Different expression patterns of carbonic anhydrase IX in oral lichen planus and leukoplakia

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ABSTRACT
Tumor hypoxia is an important indicator of cancer prognosis. Among the different genes that are up-regulated by hypoxia is carbonic anhydrase IX, which combines carbon dioxide and water to form bicarbonate and hydrogen. Although expression of this enzyme is very low in normal tissues, carbonic anhydrase IX is overexpressed in several types of cancer. The aim of the present work was to analyze carbonic anhydrase IX expression in the two most frequent potentially malignant oral disorders: oral lichen planus and oral leukoplakia. Immunohistochemical analysis of oral lichen planus and oral leukoplakia biopsies was performed using anti-carbonic anhydrase IX antibody. Samples of normal mucosa served as controls. Statistical analysis was performed by Fischer's exact test. The enzyme was detected in the epithelium of both lesions. The staining was more intense in the basal layer and decreased towards the surface in oral lichen planus. Conversely, the most intense reaction was observed in the superficial layers in leukoplakia, and staining intensity decreased towards the basal membrane. No carbonic anhydrase IX expression was seen in normal mucosa samples. Carbon anhydrase IX expression in lichen and leukoplakia epithelia shows that hypoxia plays a role in the pathogenesis of both lesions. The different distribution patterns provides further evidence of the different biological behavior of these two entities, which under certain circumstances can have similar clinical and histological features.

Key words: *Carbonic Anhydrase IX; Oral Lichen Planus; Oral Leukoplakia.*

Diferentes patrones de expresión de la anhidrasa carbónica IX en liquen plano bucal y leucoplasia

RESUMEN
La hipoxia tumoral es un importante indicador de pronóstico en cáncer. Entre los distintos genes que son activados por hipoxia, uno de los principales es la anhidrasa carbónica IX (CAIX), que combina CO_2 con H_2O para sintetizar HCO_3^- y H^+. Aunque la expresión de esta enzima es muy baja en tejidos normales, se sobreexpresa en varios tipos de cáncer. La finalidad del presente trabajo fue analizar la expresión de CAIX en las dos lesiones orales potencialmente malignas más frecuentes: el liquen plano y la leucoplasia. Se utilizó una técnica inmunohistoquímica con un anticuerpo específico contra CAIX, en biopsias de liquen plano oral y leucoplasia oral. Se utilizaron mucosas normales como controles. Se realizaron análisis estadísticos utilizando test exacto de Fischer. La identificación de la enzima fue positiva en el epitelio de ambas lesiones. En los líquenes la reacción es más intensa en los estratos basales, disminuyendo hacia la superficie. Inversamente, las leucoplasias mostraron marcación más intensa en estratos superficiales, con disminución hacia la membrana basal. Las mucosas normales resultaron negativas. La expresión de CAIX en el epitelio de líquenes y leucoplasias indica que la hipoxia juega algún papel en la patogenia de ambas lesiones. El diferente patrón de distribución es una evidencia más del diferente comportamiento biológico de dos entidades las cuales en ciertas circunstancias pueden manifestar cuadros clínicos e histológicos semejantes.

Palabras clave: *Anhidrasa Carbónica IX; Liquen Plano Oral; Leucoplasia Oral.*

INTRODUCTION

Tumor hypoxia is an important indicator of cancer prognosis, since it is associated with aggressive growth, metastasis and poor response to treatment[1]. Genes that are up-regulated by microenvironmental hypoxia through activation of Hypoxia Inducible Factor-1 (HIF-1) include glucose transporters, glycolytic enzymes, and angiogenic growth factors[2]. Carbonic anhydrases (CAs), a family of metalloenzymes that require Zn^{2+} as a cofactor, are one of the most important groups. Sixteen isozymes that differ in their subcellular localization, catalytic activity

and susceptibility to different classes of inhibitors have been identified. Some of these isozymes are cytosolic (I, II, III, VII and XIII), others are membrane bound (IV, IX, XII and XIV), two are mitochondrial (VA and VB), and one is secreted in saliva (VI). There are also three acatalytic forms, called CA-related proteins (CARPs): CARP VIII, X and XI. In humans, CAs are present in various tissues, including the gastrointestinal tract, the nervous system, kidneys, lungs, skin and eyes, among others[3], but their expression in these tissues is very low. The most reactive site was found in the basolateral surfaces of the crypt enterocytes in duodenum, jejunum and ileal mucosa[4].

