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Immunoreactivity of proliferating cell nuclear antigen in salivary gland tumours: an assessment of growth potential

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Abstract. Immunoreactivity of proliferating cell nuclear antigen (PCNA) was assessed to evaluate growth potential in surgically resected tissue specimens from 70 cases of benign and malignant salivary gland tumours. Three stage streptavidin-biotin immunoperoxidase immunostaining using monoclonal antibody to PCNA showed a heterogeneity of PCNA index and distribution. In normal salivary gland specimens, PCNA was demonstrated in the nuclei of few ductal and acinar cells. In pleomorphic adenoma a multiple nodular growth pattern was observed with positive immunoreactivity restricted to the nuclei of tubulo-ductal structures. Warthin's tumour had positive nuclei in the outer cuboidal cells of epithelial component and germinal centres of lymphoid tissue. Myoepithelioma and acinic cell carcinoma showed slightly differing values and a statistically significant difference in the value of the index was observed in tumour cell aggregates of the cribiform type of adenoid cystic carcinoma and the solid undifferentiated type and between low/intermediate and high-grade mucoepidermoid tumours. PCNA is a useful marker of tumour cell proliferation; the index correlates with the grade of malignancy in salivary gland tumours.

Key words: Proliferating cell nuclear antigen – Salivary gland – Immunoreactivity

Introduction

Proliferating cell nuclear antigen (PCNA), an auxiliary protein for DNA polymerase delta, plays an important role in the initiation of cell proliferation (Bravo et al. 1987; Wong et al. 1987; Jaskulski et al. 1988). The expression of this protein is correlated with the S-phase of the cell cycle (Kurki et al. 1986; Morris and Mathews 1989) and immunohistochemical detection of the protein has been found to be a useful marker for the proliferating fraction of cells in tissue. Significant correlations have been found between a semiquantitative scoring system for the PCNA index and parallel S-phase analysis by flow cytometry, titrated thymidine incorporation and uptake of bromodeoxyuridine (BrdU) (Garcia et al. 1989; Battersby and Anderson 1990; Dierendock et al. 1991).

PCNA immunoreactivity demonstrates the actively dividing cell in S-phase in conventionally fixed and paraffin-embedded tissue. The proliferation pattern in intact histopathological structures can be worked out (Garcia et al. 1989; Hall et al. 1990) and we have demonstrated consistent immunoreactivity in tissue specimens fixed in 10% formalin for 6–48 h and embedded in paraffin when various fixatives and both frozen and paraffin sections are compared (Tsuji et al. 1992). We now demonstrate the use of the monoclonal antibody PC10 (Dakopatts, Denmark) in the immunohistochemical analysis of the growth pattern of salivary gland tumours and compare the cell kinetic information with the grades of malignancy.

Materials and methods

Seventy cases of salivary gland tumours (20 cases of pleomorphic adenoma, 7 of myoepithelioma, 10 of Warthin's tumour, 6 of acinic cell carcinoma, 17 of adenoid cystic carcinoma and 10 of mucoepidermoid carcinoma) were selected for this study. The tumour tissues obtained surgically were fixed in 10% formalin solution for 6–12 h, dehydrated and embedded in paraffin. Sections 4 μm thick were used for routine histopathological evaluation by haematoxylin and eosin staining and for immunohistochemical studies. The histopathological diagnosis and grading of the tumours were evaluated independently by all of us and include typical cases diagnosed and graded according to the WHO international histological classification of salivary gland tumours (Seifert et al. 1990).

For immunohistochemical study the sections were deparaffinized and dehydrated in a series of graded alcohol and treated with methanol containing 0.05% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were then treated

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with normal rabbit serum (1:20) for 30 min to block nonspecific background staining. This was followed by incubation with monoclonal mouse anti-PCNA PC10 (Dakopatts, Denmark) at a dilution of 1:20 for 1 h at room temperature. After washing 3 times for 5 min each in phosphate buffered saline, the second antibody, biotinylized rabbit IgG to mouse IgG at a dilution of 1:200 was used, followed by the avidin-biotin complex (ABC) immunoperoxidase reaction (Dakopatts; dilution 1:100 for 30 min). The peroxidase reactivity was visualized by diaminobenzidine hydrochloride containing 0.05% hydrogen peroxidase.

