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Expression of apoptotic and cell proliferation regulatory proteins in actinic cheilitis

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BACKGROUND: Actinic cheilitis (AC) is a pre-malignant lesion caused by ultraviolet (UV) radiation. The apoptotic proteins p53, bax, bcl-2, and the proliferation marker Ki-67, are known to play an important role in UV-exposed skin and carcinomas, therefore, these markers were assessed in AC and compared with normal lip and oral mucosa.

METHODS: AC ($n = 13$), normal lip ($n = 7$) and oral mucosa ($n = 6$) biopsies were stained immunohistochemically for p53, bax, bcl-2 and Ki-67, to determine their expression and distribution.

RESULTS: p53 was over-expressed in AC as compared with normal lip and oral mucosa ($P < 0.003$). Although bcl-2 expression was higher in AC than in oral mucosa ($P < 0.002$), it was significantly reduced as compared with normal lip ($P < 0.04$). Bax expression remained unchanged, and Ki-67 was significantly increased in AC and normal lip as compared with oral mucosa ($P < 0.05$).

CONCLUSION: The results suggest that DNA-damaged cells by UV radiation in AC are eliminated by apoptosis.
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Keywords: actinic cheilitis; bax; bcl-2; Ki-67; p53

Introduction

Actinic cheilitis (AC) is a pre-malignant lesion of the lip with the potential to develop into invasive squamous cell carcinoma (SCC) (1). It affects mainly the lower lip of people frequently exposed to sunlight and/or artificial ultraviolet (UV) irradiation (2, 3). Histopathological changes include hyperplasia, acanthosis, or atrophy of the epithelium, thickening of the keratin covering, ulceration, and mild to severe dysplasia. In the connective tissue, elastosis and in most cases inflammation is found (2, 4).

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Immunohistochemical analysis of cell cycle regulatory proteins has been used to distinguish malignant neoplasms from reactive lesions and to predict their biologic behavior (5–7). The proapoptotic protein p53 is upregulated in injured cells as p53 maintains genomic stability through the activation of multiple downstream pathways that regulate cell cycle arrest, cell repair and apoptosis. In most carcinomas, including oral SCC, actinic keratosis, and skin carcinomas, p53 has been found over-expressed (8–10).

The bcl-2 family consists of anti-apoptotic (Bcl-2, Bcl-XL, Bcl-w, Bfl-1, Brag-1, Mcl-1, A1) and proapoptotic (Bax, Bak, Bcl-XS, Bad, Bid, Bik, Hrk) proteins (11) that are localized at the outer mitochondrial membrane, the nuclear envelope, and the endoplasmic reticulum (12). They act via protein dimerization and their expression ratio within the cell determines whether apoptosis is induced or prevented. High levels of bcl-2 in a cell, which leads to increased bcl-2 dimers, are anti-apoptotic (13). On the contrary, low bcl-2 levels and high bax levels, resulting in bax dimers, are proapoptotic (14). Dysplastic epithelia and SCC borders show strong presence of bcl-2 in most cases, while bax is rarely expressed. In addition, bcl-2 is over-expressed in poorly differentiated carcinomas, while bax is mostly expressed in well-differentiated SCC (15).

The proliferation marker, Ki-67 can be observed in the nuclei of proliferating cells, and it is a useful marker to predict growth status of a specific tissue or organ (16). It has been reported that Ki-67 expression increases in pre-malignant and malignant lesions of the oral mucosa (17–19).

Ultraviolet radiation, known for its immunomodulatory properties, plays a pivotal role in skin damage and photocarcinogenesis (20, 21). It has been recognized that UV radiation induces the formation of sunburn cells, which are apoptotic cells with increased expression of proapoptotic proteins, such as p53 and bax, and decreased expression of anti-apoptotic proteins such as bcl-2. Ki-67 expression is also increased in sunburn cells (22–27).

Although apoptotic and proliferation markers have been widely studied in UV irradiated skin, basal cell

carcinomas and SCCs (5, 8, 15, 19, 22–27), their expression in AC lesions has not been yet studied. Therefore, expression of p53, bax, bcl-2 and Ki-67 was assessed in AC and compared with normal lip and oral mucosa.

