J Oral Pathol Med (2004) 33: 269–73

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Cyclic AMP phosphodiesterase activity in human gingival carcinoma

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BACKGROUND: The phosphodiesterases (PDEs) are responsible for the hydrolysis of the second messengers, cyclic AMP (cAMP) and cyclic GMP (cGMP), to their corresponding monophosphates with a fundamental role in the transduction of the intracellular signals. At least 11 different enzymatic isoforms have been identified, which are listed according to their specificity or affinity for the substratum, identity of the amino acid sequence, cofactor, and inhibitor sensitivity. Variations in PDE activity have been found in different pathologies, and they have also been correlated to different pathological e/o physiological mechanisms, such as cellular differentiation, apoptosis, and tumor invasivity.

OBJECTIVES: In this study, we have evaluated cAMP PDE activity in patients with carcinoma of the gingiva, with the purpose of correlating differences in its development and progression. The same enzymatic activity has been used to evaluate differences between patients with lymph node involvement (group N^+), and patients without lymph node involvement (N^-).

MATERIALS AND METHODS: The analysis of PDE activity and the cAMP assay was performed by reverse-phase HPLC on samples of fresh or frozen gingival tissues. Analysis of cAMP was confirmed with the enzyme-linked immunoabsorption assay (EIA).

RESULTS AND CONCLUSIONS: The differences between control and N^- groups ($P = 0.0433$), and between control and N^+ groups ($P = 0.0156$) were statistically significant. PDE3A was also evaluated immunohistochemically in lymph-node negative and lymph-node positive cases. The differences between the two groups were statistically significant ($P = 0.0397$).

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Keywords: cAMP; oral squamous cell carcinoma; phosphodiesterase

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Accepted for publication October 2, 2003

Introduction

Cyclic nucleotide phosphodiesterases (PDEs), which hydrolyze the intracellular second messengers cyclic AMP (cAMP) and cyclic GMP (cGMP) to their corresponding monophosphates, play an important role in signal transduction by regulating the intracellular concentration of cyclic nucleotides. At least 11 families of mammalian PDEs have been defined based on sequence similarity, substrate specificity, affinity, and sensitivity to cofactors and inhibitory drugs (1). Within families, there are multiple isoenzymes and multiple splice variants of those isoenzymes. cAMP is hydrolyzed specifically by the cGMP-activated PDE of type 2, by PDE3, a cGMP-inhibited isoenzyme, and by the fourth family of PDEs. The existence of multiple PDE families, isoenzymes, and splice variants presents an opportunity for complex regulation of cyclic nucleotide levels. Moreover, current studies have shown that PDE activities are regulated by multiple inputs from other signaling systems and that they may be key factors in the cellular compartmentalization of cyclic nucleotides (2–4). Important evidences exist of PDE involvement in many physiological functions, as well as in different human pathologies (5–7). Cyclic nucleotides are involved in different cellular events, such as differentiation and apoptosis (8, 9).

Cancer is characterized by abnormal cell growth, not regulated by outside signals, and by the capacity to invade tissues, and metastasize and colonize distant sites. Carcinoma invasion is a complex process that involves directed migration and localized proteolysis. Although the mechanistic basis of invasion had been elusive, recent advances in molecular cell biology have facilitated a much more rigorous analysis of this important and critical component of cancer progression. The process of invading and metastasizing is influenced by signal transduction pathways activated by paracrine and autocrine factors.

The role of cyclic nucleotides in neoplastic growth, carcinoma invasion and malignancy, and in cellular differentiation has not been clearly defined. However, their evident importance as second messengers, together with different examples of relevant changes in PDE expression in different neoplastic tissues and cellular lines (10–12), suggest a role for them in the different phases. PDE activity

is essential for lamellae formation, chemotactic migration, and invasion in MDA-MB-435 breast carcinoma cells originally derived from a pleural effusion (13); cAMP PDE hydrolysis is involved in Tamoxifen activity, a non-steroidal anti-estrogen drug widely used in the treatment of breast cancer (14), and different experimental anti-tumor drugs are specific PDE inhibitors (15).

Human oral squamous cell carcinoma (OSCC) is a very aggressive form of tumor with a high prevalence of lymph node metastases. It can be divided into the following classes: well-differentiated, moderately well-differentiated, and poorly differentiated. Recently, the various degrees of differentiation have been associated with a decreased expression of apoptosis regulatory gene *bcl-2* and with increased apoptosis. Patients with poorly differentiated tumors are believed to have a worse prognosis than those with well-differentiated ones.