The extent of PCNA positivity was evaluated by determining the positively stained nuclei present in at least 1000 tumour cells in the active areas of the specimen using a Nikon microscope (20 objective and ×10 eye piece). The significance of PCNA positivity and tumour grades was assessed using Student's *t*-test and the results were considered significant when $p < 0.05$.

Results

In normal salivary glands ($n = 5$), the mean number of PCNA-positive nuclei was 1.1 with a standard deviation (SD) of 0.2 which were confined to the ductal system and acinar cells. Myoepithelial cells were negative in all cases.

The mean and SD of positive nuclei in various tumours are summarized in Table 1. In Warthin's tumour positive nuclear staining of varying intensity was seen in both the epithelial and the lymphoid cell component of the tumour. However, the positive nuclei were localized in the basal cuboidal cells of the tumour epithelium and in the germinal centres of the lymphoid component (Fig. 1 A, B). In monomorphic adenoma PCNA-positive nuclei were scattered in tumour cell aggregates (Fig. 1 C, D).

In pleomorphic adenoma and myoepithelioma heterogeneity of PCNA-positive nuclei was found in the tumour specimens. A positive nuclear staining was generally located focally in the epithelial sheets and luminal and outer cells of tubulo-ductal structures but the number of positive nuclei varied greatly throughout the tumour specimen. The cells in the myxoid and chondroid areas were negative. Myoepithelioma had spindle-shaped plasmacytoid and occasional clear cells with quite often a combination of both pattern of cells. Clear cells showed limited numbers of PCNA-positive nuclei in aggregates, but plasmacytoid cell nuclei were the most reactive with fine granular nuclear staining (Fig. 1 E, F).

In adenoid cystic carcinoma the pattern of positive nuclear immunoreactivity in the cribiform-tubular type of tumour was similar to that observed in pleomorphic adenoma. However, a statistically significant difference ($P < 0.001$) in PCNA index was found between the cribiform-tubular type and solid and undifferentiated adenoid cystic carcinoma. In acinic cell carcinoma few PCNA-positive nuclei were seen.

In low-grade mucoepidermoid carcinoma, epidermoid cells in the periphery of the tumour mass and the basal layer showed intense staining but the mucus-forming cells were negative (Fig. 2 A, B). In intermediate cell carcinoma, intermediate and epidermoid cells showed intense staining. However, in high-grade tumours, composed mainly of epidermoid and intermediate cells, PCNA immunoreactivity was distinct in most of the tumour cells (Fig. 2 C–F). A statistically significant difference in PCNA index was observed between high-grade and low-intermediate mucoepidermoid carcinomas ($P < 0.01$). A lymph node metastasis of high-grade carcinoma had a very high index in the metastatic cells.

Table 1. Percentage of proliferating cell nuclear antigen (PCNA)-positive cells in normal salivary gland and salivary gland tumours

	n	% PCNA positive nuclei Mean ± standard deviation
Normal salivary gland	5	1.1 ± 0.2
Warthin's tumour	10	4.9 ± 1.7
Pleomorphic adenoma	20	6.9 ± 3.4
Myoepithelioma	7	4.2 ± 3.3
Acinic cell carcinoma	6	5.9 ± 3.7
Adenoid cystic carcinoma:		
Cribiform-tubular type	15	8.9 ± 5.1*
Solid undifferentiated	2	24.9 ± 2.5*
Mucoepidermoid carcinoma:		
Low and intermediate grade	6	5.9 ± 3.7**
High grade	4	25.8 ± 9.7**