Materials and methods

Biopsies

Biopsies of the lower lip vermilion from 13 non-smoking patients with AC (10 male and three female, age range from 25 to 72 years, mean 50 ± 15) were obtained from the Archives of the Oral Pathology Laboratory, Facultad de Odontología, Universidad de Concepción. Normal lip vermilion biopsies (five male and two female, age range from 25 to 63 years, mean 37 ± 15) were used as controls for UV-exposed tissue and normal oral mucosa biopsies taken from the buccal mucosa (four male and two female, age 18–66, mean 40 ± 21) were used as controls for non-UV exposed tissue. Informed consent was obtained from all subjects and this study was approved by the Ethics Committee of the Universidad de Concepción.

All specimens were fixed in 10% buffered formalin, pH 7.4, and paraffin-embedded within 24 h. Serial sections, 4 μm thick, were taken from the tissue blocks and processed for histopathologic and immunohistochemical analysis.

Histopathologic study

Tissue sections were deparaffinized through xylene and descending grades of ethanol, and stained with hematoxylin and eosin. Histopathologic diagnosis of AC was assessed, according to epithelial changes such as hyperkeratosis, hyperplasia, acanthosis, atrophy, ulceration and dysplasia, and connective tissue alterations such as elastosis and inflammation. Microscopic study of biopsies of normal lip and oral mucosa was performed and clinically normal tissues that showed any epithelial and/or connective tissue alterations were excluded.

Immunohistochemical staining of p53, bax, bcl-2 and Ki-67

Tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubation in methanol with 3% hydrogen peroxidase for 5 min. For antigen retrieval, tissue sections were microwaved in citrate buffer (pH 6.0) at 96°C for 10 min. After rinsing in phosphate-buffered saline, slides were incubated with primary antibodies at the concentrations listed in Table 1, followed by incubation with the Immunodetector System, Biotinilated HRP/DAB (Bio.SB Santa Barbara, CA, USA). Slides were counterstained with Harris hematoxylin. Positive controls were skin SCC for p53 and Ki-67, pancreas for bax, and lymph node tissue for bcl-2. The negative controls consisted in replacing the first antibody with another primary antibody of the same IgG subclass, isotype IgG_{2b} Kappa Cromogranine (Dako, Carpinteria, CA, USA) for p53, isotype Myeloperoxidase polyclonal (Cell Marque, Hot Springs, AR, USA) for bax, isotype IgG_1 Kappa Caldesmon (Neo Markers, Fremont, CA, USA) for bcl-2 and Ki-67.

Table 1 List of antibodies and dilutions

Marker	*Type (clone)*	*Isotype*	*Source*	*Dilution*
p53	DO7	IgG_{2b}	Dako	1:100
bax	Polyclonal	–	Dako	1:100
bcl-2	124	IgG_1 Kappa	Dako	1:25
Ki-67	Polyclonal	–	Dako	1:100

Counting of p53, bax, bcl-2 and Ki-67 positive epithelial cells

Positive cells for each marker were counted separately in serial sections of AC, normal lip and oral mucosa, using a Nikon Diaphot 300 microscope equipped with an OC-M calibrated eyepiece micrometer and connected to an ImagePro analysis program 4.0.1 (Media Cibernetics, Atlanta, GA, USA). Positive and negative cells for each marker were counted at 40× magnification by two calibrated observers. A minimum of 1000 cells were counted for each marker at the basal, parabasal and suprabasal layers. Results were expressed as percentage of positive cells/1000 cells (mean ± SEM).

Statistical analysis

Data were tabulated and statistical tests were performed with JMP-IN 3.2.1 (SAS Institute Inc., Cary, NC, USA). Differences among groups were examined using one-way ANOVA and Tukey-Kramer tests. When variables did not have a normal distribution, nonparametric, Wilcoxon and Kruskal–Wallis tests were used. Differences were considered statistically significant when $P < 0.05$.

Results

Histopathologic findings

Epithelial changes observed in AC specimens were as follows: hyperkeratosis (in 10 of 13 samples), hyperplasia (four of 13), acanthosis (eight of 13), atrophy (five of 13), and ulceration (one of 13). All specimens presented solar elastosis and chronic inflammation in the connective tissue.

