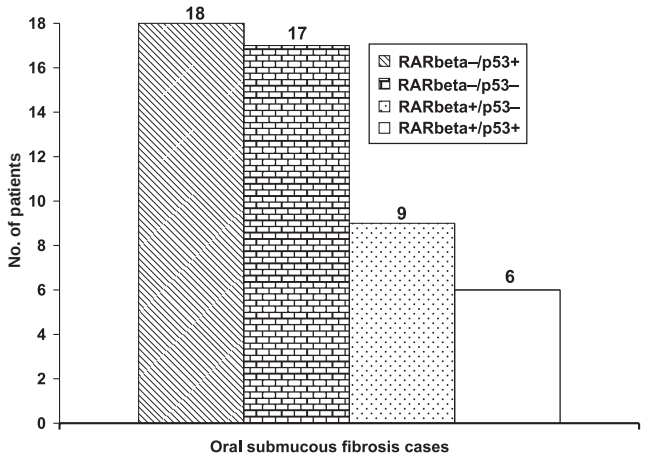

Figure 2 The relationship between expression of RARβ and p53 proteins in serial tissue sections of OSF lesions

Table 2 Correlation of p53 and RARβ expression in OSF lesions with areca nut and tobacco consumption habits

Habits	*Total cases (n = 50)*	*$p53^+$ [n (%)]*	*$RAR\beta^-$ [n (%)]*	*$p53^+$ and $RAR\beta^-$*
Consumers	44	20 (45)	31 (70)	16 (40)
Areca alone	12	8	8	5
Tobacco	1	0	0	0
Areca with tobacco	30	12	22	11
Smokers	1	0	1	0
Current consumers	40	17	30	16
Ex-consumers	4	3	0	0
Non-consumers	6	4 (67)	4 (67)	1 (17)

Current consumers: Patients who were consumers when the biopsy was performed.
Ex-consumers: Patients who stopped consuming before the biopsy was performed.
Areca alone consumers: Patients who were consumers of areca nut or panmasala or pan with areca nut.
Tobacco consumers: Patients who were consumers of the chewable form of tobacco alone. Areca with tobacco: Patients who were areca chewers along with tobacco.

noreactivity suggesting concomitant alterations in the expression of both these proteins (Figure 2). However, the inverse correlation between RARβ and p53 expression failed to reach statistical significance because of the limited sample size. Seventeen of 50 (34%) OSF lesions did not show detectable levels of both RARβ and p53 proteins. Interestingly, 41 of 50 (82%) of OSF lesions showed alterations in the expression of at least one of these proteins.

Of the 12 OSF patients who were areca chewers eight cases showed p53 immunopositivity and eight cases did not show detectable RARβ expression. Of the 30 consumers of areca with tobacco, loss of RARβ expression was observed in 22 (73%) patients and p53 protein expression was observed in 12 (40%) patients (Table 2). However, the expression of p53 and RARβ protein did not show any statistical significant association with the consumption habits of the patients.

Discussion

Low levels of RARβ have been reported in various tumor-derived cell lines as well as in several primary human tumors including carcinoma of oral cavity, prostate, pancreas and breast (Gebert *et al*, 1991; Kaiser *et al*, 1997; Xu *et al*, 1997a; Lotan *et al*, 2000). Differential expression of RARβ has been reported in normal, premalignant (leukoplakia) and malignant head and neck tissues, suggesting that decreased expression of RARβ may be associated with HNSCC (head and neck squamous cell carcinoma) development (Xu *et al*, 1994; Chakravarti *et al*, 2001). OSF is also a potentially malignant condition showing a high tendency, 7.6% over a period of 10 years (Murti *et al*, 1985), to progress to oral SCC. Therefore, it was of interest to determine if RARβ expression is down-regulated in OSF lesions. In this study, the majority of oral submucous fibrosis lesions (35/50, 70%) did not show detectable level of RARβ expression. Whether the abnormally low levels of RARβ are a cause or a consequence of OSF remains to be determined. In this context, it is worthwhile to note that all-*trans*-retinoic acid (ATRA) has been reported to inhibit type I collagen expression in human lung fibroblasts (Krupsky *et al*, 1994). Furthermore, in human skin fibrosis, treatment of cells with 13-*cis*-retinoic acid (13-cRA) or ATRA has been shown to inhibit the synthesis of collagen (Shigematsu and Tajima, 1995; Ogawa *et al*, 1998). Jiang *et al* (1995) demonstrated that treatment of cultured chondrocytes with antisense RARβ2 oligonucleotides resulted in increased synthesis of type IIB collagen. Recently, Wang *et al* (2000) showed that retinoic acid treatment resulted in decreased collagen synthesis by stellate cells in culture. Furthermore, this effect was less pronounced when the cells were treated with acetaldehyde, which decreases the expression of RARβ mRNA as well as protein. Thus, it will be worthwhile to determine the relationship between expression of RARβ and collagen in OSF lesions.

Our data on accumulation of p53 protein in OSF lesions corroborate the earlier findings of Cox and Walker (1996b) that demonstrated p53 immunopositivity in 75% of OSF cases from Nepal. Trivedy *et al* (1998) in OSF lesions from Karachi, Pakistan, reported p53 immunostaining in 15 of 20 (75%) of OSF specimens, three of six SCC arising from OSF and 14 of 21 of SCC not arising from OSF. In view of the high frequency of p53 alterations reported in these studies, p53 immunostaining of tissue biopsy specimens was proposed to be a biomarker of DNA damage in this potentially malignant condition. Previous reports from our laboratory as well as several other groups have shown that accumulation of p53 protein is a frequent event in oral potentially malignant lesions (leukoplakia) and oral squamous cell carcinomas in patients who are heavy consumers of betel and/or tobacco (Kaur *et al*, 1994, 1998).

The intriguing feature of this study was the alteration in expression of RARβ and/or p53 in majority (41/50, 82%) of OSF cases. Interestingly, 18 of 50 (36%) OSF

lesions demonstrated concomitant alterations in both these proteins, suggesting that alteration in p53 and RARβ expression may not be mutually exclusive events in OSF. In light of the high frequency of RARβ and/or p53 alterations observed in OSF tissues, it will be worthwhile to determine whether the OSF lesions harboring concomitant alterations in RARβ and p53 are at higher risk of transition to malignancy in comparison with lesions that do not harbor these alterations in a long-term follow-up study of OSF patients.

Acknowledgements

This research work is supported by a grant from Department of Biotechnology, India. The technical assistance of Ms Neeru Gupta and Ms Meenu Balla is gratefully acknowledged.

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