* $P < 0.001$; ** $P < 0.01$

Discussion

Immunohistochemical methods of assessing cell proliferation with their particular advantage of maintenance of cellular and tissue architecture have been used in a wide variety of pathological conditions. In an earlier report, Tsuji et al. (1992) have shown a statistically significant variation in PCNA index in well and moderately differentiated squamous cell carcinomas of the oral cavity but a wide variation in poorly differentiated tumours of the oral cavity, without significant effect on the clinical outcome of the treatment response. A high labelling index in premalignant lesions has been found to correlate with the degree of dysplasia in the oral mucosa and skin (Tsuji et al. 1992). Shrestha et al. (1992) have shown a significant correlation of PCNA index with c-*erb*B-2 oncoprotein and epithelial growth factor receptor status as well as poor survival and disease free survival in primary breast cancer patients. Similar studies have shown a correlation of PCNA index with clinical outcome in gastric carcinoma (Jain et al. 1991), gastrointestinal lymphoma (Woods et al. 1991), haemangiopericytoma (Yu et al. 1991) breast cancer (Aaltomaa et al. 1992), transitional cell bladder cancer (Lipponen and Eskelinen 1992) and prostatic carcinoma (Harper et al. 1992). However, others have found no significant outcome with the PCNA index in breast cancer (Leonardi et al. 1992) and glioblastoma (Figge et al. 1992). This is the first study of PCNA immunoreactivity in salivary gland tumours. The primary treatment in our patients was surgery, but there was no uniformity in post-surgical management and in many cases radiotherapy and/or chemotherapy were employed. The index we have determined was correlated with the degree of malignancy of the tumour