Expression and distribution of apoptotic markers

p53

Immunohistochemical detection of p53-positive cells showed that AC samples had the highest levels of p53 expression (53.8 ± 2.6%) as compared with normal lip (34.6 ± 3.6%) and oral mucosa (1.8 ± 1.0%) ($P < 0.003$, Wilcoxon and Kruskal–Wallis) (Figs 1 and 2). Normal lip p53 expression was also elevated as compared with oral mucosa ($P < 0.003$). Positive immunohistochemical staining was localized at the nucleus of basal (13 of 13), parabasal and suprabasal (four of 13) cells (Fig. 2).

bcl-2 family

Immunohistochemical detection of bcl-2 at the epithelium showed a significant reduction of bcl-2 expression

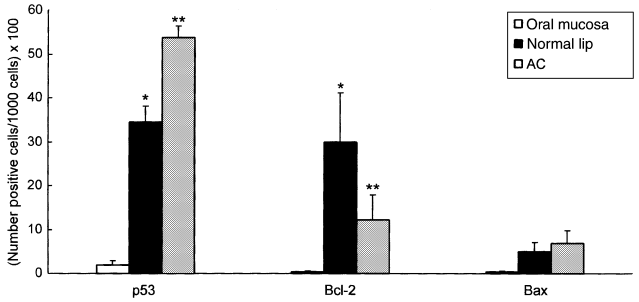

Figure 1 Expression of apoptotic markers in actinic cheilitis (AC), normal lip and oral mucosa. The apoptotic markers p53, bcl-2 and bax were detected by immunohistochemistry. For each marker, results are expressed as percentage of positive cells/1000 cells ± SEM ($n = 13$ for AC, $n = 7$ for normal lip, and $n = 6$ for oral mucosa). *$P < 0.05$ (Wilcoxon and Kruskal–Wallis) for p53 and bcl-2 in normal lip as compared with oral mucosa. **$P < 0.05$ for p53 and bcl-2 in AC as compared with normal lip and oral mucosa.

in AC (12.2 ± 5.8%), as compared with normal lip (30.0 ± 11.1%), but higher bcl-2 expression than oral mucosa (0.3 ± 0.3%) ($P < 0.04$, Wilcoxon and Kruskal–Wallis) (Figs 1 and 3). Bcl-2 expression in normal lip was higher than oral mucosa ($P = 0.0019$). Positive bcl-2 staining was intracytoplasmatic and was distributed mainly in basal cells. In addition, abundant bcl-2 positive staining was found in lymphocytes in the connective tissue of all AC samples (Fig. 3C,c).

Immunohistochemical bax staining showed a tendency for higher bax expression in AC (6.8 ± 3.0%) and normal lip (5.0 ± 2.1%) than in oral mucosa (0.3 ± 0.3%), however, no statistically significant differences were found (Fig. 1).

Expression and distribution of Ki-67

Immunohistochemical detection of Ki-67 was mostly found in the nucleus of basal epithelial cells in AC, normal lip and oral mucosa samples (Fig. 4). Ki-67 expression in parabasal layers was found sporadically in AC. A significant increase in Ki-67 expression was found in AC (44.6 ± 6.0%) and normal lip (45.3 ± 3.5%) as compared with oral mucosa (25.0 ± 1.6%) ($P < 0.05$, ANOVA and Tukey-Kramer), with no differences between normal lip and AC (Fig. 5).

Discussion

Ultraviolet light is known to be responsible for UV-induced genotoxicity (20, 28), but its role in triggering apoptosis is not yet completely understood. Whereas there are several reports on this issue regarding its effect on the skin, there are no investigations on AC lesions. The aim of this study was to assess the role of apoptotic and cell proliferation markers in AC.

Expression of p53 was significantly increased in AC as compared with normal lip and oral mucosa. p53 was also increased in normal lip as compared with oral mucosa. Similar results have been found in UV-irradiated mouse keratinocytes, human epidermis and basal

