Assessment of c-Jun, c-Fos and cyclin D1 in premalignant and malignant oral lesions

Eveline Turatti§, Adriana da Costa Neves†, Marina Helena Cury Gallottini de Magalhães† and Suzana Orsini Machado de Sousa†

§Department of Oral Pathology, University of Fortaleza, Brazil
†Department of Oral Pathology, School of Dentistry, University of São Paulo, Brazil

(Received 15 December 2004 and accepted 9 April 2005)

Abstract: Some oral cancers are known to develop from dysplastic oral epithelium. In the present study, the expression of c-Jun, c-Fos, and cyclin D1 proteins in oral epithelial lesions with different degrees of dysplasia, and in oral squamous cell carcinomas (OSCCs) was evaluated. Eighteen cases of mild dysplasia, 23 cases of moderate to severe dysplasia and 24 OSCCs were studied immunohistochemically. Additionally, 15 sections of oral mucosa without any evidence of dysplasia were included in the study. Results: c-Jun expression increased according to the degree of oral dysplasia, with the greatest expression found in OSCC. c-Fos expression was intense in normal mucosa, reduced in mild dysplasia and high in moderate to severe dysplasia and in OSCCs. Cyclin D1 was expressed in only a few cases of moderate to severe dysplasia and in most of the OSCCs. Statistical analysis showed a correlation between the three proteins and the degree of epithelial alteration. The present results indicate a possible role of c-Jun and c-Fos in malignant transformation of oral mucosa. (J. Oral Sci. 47, 71-76, 2005)

Keywords: AP-1 proteins; cyclin D1; oral squamous cell carcinomas.

Introduction
Uncontrolled cell proliferation is the hallmark of cancer, and neoplastic cells have abnormalities of genes that regulate their cell cycles. Many genes directly control the expression of other genes, and are involved in cell processes such as proliferation, differentiation and cell death. Among genes that control the expression of others are the immediate early genes of the Fos and Jun families (1).

Fos and Jun proteins form the composite transcription factor activating protein-1 (AP-1), a mitogen-activated transactivator important for cell proliferation and differentiation, indicating that these proteins play specific roles during the cell cycle (2-7).

Thus, it is noteworthy that many genes encoding components of the cell cycle have an AP-1 binding site in their promoters. Cyclin D1 is an example of one of these genes that may directly link AP-1 to cell cycle progression, since it is related to G1 progression, and can be induced by treatments that increase AP-1 activity (8-13).

The aim of this study was to compare the immunexpression of c-Jun, c-Fos and cyclin D1 in oral premalignant and malignant lesions, using commercially available antibodies and paraffin-embedded sections.

Materials and Methods
The specimens were selected from the archives of the Surgical Oral Pathology Service at the University of São Paulo, and included tissue from 41 cases clinically diagnosed as leukoplakias, which were histologically classified as presenting mild dysplasia (18 cases) or moderate to severe dysplasia (23 cases) and 24 cases that were diagnosed as invasive oral squamous cell carcinomas.
Fifteen cases of normal oral mucosa were also evaluated as controls (Table 1). All tissue samples came from the tongue. Oral dysplasia was graded using the WHO (14) list of histological changes, and was considered mild when up to two epithelial changes were present, and moderate to severe when more than two epithelial changes were present. The most atypical sites of the submitted biopsies were chosen to be graded.

Biopsy material had been previously fixed in 4% buffered formaldehyde and was embedded in paraffin. Sections 3 µm thick were cut and mounted on poly-L-lysine-coated glass slides, and air-dried overnight at room temperature. After deparaffinization in xylene the sections were rehydrated through graded ethanol, and then treated for antigen retrieval, which consisted of 30 min in a steamer with the sections immersed in 0.1M citrate buffer (pH 6.0). Next, the sections were immersed in 3% hydrogen peroxide in methanol (V/V) for 15 min in order to quench endogenous peroxidase activity. The sections were then washed in Tris-buffered saline and incubated with normal 1% bovine serum albumin (BSA) in Tris for one hour to reduce non-specific antibody binding. After being washed in Tris, the sections were incubated with primary antibodies against c-Jun (clone H79*, 1:50, 60 min), c-Fos (clone: H125*, 1:100, 60 min), and cyclin D1 (clone 16P04**, 1:50, 60 min) (*Santa Cruz Biotechnology; **Neomarkers). They were then thoroughly washed in Tris and exposed to streptavidin-biotin complex (Dako, Carpinteria, CA) according to the manufacturer’s instructions. Diaminobenzidine (Dako, Carpinteria, CA) was used as a chromogen, followed by counterstaining with Mayer’s hematoxylin. Sections were dehydrated in ethanol, cleared in xylene and mounted in Permount (Fischer Chemicals, New Jersey).