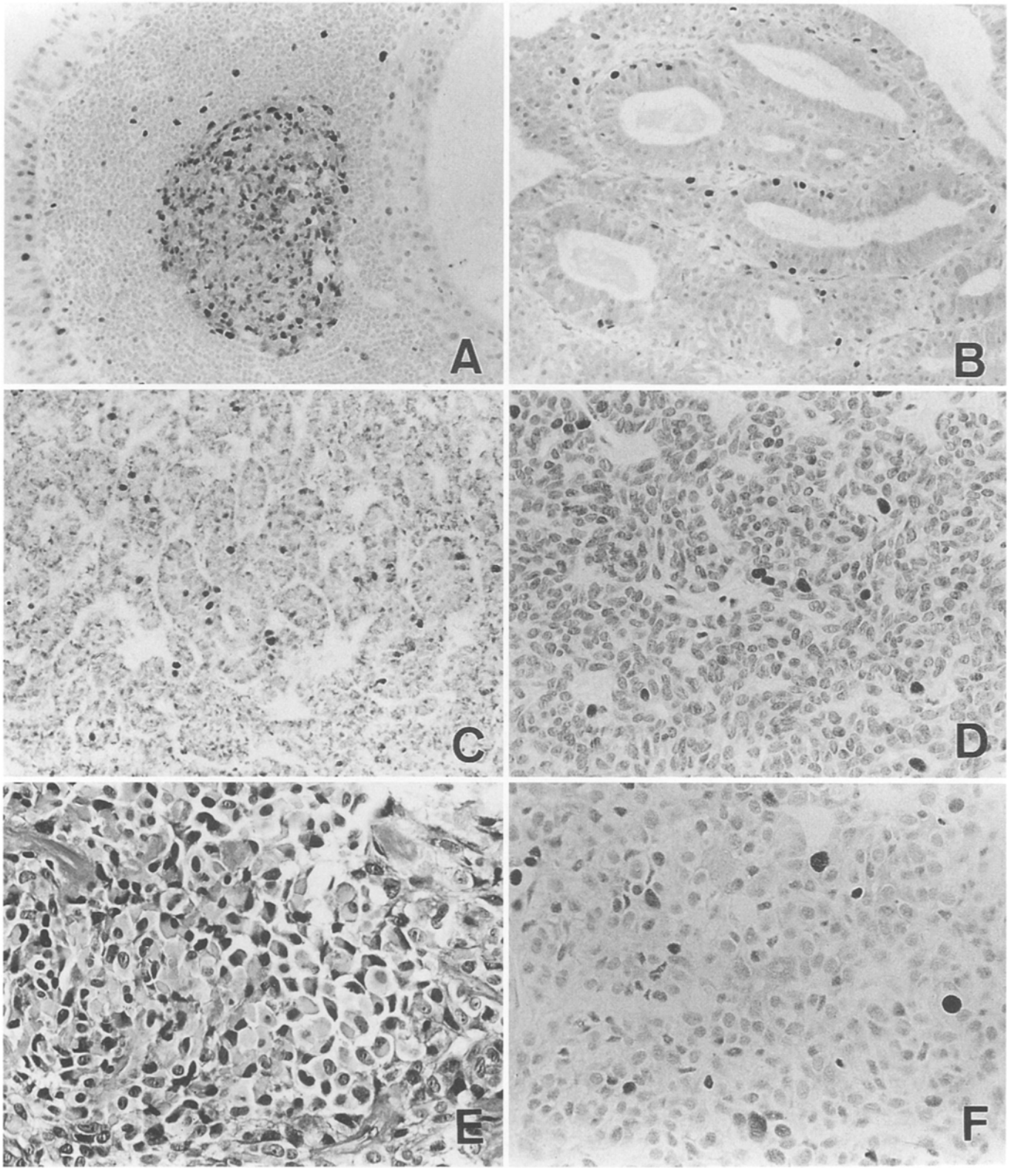

Fig. 1 A–F. A Lymphoid follicles in Warthin's tumour stain markedly and numerous cells in lymphoid tissue react strongly to proliferating cell nuclear antigen (PCNA). ×100. **B** Basal cell nuclei of Warthin's tumour epithelium label intensely with PCNA but the columnar apical cell nuclei are devoid of staining. ×100. **C** PCNA-positive nuclei are distributed in the basal cells of tubular structure in monomorphic adenoma. ×100. **D** Basal cell adenoma shows few cells containing PCNA-positive nuclei. ×200. **E** Histological appearance of plasmacytoid cells. H & E, ×200. **F** Few PCNA-positive nuclei are present in the plasmacytoid focus. ×200

