Epithelial cell proliferation in oral lichen planus

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Abstract. Although the pathogenesis of oral lichen planus (OLP) is not clear, a small proportion of cases with OLP are reported to transform to cancer. We examined the epithelial cell proliferation status of OLP to relate the labelling index to microscopic features surveyed routinely in pathology. Mucosal biopsies obtained from 44 cases diagnosed with OLP with an intact oral epithelium and 10 normal control specimens from Japanese subjects were immunohistochemically stained with MIB and p53 antibodies. The Ki67 labelling index (LI) was significantly higher in OLP compared with normal controls. A particularly large number of OLP lesions (64%) were p53 positive. No association was, however, found with p53 expression and the Ki67 LI. Atrophic and flat epithelia had a quantitatively higher LI, which did not significantly differ from acanthotic biopsies. Increased cell proliferation in OLP is likely to be a secondary phenomenon due to the damage inflicted on keratinocytes by infiltrating mononuclear cells in the submucosa.

Keywords: cell proliferation, Ki67, lichen planus; oral epithelium, p53.

INTRODUCTION

Oral lichen planus is a chronic inflammatory disorder that primarily affects skin and oral cavity. Although the cause of this condition remains unknown (Lozada-Nur & Miranda 1997), the underlying immunopathologic mechanisms that result in the migration of chronic inflammatory cells to the involved dermal and mucosal sites have been well described (Porter *et al.* 1997). In the oral cavity, most published studies have examined the role of cytokines and lymphokines in the evolution of this lesion (Williams 1996), which may result in a significant damage to the overlying oral epithelium. The symptoms arising in oral lichen planus are indeed secondary to this epithelial damage that in some instances result in its loss. Due to the chronicity of the disorder, the epithelium continuously undergoes regeneration following repeated events of liquefaction that affect the basal keratinocytes. Renewing epithelia are well suited to investigations into damage response mechanisms and the processes involved in pathological

changes (Potten 1997). However, epithelial cell kinetics of this condition has not received much attention.

The aims of this study were to examine the state of cell proliferation in oral lichen planus and relate this to the clinical and histomorphologic characteristics in a group of Japanese subjects. We also examined the status of p53 protein expression in these tissues to look for any association of p53 overexpression with increased or decreased cell proliferation in the progenitor cells.

METHODS

Sample

All specimens were obtained by incisional biopsy of lesional tissue from the oral cavity of patients treated at the School of Dentistry, Aichi-Gakuin University from 1990–99. A diagnosis of lichen planus was based on the criteria described by the World Health Organization (WHO 1978) and all biopsies were obtained at their first visit to the hospital before initiating treatment. The tissues were fixed in 10% buffered formalin for 24 h, dehydrated and embedded in paraffin. Consecutive sections of the biopsy material were stained with haematoxylin and eosin for routine histopathological examination or for immunohistochemical studies. Ten nonsmoking Japanese volunteers provided normal oral mucosal biopsies from their buccal cavity for comparison. These specimens were treated in the laboratory in the same way as lesional tissues.

Immunohistochemical staining

Immunohistochemical staining of Ki67 and p53 was performed on paraffin sections mounted on poly-L-lysine coated glass slides. The streptavidin–biotin–peroxidase method used in this study has been described in detail from our laboratory (Warnakulasuriya & Johnson 1992). Briefly, 6- μ m sections were dewaxed and endogenous peroxidase was blocked by 20 min pretreatment with 0.5% H₂O₂ in 70% methanol. Following rehydration nonimmune goat serum (1 : 30) was used to block nonspecific staining. The primary antibody used for Ki67 detection was MIB-1 (Immunotech, Marseille, France; monoclonal mouse; dilution 1 : 100) and that for p53 detection was Do-7 (DAKO A/S, Glostrup, Denmark; monoclonal mouse; dilution 1 : 100). Tissues were then incubated with biotinylated antimouse IgG and avidin–biotin–peroxidase complex (Vector Laboratories Inc, Burlington, USA) in sequence, with 0.05% diaminobenzidine as substrate. Sections were lightly counterstained with Harris' haematoxylin.

Negative laboratory controls included omitting the primary antibodies, thus reacting sections with diluent buffer only, and the use of irrelevant mouse monoclonal antibody (IgG isotype) with each staining set. A known positive control specimen from a colorectal cancer expressing p53 and bearing a *p53* gene mutation, and standardized positive control blocks from an intra-oral carcinoma for Ki67 expression were included.