These enzymes catalyze the reversible hydration of carbon dioxide to bicarbonate

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

Membrane-bound CAs, including carbonic anhydrase IX (CAIX), have an extracellular active site and can provide H^+ or HCO_3^- ions formed during catalytic turnover for various physiological processes, such as extracellular acidification. Several isoforms of CA are thus involved in essential cell processes, like respiration and pH regulation, electrolyte balance, bone resorption, calcification and biosynthetic reactions requiring HCO_3^- as a substrate[5]. CAIX specifically, is important because together with its isoform XII, it is involved in malignant transformation processes[6].

CAIX is overexpressed in a variety of solid malignant tumors, including renal carcinoma and particularly clear cell adenocarcinoma[7-8], cervical carcinoma[9], ovarian carcinoma[10], oesophagal carcinoma[11], bladder carcinoma[12], non-small cell lung carcinoma[13] and mesothelioma[14]. In addition, overexpression of CAIX has been found to correlate with greater tumor aggressiveness[15].

CAIX has several functions in tumor cells. As it extrudes H^+ ions to the extracellular environment, it maintains the latter acidic while making intracellular pH slightly alkaline by incorporating HCO_3^- ions**.** This combination not only favors tumor cell growth but also facilitates events such as cell transformation, chromosomal rearrangements, extracellular matrix breakdown, migration and invasion, protease activation, and growth factor synthesis[16].

In addition, both *in vitro*[17] and *in vivo*[18] studies have shown CAIX to be a useful marker of hypoxia, exhibiting a pattern of expression around regions of necrosis. This distribution is similar to that of pimonidazol, the most validated exogenous marker of hypoxia. Few studies have addressed the expression pattern of CAIX in premalignant lesions. CAIX expression has been shown to increase in bronchial premalignant lesions[19], in breast[20], uterine cervix[21] and skin dysplasias[22], and in oral leukoplakia[23]. The aim of the present work was to study and compare immunohistochemical expression of CAIX in oral lichen planus (OLP) and oral leukoplakia, since both lesions have been classified as potentially malignant disorders, but differ greatly in their risk for malignant transformation.

MATERIALS AND METHODS

The study comprised 37 biopsies corresponding to cases with proven clinical and histopathological diagnosis of OLP (23 cases) and homogenous leukoplakia (14 cases), which were retrieved from the archives of the Oral Pathology Department of the School of Dentistry, University of Buenos Aires. The study was approved by the Ethics Committee of the School of Dentistry of the University of Buenos Aires, Argentina (Res CD 325/02).

All diagnoses were established following diagnostic criteria of the World Health Organization (WHO)[24] and modified WHO diagnostic criteria[25]. Nine of the 23 OLP cases were reticular lichen planus, and the remaining cases were the erosive or atrophic type of lichen. Two cases of leukoplakia showed moderate dysplasia. The specimens had been fixed in 10% formalin with PBS and embedded in paraffin. Seven specimens of normal oral mucosa obtained during surgery of deep-seated lesions were also studied. Endogenous peroxidase was blocked by immersion in 0.5% H_2O_2 in methanol for 30 min. Antigen retrieval was performed with Citra (Biogenex, Fremont, California, USA) in 3 cycles of 4 minutes each, at 400 W microwaves. The sections were then incubated overnight with the primary antibody, anti-human CAIX, raised in rabbit (Santa Cruz Biotechnology, Dallas, Texas, USA). Antibody binding sites were visualized using a streptavidin-peroxidase detection kit (Kit Multilink, Biogenex, Fremont, California, USA), and incubation in 3,3-diaminobenzidine substrate and 1% nickel chloride as intensifier. A section of a single block of a reactive oral squamous cell carcinoma was included in each staining batch as positive control. Sections in which the primary antibody was omitted were used as negative control.

The percentage of cases showing positive staining in the different localizations was analyzed using Fisher's exact test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

CAIX expression was detected in 16 out of 23 cases of lichen and 12 out of 14 cases of leukoplakia, whereas all normal mucosa samples were negative. Difference in frequency was not satistically significant.

OLP and leukoplakia showed different expression patterns. In all positive lichen samples, CAIX was expressed in the basal layers of the epithelium A weaker reaction in superficial layers was also seen in 4 cases, whereas the staining was intense in the superficial and middle layers of 10 leukoplakia cases and only 4 cases were positive in the basal layers, In addition, CAIX was expressed mainly on the cell membrane (14 out to 16 cases on the membrane only) in lichen planus, but was observed on the membrane (5 cases), in the cytoplasm (5 cases), or both localizations (2 cases) in leukoplakia (Fig. 1).

Statistical analysis of results showed significant differences between leukoplakia and lichen when comparing localization of staining on the membrane or in the cytoplasm ($p < 0.05$), and in the layers expressing CAIX ($p < 0.02$).

The presence or absence of CAIX expression and the slight differences in staining intensity were not associated with type of OLP or with the presence or absence of dysplasia in leukoplakia.