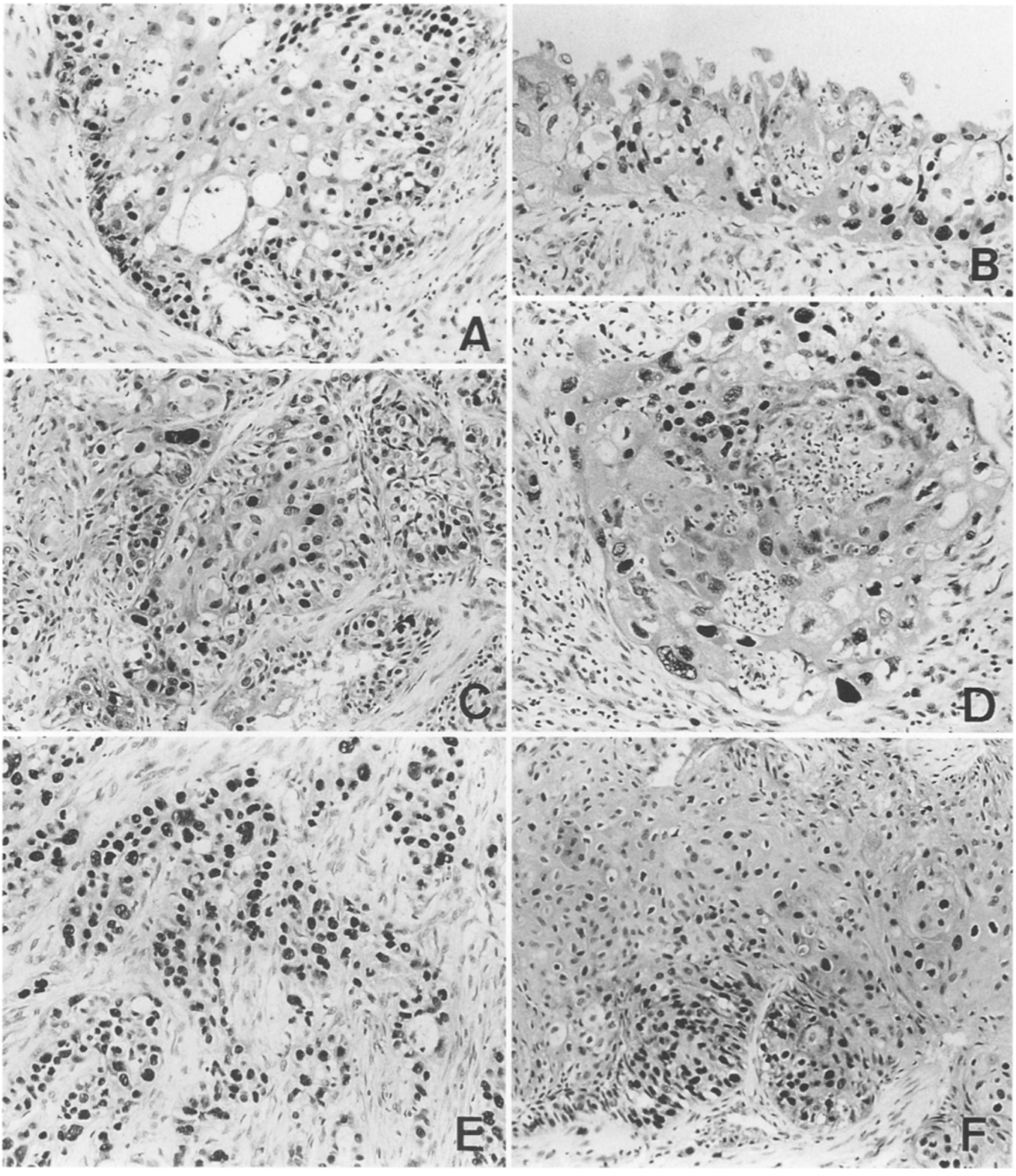

Fig. 2A–F. PCNA distribution in mucoepidermoid carcinoma, ×200. **A** Low-grade tumour mass; PCNA-positive cells are distributed in peripheral cells and mucus-forming cells have no reaction. **B** Low-grade tumour. Epidermoid cells in the basal layer are strongly positive. **C, D** Intermediate grade tumour. Malignant tumour cells show abundant reaction product of PCNA. **E** High-grade malignant tumour. Most of the tumour cell nuclei are markedly labelled. **F** High-grade tumour cells. The epidermoid tumour cells in the basal area have a high index of PCNA-positive nuclei

and its known biological behaviour as reported in breast cancer (Aaltomaa et al. 1992), haemangiopericytoma (Yu et al. 1991), gastrointestinal lymphoma (Woods et al. 1991), prostatic carcinoma (Harper et al. 1992; Visakorpi et al. 1992), non-small cell carcinoma (Theunissen et al. 1992), gastrointestinal stromal tumours (Yu et al. 1992) and acute leukaemia (Ito et al. 1992).

Benign salivary gland tumours are slow growing neoplasms. In this study, pleomorphic adenoma and myoepithelioma had a low labelling index, with positive nuclei confined to epithelial sheets and duct-like structures showing that the proliferating fractions are localized in those structures. A wide variation in the labelling pattern in the tumour cell aggregate of pleomorphic adenoma in different areas of the same tumour specimens, however, suggests multiple focal growth areas in the tumour. However, the myxoid, hyaline and chondroid areas had no active cell proliferation determined by PCNA and PCNA immunoreactivity in squamous metaplastic regions was similar to that in the premalignant dysplastic lesions of oral mucosa as reported by Tsuji et al. (1992). Warthin's tumour had actively dividing cells in the lymphoid follicles in addition to a proliferating fraction of cuboidal cells in the basal epithelial component. It is suggested that growth occurs in the basal layer of the epithelium and in the lymphoid cells, and that an interaction between the two cell groups is responsible for the tumour growth.

The labelling index of PCNA in acinic cell and adenoid cystic carcinoma was found to correspond with the usual slow growth of these tumours. However, the prognosis of adenoid cystic carcinoma varies according to the histopathological sub-type and recurrences and metastases are more frequent in the solid undifferentiated type than in the cribiform type. This behaviour in the former type is related to a high index of proliferating fraction found in the present study. Acinic cell carcinoma usually contains well-differentiated cells and these tumours usually have a better prognosis, with a 5-year survival rate reported as 85% (Seifert et al. 1990).

The PCNA index was also well correlated with the histological grade in mucoepidermoid carcinoma. Mucus-secreting cells, which are well differentiated in low and intermediate grade tumours, do not proliferate, whereas epidermoid and intermediate cells are the major proliferating component. However, in high-grade tumours both the mucus and epidermoid cells proliferate and show a high index, reaching more than 50%. We conclude that the proliferating fraction revealed by PCNA immunohistochemistry is related to the histological grade of the tumour. It may be helpful in providing objective support for the histopathological grading, especially in cases with borderline histology.

Cellular proliferation rates are not the only factor determining malignant behaviour in a tumour; the patterns of altered morphological differentiation are equally important. However, the PCNA index may be valuable as a prognostic indicator in salivary gland tumours, and on the basis of this study, it is concluded that PCNA immunoreactivity in salivary gland tumours may be an important adjunct to histologically based tumour classifications. It plays an important role in assessing prognosis and planning multimodal therapeutic approaches by distinguishing the less and more aggressive tumours. However, its prognostic value must be confirmed by follow-up studies.

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