Evaluation and statistical methods

The following histological criteria were assessed using the haematoxylin and eosin sections: epithelial thickness (graded as normal for site, atrophic or acanthotic), pattern of keratinization (ortho, para or non keratinized), rete ridge morphology (flat, normal for site or hyperplastic; saw-toothed or not) and the density of lymphocytic infiltrate (semiquantitatively expressed as +, ++ or +++). Ki67-positive cells in the basal epithelium were counted by one observer using a

microscopic graticule that allowed the measurement of the length of the basement membrane of the evaluated tissues. More than 10 consecutive grid lengths were counted in each section under $\times 400$ magnification. Ki67 index was estimated as positive cells/mm. p53-stained slides were evaluated as positive or negative. Equivocal p53 staining when less than 5% of cells within a grid area showed immunopositive cells were included in the negative category.

Statistical analysis was performed using SPSS statistical package. Both the Student's *t*-test and χ^2 test were applied to assess statistical differences between the groups classified by histopathological assessments against Ki67 labelling indices. A value of $P < 0.05$ was considered significant.

RESULTS

The study group comprised 44 subjects who had histologically proven oral lichen planus. Any subject with overt ulceration and any biopsies that showed ulceration and separation of the overlying epithelium that hindered any systematic evaluation of epithelial tissues were also excluded from the study. There were 17 men and 26 women (one not recorded), with a mean age of 54.7 ± 7.8 years (range 26–72). The mean age of the control sample was 50.3 ± 8.6 years (range 40–67) was slightly lower because a younger group attending the same surgical unit volunteered to provide normal tissue.

The clinical lesions were characterized as 25 being predominantly reticular in appearance and 19 being atrophic lichen planus. Of 44 biopsies, 38 were obtained from lesional buccal mucosa, two from lip, one from palate and one from tongue. All control samples were also taken from buccal mucosa.

Of the 44 OLP specimens, 36 were parakeratinized, 19 showed atrophy and 26 demonstrated a flat epithelial/connective tissue interphase. Only seven biopsies had the feature of saw-tooth appearance of the rete structures. Heavy lymphocytic infiltrate distributed in the form of a band underneath the basement membrane (Table 1) was seen in 25 samples.

Figure 1(A–D) shows the immunological staining of oral lichen planus tissues with MIB-1 antibody for Ki67 antigen. Generally staining was homogenous, but some focal staining of cells was noted in rete ridges with a saw-tooth appearance (Fig. 1C), suggesting budding of proliferative cells out of a flat epithelium. In areas where basal cells had undergone liquefaction, the immediate parabasal layer showed Ki67-immunopositive cells.

The Ki67 index of the OLP specimens was not associated with age or sex of patients. The mean Ki67 proliferative index of lichen planus specimens (42.04 ± 33.56) was significantly higher ($P = 0.03$) than the control samples (28.23 ± 15.61) used for this study. The atrophic OLP specimens had slightly higher mean Ki67 indices compared with biopsies showing acanthosis but the difference was not significant ($P = 0.275$). This feature of higher cell proliferation in atrophic OLP was also reflected in flat epithelia showing a higher LI compared with those showing rete ridge hyperplasia. The density of lymphocytic infiltration in the lamina propria did not influence the epithelial Ki67 index (Table 1).

Twenty-eight (64%) of the 44 OLP biopsies expressed p53 protein in basal epithelial cells as determined by Do-7 antibody, which detects both wild type and the mutated protein. In two of the biopsies, equivocal staining was found and these were categorized in the p53-negative group. A clear lack of association of p53 expression (+ or –) with the Ki67 index is illustrated in Fig. 2. The mean Ki67 index of the p53-positive group (44.74 ± 35.04) was not significantly different ($P = 0.33$) from the p53-negative specimens (37.33 ± 31.44).

Table 1. Microscopic features of oral lichen planus and Ki67 expression

	Ki67 (cells/mm)			Mean Ki67 index	P-value
	≤ 20	> 20–40	> 40		
Sex					
Male	4	7	6	40.5 ± 29.2	0.299
Female	11	6	9	37.2 ± 32.5	
Age					
Under 50 years	3	3	4	41.7 ± 36.7	0.350
50 years and above	12	10	12	38.2 ± 29.5	
Clinical diagnosis					
Keratotic/Reticular	8	8	9	42.6 ± 34.2	0.662
Atrophic	7	6	6	38.9 ± 32.6	
Thickness					
Acantosis	4	3	2	26.8 ± 14.6	0.275
Atrophy	5	6	8	43.1 ± 33.6	
Normal thickness	6	4	6	39.3 ± 39.6	
Keratinization					
Para-keratinized	12	12	12	43.9 ± 41.3	0.266
Ortho- or non-keratinized	3	1	4	39.5 ± 31.9	
Rete structures					
Flat	7	8	11	46.7 ± 35.9	0.209
Hyperplasia	8	5	5	36.5 ± 30.1	
Saw toothed rete					
No	14	9	14	40.9 ± 33.3	0.742
Yes	1	4	2	47.9 ± 37.2	
Lymphocytic infiltrate					
Mild	4	3	2	33.8 ± 27.7	0.328
Moderate	5	3	2	32.8 ± 31.3	
Heavy	6	7	12	48.7 ± 35.9	

DISCUSSION

We were interested to examine whether the proliferative activity of the oral epithelium in lichen planus is altered due to the underlying inflammation following immunological reactions in its submucosa. These immunological mechanisms causing liquefaction of keratinocytes may render these epithelial cells more susceptible to genetic damage. Although the risk is small, malignant transformation of OLP has been reported (Holmstrup 1992).