The aim of this study was to evaluate cAMP PDE activity in normal and neoplastic tissues in order to correlate its activity with grade of differentiation, and presence or absence of lymph node metastases.

We evaluated the enzymatic activity in 43 gingiva specimens; 21 specimens were with OSCC, while 22 were normal gingival tissues. We tried to assign to each cAMP-specific PDE family the right entity of activity expression, evaluating enzymatic hydrolysis in the presence of cGMP, which specifically activates PDE2, inhibits PDE3, and does not influence PDE4.

Materials and methods

Samples

Twenty-one cases of human OSCC of the gingiva were used in the present study. Ten patients presented negative lymph nodes (N^-), while in 11 patients, the lymph nodes were positive (N^+). As control tissues, 22 specimens removed during third molar extractions were used.

Partial purification of PDEs

The tissue samples were weighed and homogenized with 10 mM K_1K_2 phosphate buffer (pH 7.00) in a ratio 1 : 10 (w/v) utilizing a potter Model T25 basic (Ikalabortechnik, Germany). The homogenate was treated three times in ultrasonic bath model Sonic 18–35 (Simply, Italy) for 5 s at 25°C. The homogenate was centrifuged for 30 s at 108 ***g*** in a Mikro 22R (Hettich Zentrifugen, Germany). Supernatant was used for PDE assay and protein determination.

cAMP PDE assay

The enzymatic reaction was carried out using the method of Spoto et al. (16, 17) with minor modifications: 0.1 M Tris-HCl buffer (pH 8.3), 10 mM $MgCl_2$, 0.1 M KCl at 37°C. The reaction was initiated by the addition of 44 μM of cAMP. To evaluate PDE2 and PDE3 activities, 6 μM of cGMP was added. Control experiments were performed using a commercial preparation (Sigma), where the enzyme concentration was 0.4 μM. The time-course of reaction was 60 min. The reaction was terminated by adding HCl to final 0.01 M and transferring the tubes with the reaction mixture in a boiling water bath for 5 min. The sample was then centrifuged for 10 min at 16 000 ***g***, and the supernatant was filtered through a nylon-66 filter (0.2 μm; Rainin Corporation). The clear filtrate obtained was used directly for HPLC assay or stored at −80°C.

Analysis of cAMP by reverse-phase HPLC

The HPLC system was from Shimadzu and consisted of a LC-9 A pump, a variable wavelength spectrophotometer SPD-10AV vp measuring at 254 nm, an auto injector SIL-10 A xl, and a system controller SCL-10A. The column used was a 5-μm Li-Chrospher 100 CH 18/2 Merck (250 mm × 4 mm). The mobile phase employed for the separation of nucleotides consisted of 200 mM ammonium acetate (pH 6.0) with 1.5% acetonitrile (v/v). The flow rate was 1 ml/min; the detection was performed at 254 nm. Peak identities were confirmed by co-elution with standards. Quantitative measurements were carried out by comparison using standard solutions of known concentrations. Analysis was confirmed with the cAMP enzyme-linked immunoabsorption assay (cAMP EIA kit; Biomol, USA) following the manufacturer's recommendation, using non-acetylated cyclic nucleotides as a standard and acetylcholine esterase-linked cAMP as a competitor. Tissue samples should be frozen in liquid nitrogen. The tissues were ground to a fine powder under liquid nitrogen in a stainless steel mortar. After the nitrogen evaporated, the frozen tissue and the homogenate were weighed in 10 volumes of 0.1 M HCl. It was centrifuged at 600 ***g*** at room temperature. The samples were diluted in the 0.1 M HCl provided for the assay.

Analysis of PDE activity by reverse-phase HPLC

The analysis was performed as described by Spoto et al. (16, 17). In some experiments, the presence in the homogenate of oxidase or/and phosphatase produced products of degradation of the nucleotide-monophosphates. From cAMP PDE were obtained adenosine-monophosphate and inosine with a quantity equivalent to the decrease of cyclic AMP (18).

PDE3A rabbit polyclonal antibody

Immunostaining for PDE3A was performed using the alkaline phosphatase–anti-alkaline phosphatase method (APAAP) with a rabbit polyclonal antibody (Santa Cruz, CA, USA). Sections of 4 microns were cut and mounted on poly L-lysine-coated slides. Paraffin sections were de-waxed by xylene, re-hydrated, and at the end, washed in Tris-buffer (pH 7.6) for 10 min. PDE3A required proteinase-K predigestion in a working solution of 0.4 mg/ml (Dako) for 10 min at room temperature. The following steps were optimized by automatic staining (Dako, TechMate 500, Glostrup, Denmark). Sections were incubated with primary antibody solution for PDE3A at a dilution of 1 : 400 for 25 min at room temperature. Slides were rinsed in buffer (Buffer Kit, Dako) and immunoreaction was completed with the APAAP kit (Dako).