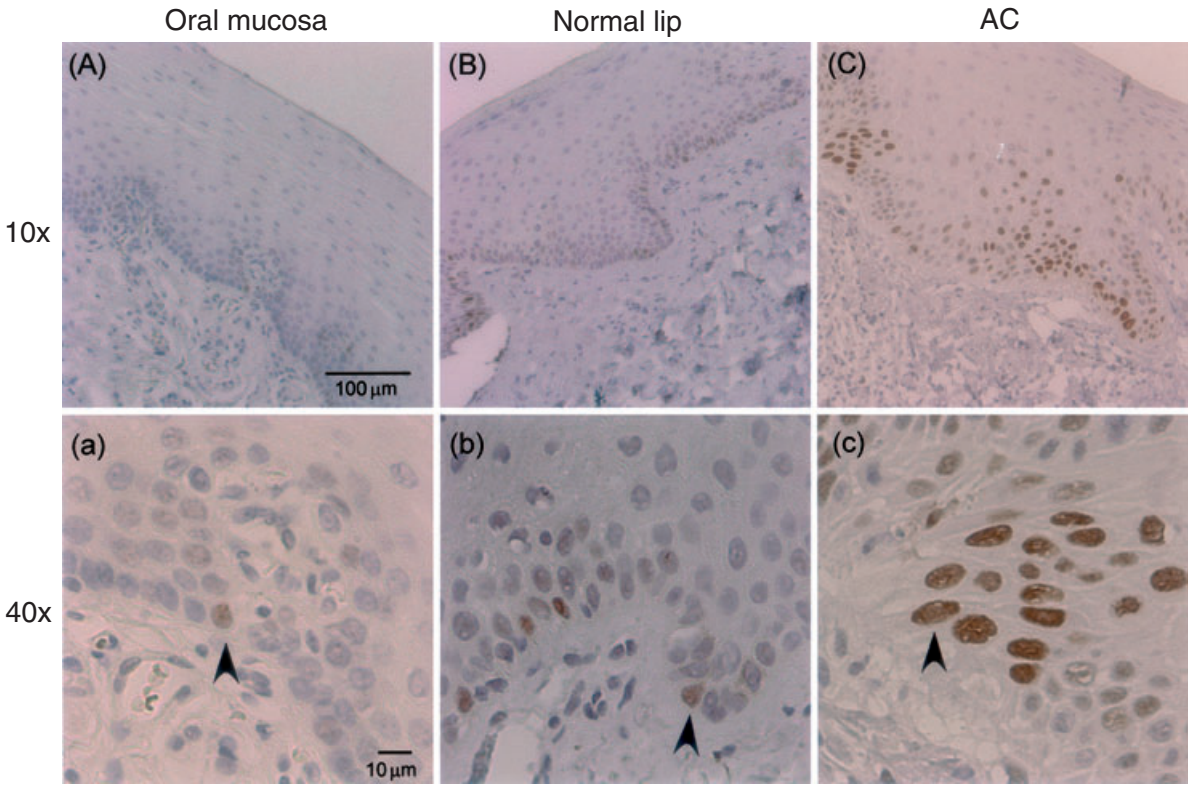

Figure 2 Immunohistochemical staining of p53. Sample slides immunostained for p53 are shown in (A) and (a) for oral mucosa, (B) and (b) for normal lip, and (C) and (c) for AC. Arrowheads indicate p53-positive cells.