A review of the range of methods of measuring cell proliferation of oral mucosa (Warnakulasuriya & Johnson 1996) suggests that immunohistochemical detection of Ki67 can be used as an operational marker to label cycling cells at this site. Before the advent of immune markers, Walker's group (Walker & Dolby 1974) determined *in vitro* tritiated thymidine labelling index of lichen planus. In their study, the total labelling activity of LP was comparable with that of normal mucosa and most proliferation occurred in the basal layers. Subsequently, using the PCNA index, Walker's group reported the growth fraction of LP to be higher than normal mucosa or other oral keratoses (Schifter *et al.* 1998). Conflicting results were reported using

Ki67 labelling by other groups, suggesting that cell proliferation in OLP was either similar to normal oral mucosa (Bloor *et al.* 1999) or higher (Girod *et al.* 1998). The present study confirms a significant difference found in the Ki67 cell proliferation index of lichen planus samples and normal mucosa. This confirms that OLP could have a secondary proliferative disorder probably due to repeated breakdown of the cycling cells leading to a heightened state of proliferation; indeed, some variations in the thickness of the epithelium in OLP are encountered by light microscopy. However, no significant differences in Ki67 index were found whether the epithelium was atrophic, acanthotic or normal for site. The mean Ki67 labelling index derived for OLP (42.04 ± 33.56) was lower than that reported for oral epithelial dysplasia (51.55 ± 20.75) using the same unit of measurement (labelled cells/mm basement membrane length) (Oliver *et al.* 2000). The difference is in the expected direction in that dysplastic lesions have a higher growth fraction.

A particularly large number of OLP lesions were p53-positive. This finding, although surprising for a non-neoplastic condition, has also been reported in several earlier studies that examined p53 expression in OLP (Dekker *et al.* 1997; Girod *et al.* 1998; Schifter *et al.* 1998). As only a very small proportion of oral lichen planus cases eventually transform to cancer this finding of abundant p53 expression needs further evaluation. It is unlikely that overexpression of the protein in this condition is due to any gene mutation and this is more likely to be the overexpression of the wild type protein that is bound to other molecules. An association of p53 overexpression and increased cell proliferation as assessed by Ki67 was reported in oral epithelial dysplasia (Girod *et al.* 1993) and indeed in oral carcinomas (Warnakulasuriya & Johnson 1994). In OLP it appears that although the p53 protein is immunohistochemically detectable it is not influencing epithelial cell proliferation (Fig. 2). As p53 is overexpressed in 65% of the OLP cases it will not be a suitable prognostic marker to determine the precancerous nature of these lesions, considering that the probability of neoplastic transformation of OLP is very low.

In recognized precancerous states of the oral mucosa such as oral leukoplakia, epithelial cell proliferation is nearly double that of the normal oral epithelium (Warnakulasuriya & MacDonald 1995). The finding of a significantly higher state of cell proliferation in OLP compared with the normal oral mucosa raises the question whether this feature could be an important early step in some cases of OLP that eventually transform to malignancy. This alteration, if sustained over a period of time, may give rise to initiated clones that display a growth advantage over normal cells. Proliferating cells have several mechanisms for repairing damaged DNA to reduce the number of genetic alterations. Overexpression of wild-type p53, as now reported in several studies on this disorder, may be one such cell cycle control mechanism. To our knowledge, apart from p53, other molecules such as pRB, p21 and p27 involved in cell cycle regulation (Warnakulasuriya *et al.* 1998; Todd *et al.* 2002) have not been examined in detail in OLP. Apoptosis-associated cell death, however, is reported to be slight (Dekker *et al.* 1997; Bloor *et al.* 1999). The paracrine growth promotion and apoptotic influences due to lymphocyte-mediated keratinocyte damage in this chronic inflammatory disorder require further study.

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Figure 1. Ki67 labelling of oral lichen planus. (A, B) atrophic and acanthotic OLP; (C) increased labelling noted in saw toothed rete structures; (D) control normal buccal mucosa (×100).

Figure 2. A scattergram showing Ki67 LI values for p53-positive and p53-negative OLP and 10 control samples.

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