Protein content

Protein content was determined using a bicinchoninic acid protein determination kit from Sigma with bovine serum albumin as a standard.

Data processing

Fisher's protected least significant difference (PLSD), Scheffe, Bonferroni/Dunn were used to evaluate the

presence of statistically significant differences in the HPLC experimental data. The logistic regression-dependent variable was used to evaluate the immunohistochemical results.

Results

The mean value of HPLC PDE activity in the controls was of 25.9 nmol/mg of proteins ± 3.42 SD. Immunohistochemistry showed that the control specimens were slightly PDE3A-positive, predominantly at the superficial layers of epithelium (Fig. 1).

The HPLC enzymatic activity for the group of N^- specimens was of 40.56 nmol/mg of proteins ± 1.71 SD. In the N^- specimens, in 4 of 10 cases (40.0%) PDE3A positivity (Fig. 2) was less than 60%; in four cases (40.0%) the positivity was higher than 50%, and two cases (20.0%) were negative (Fig. 3).

The mean value in the N^+ group was 43.08 nmol/mg of proteins ± 8.46 SD. In the N^+ specimens, in six cases (54.5%) the PDE3A positivity (Figs. 4 and 5) was less than 50%; in two cases (18.2%), the positivity was higher than 50%, and three cases (27.3%) were negative. Statistically significant differences were found between the activity of cAMP PDE in the control specimens and in N^+ patients ($P = 0.0156$), and in control specimens and in patients with negative lymph nodes ($P = 0.0433$). The specific activity was also evaluated of PDE2 and PDE3, respectively, stimulated and inhibited by cGMP. The analysis of the PDE2 has

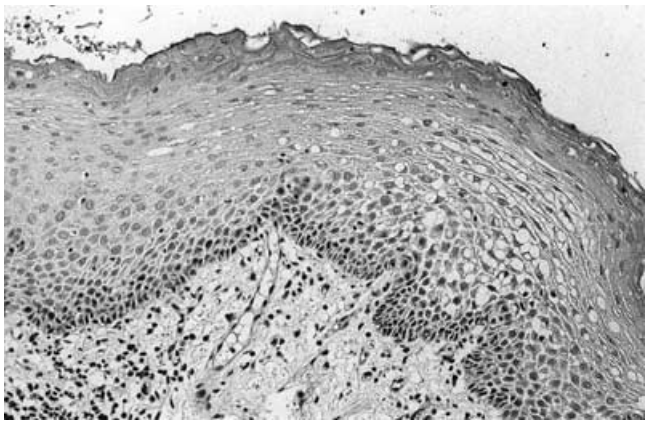

Figure 1 Control mucosa with a slight positivity of the superficial layers of the epithelium PDE3A 160×.

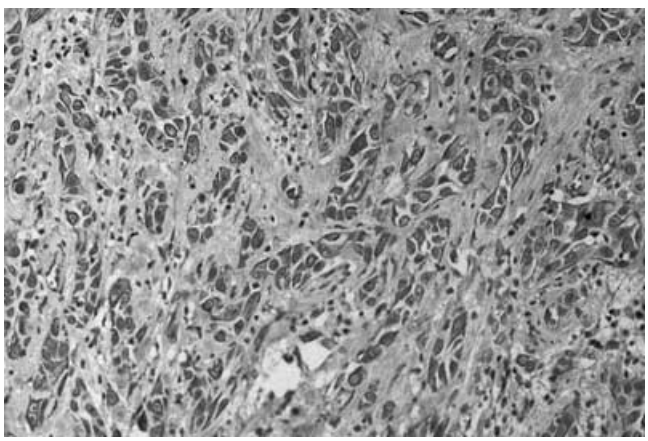

Figure 2 Squamous cell carcinoma (N^-). Positivity in less than 60% of the neoplastic cells PDE3A 200×.