Only nuclear staining was considered for all three antibodies, based on a previous study (15) that had shown cytoplasmic expression of c-Jun only in normal oral mucosa. The number of positive cells in all sections was assessed by a single observer (E.T.) using a video monitor attached to a light microscope. The sections were first examined at an ocular magnification of ×100, and then a representative field of the section was chosen randomly and viewed at ×400 (positive cells were counted from among 500 tumor cells).

Data obtained for the expression of c-Jun, c-Fos and cyclin D1 for each case were subjected to calculus of mean number of positive cells, dispersion or scattering measures (standard deviation) and percentages. Statistical analysis was carried out using different methods and a P value less than 0.05 denoted the presence of a statistically significant difference. Comparisons of the mean numbers of cells positive for c-Fos, c-Jun and cyclin D1 and the histological types of lesions were analyzed using the Kruskal-Wallis test. Multiple comparisons were analyzed using the Dunnett T3 test. For comparisons among the three markers, Spearman’s correlation test was applied.

**Results**

Table 1 shows the number and percentages of cases showing a reaction with each antibody. Figure 1 (A-I) illustrates the immunohistochemical aspects of the reactions.

No nuclear expression of c-Jun was seen in normal oral mucosa, whilst positivity was seen in 44.4% of the cases with mild dysplasia, 69.5% of those with moderate to severe dysplasia, and 87.5% of OSCCs. Compared with normal mucosa, the mean number of positive cells was significantly higher in mild dysplasia (P = 0.031), moderate to severe dysplasia (P = 0.001), and in OSCC (P = 0.001). Positivity for c-Fos was detected in 27.7% of the cases classified as mild dysplasia, 60.8% of cases showing moderate to severe dysplasia and 79.1% of OSCCs. Eleven (73.3%) out of the 15 normal mucosa controls stained positively for c-Fos. Statistical analysis of the mean numbers of positive cells showed that the expression of c-Fos in mild dysplasia was significantly lower than in normal oral mucosa (P = 0.001), moderate to severe dysplasia (P = 0.018) and OSCC (P = 0.001). Cyclin D1 was expressed by 66.6% of OSCCs and 8.6% of cases showing moderate to severe dysplasia, and was negative in normal mucosa. Eleven (73.3%) out of the 15 normal mucosa controls stained positively for c-Fos. Statistical analysis of the mean numbers of positive cells showed that the expression of c-Fos in mild dysplasia was significantly lower than in normal oral mucosa (P = 0.014), mild dysplasia (P = 0.014), and moderate and severe dysplasia (P = 0.043) (Table 2 and Figures 2-4).

The Spearman correlation test showed that the correlations between c-Jun and c-Fos, cyclin D1 and c-Jun, and cyclin D1 and c-Fos were positive and statistically significant, the coefficients of correlation and significance being r = 0.33 and P = 0.003; r = 0.48 and P < 0.001; and r = 0.24 and P = 0.032, respectively (Figs. 5-7).
Discussion
In a previous study (15) we focused on the expression of c-Fos and c-Jun in normal oral mucosa and OSCC. The most striking feature we found was that the epithelium next to areas of malignant transformation showed nuclear expression of c-Jun, in contrast to normal mucosa that

Fig. 1 A-C Oral mucosa exhibiting mild dysplasia. A) Nuclear and cytoplasmic expression of c-Jun. B) c-Fos is expressed in the nuclei (brown) of a few basal and suprabasal cells. C) Cyclin D1 is negative. Strep. × 250 (original magnification).
D-F Moderate to severe dysplasia. D) Expression of c-Jun in the nuclei and cytoplasm of suprabasal cells. E) c-Fos expression in the nuclei of most epithelial cells (brown nuclei). F) Cyclin D1 is expressed in some epithelial cells (brown nuclei). Strep. × 250 (original magnification).


Fig. 2 Mean number of cells positive for c-Jun, and 95% confidence interval, according to diagnosis.

Fig. 3 Mean number of cells positive for c-Fos, and 95% confidence interval, according to diagnosis.

Fig. 4 Mean number of cells positive for cyclin D1 and 95% confidence interval, according to diagnosis.
showed only a cytoplasmic reaction. c-Fos was present in the nucleus of normal mucosa, but its apparent expression decreased in the least differentiated cases of OSCC. Thus, in the present study we aimed to verify the expression of these proteins in premalignant lesions, in comparison with the expression in OSCC. Cyclin D1 expression was also evaluated to determine if an increase in its expression is followed by an increase in c-Jun and c-Fos expression, since previous studies had shown significant overexpression of cyclin D1 in cases of oral cancer (16). Furthermore, it is known that c-Jun activates the cyclin D1 promoter (10, 13). As cytoplasmic c-Jun expression is regularly seen in normal mucosa, only nuclear expression was considered for statistical analysis.