DISCUSSION

The importance of studies on CAIX expression in malignant tumors lies mainly in its usefulness as a marker of hypoxia, which in turn influences tumor response to radio and chemotherapy[1]. Overexpression of CAIX has also been associated with more aggressive tumor behavior, irrespective of type of treatment[15]. Hence, it has been posited that determination of CAIX could be used as a marker of malignancy.

The availability of a tumor malignancy marker prompts investigating in which stage of malignant transformation induction of the gene encoding for expression of the marker occurs, with the aim to obtain a marker of early cancerization prior to neoplastic development. In this regard, increased CAIX activity has been demonstrated in premalignant lung lesions[19], breast ductal hyperplasia[20], and skin premalignant lesions[22]. Increased CAIX activity has also been detected in dysplastic epithelium adjacent to oral carcinomas[23] and dysplastic epithelium in oral leukoplakia[26].

Yang et al [27] demonstrated the presence of CAIX in plasma of oral cancer patients, and found increased expression of the enzyme and its mRNA not only in tumor stroma fibroblasts but also in fibroblasts of oral submucous fibrosis associated with areca nut chewing, a potentially malignant disorder.

However, a given molecular change cannot be considered in itself indicative of malignant transformation simply because that same change is more marked in malignant tumors, but rather a number of factors must come together for the risk of malignant transformation to increase. Oral leukoplakia carries an increased risk of cancer development. According to the literature, the annual malignant transformation rate of leukoplakia ranges from 2 to 3%[28], whereas the potentially malignant character of OLP is still under discussion, and most authors estimate the annual malignant transformation rate of OLP to be less than 0.7%[29]. The increased expression of CAIX observed in oral lichen and leukoplakia in the present work is therefore an indicator of an interesting biological behavior of these lesions, rather than a marker of increased risk of malignant transformation.

Although OLP and leukoplakia are recognized as two distinct pathological entities, some clinical and

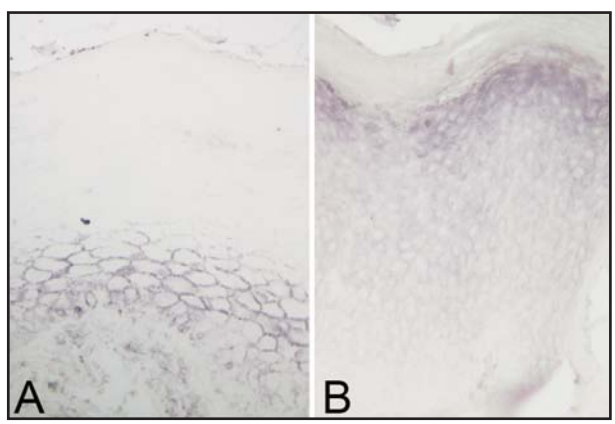

Fig. 1: CAIX immunohistochemical reaction. X 400.
A: Biopsy of oral lichen planus. Staining is more intense in cell membranes of the basal and suprabasal layers of the epithelium. B: Biopsy of leukoplakia. Staining is more intense in the superficial layers, and becomes fainter towards the suprabasal and basal layers of the epithelium.

histopathological features may pose differential diagnosis. In addition, the etiopathogenesis of both lesions was not yet fully clarified. Both entities undergo hypoxic stress. In fact, Pérez-Sayans et al[26] detected CAIX expression in oral leukoplakia; the authors associated their finding with the premalignant nature of the lesions, since they observed higher expression in lesions with dysplasia. Ding et al[30], found that expression of HIF1-α was higher in RNA isolated from lichen planus than in controls. HIF1-α is the main gene activated by hypoxia, and regulates transcription of a number of genes, including CAIX.

An interesting aspect is the markedly different expression pattern of CAIX in the entities studied here. CAIX expression was most prominent in the basal layers of the epithelium, and decreased towards the surface layers in lichen planus, whereas in leukoplakia, staining intensity increased from the basal cells towards the granular layer, where expression was strongest. It is known that OLP is an immune process mediated by T lymphocytes that are present in dense subepithelial infiltrates and cause alteration of the basal keratinocytes[31]. Hypoxia in the basal layers of the epithelium, as shown by higher intensity of CAIX expression, might mediate T lymphocyte recruitment and contribute to the onset of the disease. Leukoplakia is mainly triggered by external agents, such as smoking or alcohol consumption, which lead to an acanthotic response of the epithelium. This response, in turn, separates the epithelial cells from the blood supply, inducing higher CAIX expression in the superficial layers.

CONCLUSION

The different expression patterns of CAIX in oral lichen and leukoplakia provides further evidence of the differences in the biological behavior of both entities, and may be a contribution to the study of their pathogenic mechanisms.

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