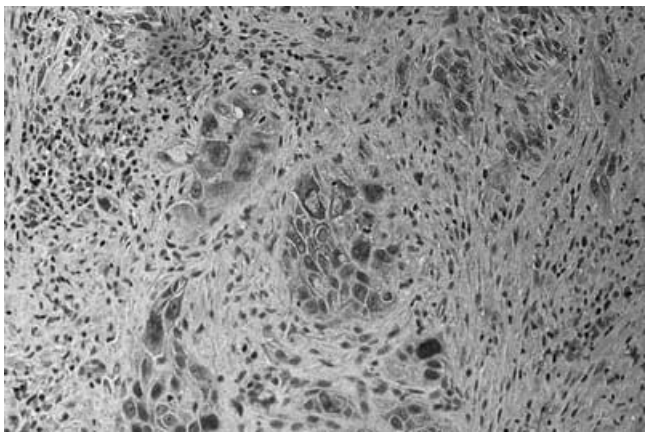

Figure 3 Poorly differentiated squamous cell carcinoma (N^-). Focal positivity (less than 10%) of the neoplastic cells PDE3A 200×.

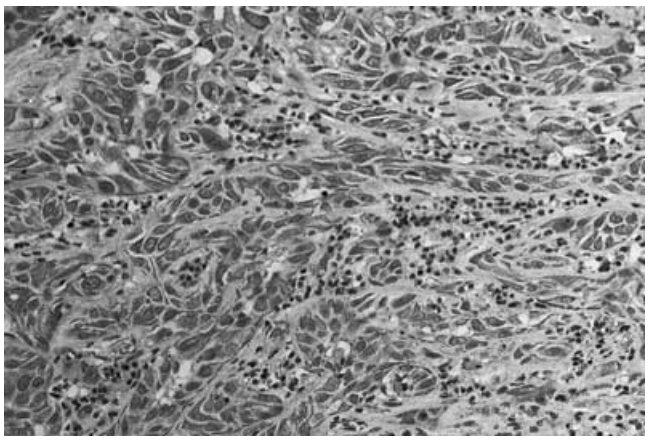

Figure 4 Squamous cell carcinoma (N^+) with positivity of about 40% of the neoplastic cells PDE3A 200×.

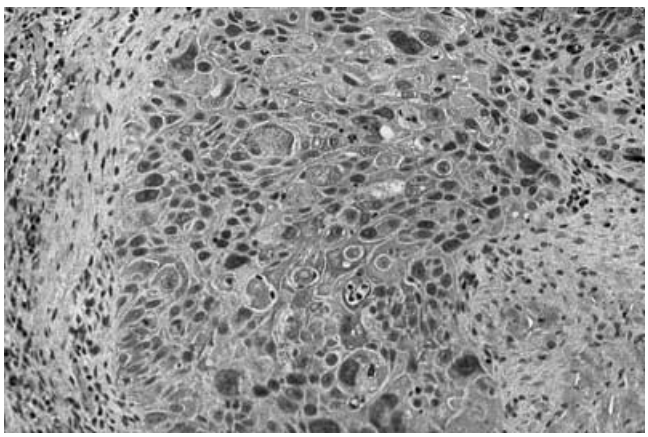

Figure 5 Areas of poorly differentiated squamous cell carcinoma (N^+) with a positivity of the neoplastic cells in about 30% PDE3A 200×.

shown a positivity only in 50% in controls and in the N^- and N^+, and such values were not statistically significant.

The PDE3 analysis has shown a positivity in 30% in controls and N^+, while in 12% of the N^-, such values were not statistically significant. It seems particularly interesting to observe that the content of the cyclic adenylate was always absent in presence of the tumor and in the controls, or however, inferior to 0.2 nmol/mg, which is the instrumental limit of analysis.

Discussion

Cyclic AMP is an essential second messenger for cellular signal transduction, generated by a protein-linked receptor. Together with cGMP, it is involved in many physiological processes. The intracellular concentrations of both these cyclic nucleotides are always correlated to the tissues in need of them. This means that their metabolism is finely regulated by a complex system of enzymes involving different families of PDEs.

Particularly, cAMP is hydrolyzed quite entirely by PDE4 and, with minor rate and less specificity, by PDE1, PDE2, PDE3, PDE10, and PDE11. cGMP acts as a competitive inhibitor for the hydrolysis of cAMP by PDE3. Moreover, the hydrolysis of cAMP by PDE1 results to be a calmodulin-stimulated activity. Also PDE7 and PDE8 are specific for the cAMP, but they have been isolated mainly in the skeletal muscles, in the heart, and in the T cells; the first one in the testicles and the second one in the eyes. The PDE10 and PDE11 are present in other tissues (19).

The aim of this study was to evaluate differences in cAMP PDE activities in normal or OSCC specimens in order to find correlations, if any, with its degree of differentiation and presence of lymph node metastases.