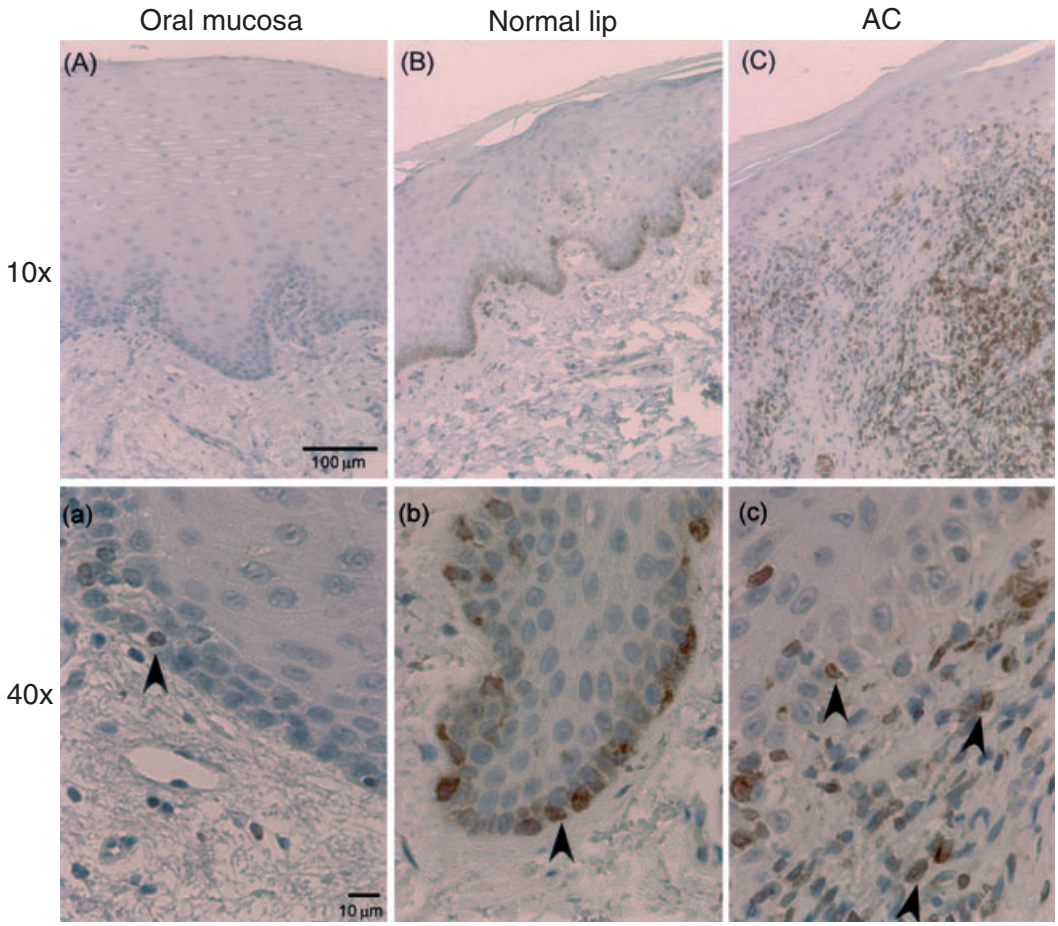

Figure 3 Immunohistochemical staining of bcl-2. Sample slides immunostained for bcl-2 are shown in (A) and (a) for oral mucosa, (B) and (b) for normal lip, and (C) and (c) for AC. Arrowheads show bcl-2-positive cells.

cell carcinoma (23–25, 29). This may be related to the intensity of DNA damage caused by UV exposure. UV-induced apoptosis is a complex event involving different pathways. DNA damage appears to be the predominant factor determining whether a cell undergoes apoptosis, but it is definitely not the only one (30). The role of p53 may be of inducing apoptotic death of pre-malignant cells. The fact that p53 was expressed in basal keratinocytes and not in differentiated cells in most samples, supports the potential role of p53 in preventing early carcinogenesis (22). However, as there were four cases of AC with p53 expression in the suprabasal layers, it could indicate a higher risk of malignant transformation in these cases of AC (31).

Study of p53 expression in tissues not exposed to UV radiation such as oral mucosa has been controversial. While Crosthwaite and cols. (1996) (32) have not found any p53 expression in oral mucosa, Fabbrocini and cols. (2000) (33) showed a 20% of expression. The results from this study showed a higher level of expression of positive basal cells (34.6%), which may be because of higher sensitivity of the immunohistochemical technique used.

The data also showed that bcl-2 was significantly decreased in AC compared with normal lip. Similar results have been previously reported in UV-exposed mouse skin and human epidermis, as well as in DNA repair-deficient fibroblasts (26, 34, 35). This suggests that UV itself could modulate bcl-2 expression. UV induces down-regulation of bcl-2, which could be an important mechanism of UV-induced apoptosis. However, no significant differences in bax expression were found in AC, normal lip and oral mucosa, although bax expression had a tendency for higher expression in AC and normal lip. Therefore, low bcl-2 and unchanged bax suggests a balance in favor of apoptosis in AC.

Ki-67 is a nuclear antigen expressed in G1, S, G2 and M phase of the cell cycle and absent in quiescent cells (G0) (36). In oral mucosa, it has been shown that the number of Ki-67-positive cells increases according to epithelial proliferation and dysplasia, which may implicate Ki-67 as a marker of the presence and severity of epithelial dysplasia (17, 37). Expression of Ki-67 is increased in UV-irradiated skin (23, 29), which is in agreement with the present results, showing a significant increase of Ki-67 expression in tissues exposed to UV light such as AC and normal lip, as compared with oral mucosa. However, no differences in Ki-67 expression were found between normal lip and AC, which should have different degrees of actinic damage, and therefore different Ki-67 expression, according to what has been previously reported.