Positive expression of c-Jun was seen in almost 70% of cases of moderate to severe dysplasia, compared with Table 2 Mean numbers of cells showing a positive reaction with each antibody according to histological diagnosis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Normal mucosa</th>
<th>Mild dysplasia</th>
<th>Moderate/severe dysplasia</th>
<th>OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Jun</td>
<td>0.00 (0.00)</td>
<td>22.24 (29.73)</td>
<td>45.08 (35.35)</td>
<td>61.36 (33.28)</td>
</tr>
<tr>
<td>c-Fos</td>
<td>56.75 (38.60)</td>
<td>8.33 (15.81)</td>
<td>33.41 (33.03)</td>
<td>39.4 (29.23)</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>1.46 (3.98)</td>
<td>11.94 (17.22)</td>
</tr>
</tbody>
</table>

* significant level according to Kruskal-Wallis test
**c-Jun ⇒ MN ≠ AL (p = 0.031); MN ≠ AM/I (p < 0.001); MN ≠ CA (p < 0.001);
       AL = AM/I (p = 0.165); AL ≠ CA (p = 0.002); AM/I = CA (p < 0.495)
**c-Fos ⇒ MN ≠ AL (p = 0.001); MN = AM/I (p = 0.315); MN ≠ CA (p = 0.594);
       AL ≠ AM/I (p = 0.018); AL ≠ CA (p = 0.001); AM/I = CA (p = 0.985)
**Cyclin D1 ⇒ MN = AL (p = 1.000); MN = AM/I (p = 0.418); MN ≠ CA (p = 0.014);
       AL = AM/I (p = 0.418); AL ≠ CA (p = 0.014); AM/I ≠ CA (p = 0.043)

** statistical significance of Dunnett-T3 test
75

87.5% of cases of OSCC. In contrast, less than 50% of the lesions diagnosed as mild dysplasias expressed c-Jun in the nucleus. Further studies may correlate this finding with malignant transformation, or to an actual potential for malignant transformation in these lesions. c-Jun nuclear expression in dysplastic epithelium could be interpreted as an early mechanism of cell cycle disturbance, since other authors have shown that the expression of c-Jun increases early in carcinogenesis (17). Also, fibroblasts from embryos lacking c-Jun show slower growth kinetics with reduced expression of cyclin D1 (9).

c-Fos was expressed in dysplastic lesions as well as in OSCCs. This finding cannot be related to malignancy since in normal mucosa it is also present in most of the cells. However, in lesions graded as mild dysplasia, a lack of c-Fos was noted, and even though no conclusion can be drawn from the present study, it could be a factor that inhibits cancer progression. In skin tumors, c-Fos is necessary for malignant transformation (8).

As expected, cyclin D1 was expressed in most cases of OSCC (67%), and in a few cases of moderate to severe dysplasia (8.6%). The finding of increased cyclin D1 protein expression in OSCC is consistent with previous studies that showed frequent amplification of the cyclin D1 gene, and overexpression of cyclin D1 protein in OSCC (18).

Our results clearly show that the expression of both c-Jun and c-Fos is increased in moderate to severe dysplasia, whereas the increase in cyclin D1 is detected later in only a few dysplastic lesions. A statistically significant correlation between cyclin D1 and the members of the AP-1 family, c-Jun and c-Fos, was found. This finding is important since many studies have shown overexpression of cyclin D1 in head and neck cancers, but were unable to clarify the mechanism involved. Other studies have attempted to isolate the point in the cell cycle at which the AP-1 transcriptional factors act. The cyclin D1 gene appears to be a target gene for these factors (8,10,12,13,18). It is still not known if one protein influences the others; however, it is known that overexpression of cyclin D1 shortens the G1 phase of the cell cycle, and it is also known that c-Fos and c-Jun, as part of the AP-1 transcription complex, are important regulators of the G0-G1 transition of the cell cycle (10). Further studies may confirm a relationship between these proteins.

In summary, the present findings indicate that the major components of AP-1 transcriptional factor and cyclin D1 are altered in dysplastic epithelium and in OSCC. It can be assumed that damage occurring in some of these proteins could influence the function of other genes involved in the cell cycle. Further studies should be undertaken to investigate the hypothesis that c-Jun and c-Fos are necessary for malignant transformation in oral cancer.

References

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