We were able to find cyclic AMP PDE activity in all the specimens analyzed in the present work, both in the cases of control and OSCC specimens.

We observed a large increase of PDE activity expression in OSCC specimens when compared to control tissues, and these values were statistically significant. These data are different from those reported by Curtis-Prior et al. (20) where the enzyme activities were three to five times greater in normal than in carcinomatous lungs. The cAMP PDEs have been reported to be decreased in solid tumors (21).

Moreover, the enzymatic activity in N^+ patients was found to be higher than that found in N^- patients. These results show that this enzyme, identifiable with the PDE2, PDE3, and PDE4, probably plays an important role in the progression of OSCC.

The statistically significant differences in PDE expression between non-neoplastic tissues and those affected by OSCC in N^- patients (mean difference = 14.65 nmol/mg of proteins; P-value = 0.0433) represent an evidence of involvement of PDE4 in the initial neoplastic transformation of tissues, probably connected to a down-regulation of those mechanisms, which are usually finely controlled by cAMP and its metabolites. The great change, also statistically significant, in PDE activity, which follows to a second stage of development of this type of cancer, clearly indicates a stimulating response of PDE4 and PDE3 to the pathological changes; this response induces an over-expression which redraws PDE activity to basal similar values.

No determination of the cyclic adenilic nucleotide and the contemporary presence of PDE3 and PDE4 can mean that the cAMP is under the limit of experimental dosing. Pazouki et al. (22) showed a significant increase in vascularity during the transition from normal tissue to dysplasia, and to early and late carcinomas. This fact could suggest that angiogenesis may be an early step in oral tumor progression (22) and has the ability to increase the permeability of the microvasculature to circulating macromolecules (23).

In carcinomas of the gingiva, we have observed an increase of cAMP PDE activity expression (40.56 nmol/mg of protein $\pm$ 1.71 SD) as compared to non-neoplastic tissues (25.91 nmol/mg of protein $\pm$ 3.42 SD). On the other hand, the enzymatic activity in N^+ patients was higher (43.08 nmol/mg of protein $\pm$ 8.46 SD) than in N^- patients.

In a previous paper from our laboratory (24), which correlated the cGMP PDEs with OSCC, we found that the values in the control and N^+ specimens, which were very similar, were three times higher than those of the N^- group. While comparing the mean values of the cGMP PDE with those of the cAMP PDE referred to the same groups, we noticed that the activity cGMP-PDE was more than 25% in comparison to that cAMP PDE in the controls. As far as it concerns the N^- group, there is an inversion of tendency that shows the value of activity cAMP PDE more than three times higher in comparison to the cGMP PDE and 18% in the N^+ group. Another statistically significant difference was the presence of endogenous cGMP is signalled (in the N^+ and N^- about double values in comparison with those found in the controls). Endogenous cAMP was not present or however inferior to 0.2 nmol/mg of proteins.

Conclusion

These results show that these enzymes most probably play a role in the progression of OSCC.

Cyclic AMP inhibits proliferation in several cell lines. cAMP delays the G_1/S transition in human vascular smooth muscle cells. In addition, exisulind, which inhibits cAMP-specific PDE, induces apoptosis in human colon cancer cells *in vitro*. Furthermore, recent studies suggest that uroguanylin induces apoptosis in T84 and Caco2 human colon cancer cells. Thus, some cell systems, including human intestine, adenylate cyclases, and cAMP, may regulate cell proliferation and/or apoptosis.

The present study examined the role of PDE2, PDE3, PDE4, and cAMP in regulating proliferation in OSCC. The precise mechanisms by which PDE2, PDE3, PDE4, and cAMP delay progression of OSCC through the cell cycle remain incompletely defined.

In conclusion, agonist activation of adenylate cyclase C (AC-C) regulates the proliferation of OSCC by slowing progression through the cell cycle without inducing cell death. These data suggest that PDE3, PDE4, and cAMP may play a role in regulating the transition between gingival cell proliferation and their differentiation. Additionally, they support the suggestion that AC-C agonists may represent novel cytostatic agents for the prevention and treatment of OSCC.

The results of the present study show a significant variations of activity of the cAMP PDE, mainly related to the second, third, and fourth enzymatic family, in the development and progression of OSCC of the gingiva. A more profound knowledge of the role of such enzyme and his substratum, the cAMP, could open the road to new treatments or diagnostic techniques.

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Acknowledgements

We thank Alessandro Contento for the purification, HPLC analysis of cAMP, and PDEs.