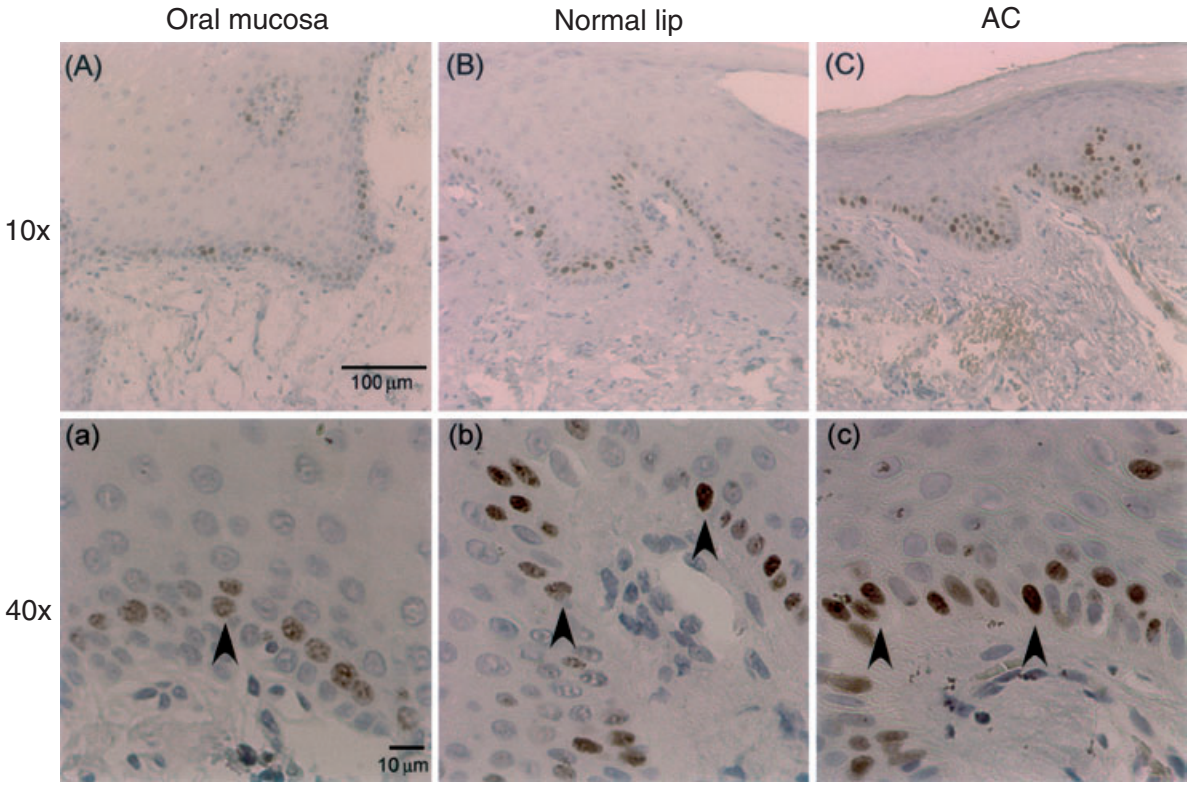

Figure 4 Immunohistochemical staining of Ki-67. Sample slides immunostained for Ki-67 are shown in (A) and (a) for oral mucosa, (B) and (b) for normal lip, and (C) and (c) for AC. Arrowheads indicate cells positive for Ki-67.

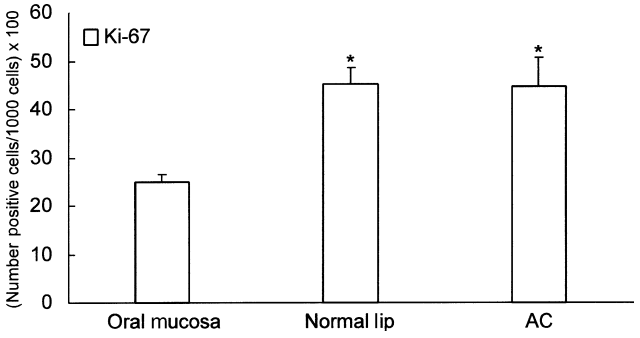

Figure 5 Ki-67 Expression in AC, normal lip and oral mucosa. Ki-67 expression was detected by immunohistochemistry. Results are expressed as percentage of positive cells/1000 cells ± SEM ($n = 13$ for AC, $n = 7$ for normal lip, and $n = 6$ for oral mucosa). *$P < 0.05$ (ANOVA and Tukey Kramer) as compared with oral mucosa.

In summary, the results showed that in AC there is an increase in p53 expression as compared with both normal lip and oral mucosa, and a decrease in bcl-2 expression as compared with normal lip, whereas bax expression remained unchanged. Therefore, apoptosis of damaged cells was favored in the AC samples analyzed, where no histopathological signs of malignant transformation were observed. This suggests that p53 and bcl-2 may play an important role in regulating malignant transformation in AC. Further studies should analyze the changes in expression of apoptotic and proliferation markers in AC samples with epithelial dysplasia.

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