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Proliferating cell nuclear antigen in malignant and pre-malignant lesions of epithelial origin in the oral cavity and the skin: an immunohistochemical study

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Summary. Proliferating cell nuclear antigen (PCNA) is a nuclear protein synthesized in the late G_1 and S phase of the cell cycle and immunohistochemical detection of the protein represents a useful marker for the proliferating fraction of cells in tissue specimens. A series of malignant and pre-malignant lesions of the oral cavity and skin were evaluated by the streptavidin biotin immunoperoxidase method for detection of this protein. Monoclonal anti-PCNA antibody (PC 10) labelled proliferating cells in all cases with varying intensity of nuclear staining. In squamous cell carcinoma ($n=48$), PCNA positivity correlated with the differentiation and atypia of the tumour cells; however, in poorly differentiated tumours, the relationship between PCNA expression and proliferation was lost. Basal cell carcinoma showed an increased growth fraction in tiny epithelial nests (mean 43.8, SD 6.0, $n=20$) than in neoplastic basal cells (mean 30.1, SD 6.9, $n=8$). The growth fractions were significantly higher in the pre-malignant lesions (leukoplakia, mean 22.3, SD 7.7, $n=14$; Bowen's disease, mean 45.2, SD 11.7, $n=12$; senile keratosis, mean 41.2, SD 7.0, $n=12$) than in the normal mucosa (mean 9.8, SD 4.9, $n=10$), suggesting that cellular growth fractions correlate with the degree of dysplasia in pre-malignant lesions.

Key words: Immunohistochemistry – Proliferating cell nuclear antigen – Cancer – Oral pre-cancer

Introduction

Proliferating cell nuclear antigen (PCNA) was originally detected by Miyachi et al. (1978) during experiments with serum from patients with systemic lupus erythematosus which reacted to nuclei of proliferating cells but failed to show reactions with resting cells in normal human tissues. It has been identified as a 36 kDa nuclear auxiliary protein required for leading strand DNA synthesis by polymerase delta (Bravo et al. 1987). The ability of PCNA-specific antibodies to inhibit inducible DNA synthesis in isolated quiescent nuclei (Wong et al. 1987), and PCNA antigen oligodeoxynucleotides to inhibit proliferation of BALB/c3T3 cells underscores the important role of this protein in DNA replication (Jaskulski et al. 1988). The human PCNA gene has a 261-amino acid polypeptide with a high content of asparate and glutamic acid (Almendral et al. 1987).

Immunological methods of assessing cell proliferation have the advantage of simultaneous analysis of histopathology and cell proliferation features. The distribution of PCNA in the cell cycle increases through G_1, peaks at the G_1/S phase interphase and decreases through G_2 phase reaching levels which are virtually undetectable in M phase and in quiescent cells using immunocytological methods. This makes it a useful marker for proliferating cells (Kurki et al. 1986, 1988; Morris and Mathews 1989). A monoclonal Ki-67 antibody that recognizes proliferation-associated proteins has been reported to be of value in assessing cell proliferation using immunohistochemical methods; however, it usually requires cryostat sections of snap-frozen materials or specially prepared histological material (Coltrera and Gown 1991; van Dierendonck et al. 1991). In this study we describe the application of a new monoclonal antibody PCNA PC10 (Dakopatts, Denmark) that recognizes PCNA in conventionally fixed and processed histological material to assess the index and pattern of cell proliferation and its correlation with the nuclear atypia and tumour grade. PCNA PC10 index was also evaluated for basal cell carcinoma, pre-malignant lesions and malignant tumours of epithelial origin.

Materials and methods

A total of 48 cases of oral squamous cell carcinoma, 20 cases of basal cell carcinoma of skin, 14 cases of oral leukoplakia, 12 cases

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of senile keratosis, 8 cases of Bowen's disease and 8 cases of condyloma acuminatum of skin were studied. The tissues of these lesions were fixed in 10% formalin solution for 12 h, dehydrated and embedded in paraffin. Serial sections were cut at 4 μm and the deparaffinized sections were stained with haematoxylin and eosin for routine histopathological examination and for PCNA immunohistochemistry.

The endogenous peroxidase was blocked by methanol containing 0.3% hydrogen peroxide for 30 min. The sections were incubated with normal rabbit serum at a dilution of 1:20 for 30 min for non-specific background blocking. This was followed by incubation with monoclonal mouse anti-PCNA, PCNA PC10 (Dakopatts) at 1:20 dilution for 30 min at room temperature. The second antibody was biotinylated affinity associated rabbit IgG to mouse IgG at a dilution of 1:200 (Dakopatts), in which the sections were incubated for 30 min at room temperature. Finally, the sections were treated with the streptavidin biotin complex (1:100, 30 min) from Dakopatts. Diaminobenzidine hydrochloride (0.02% 3-3'DAB)/0.05 M tris buffer solution (pH 7.6) containing 0.05% hydrogen peroxide was used as chromogen to visualize the peroxidase activity. Each step was followed by phosphate-buffered saline washing.

The optimal dilution of each antibody was determined by using serial dilution of each antibody on sections of normal oral mucosa. The dilution (1:20) at which the greatest amount of nuclear staining occurred with little or no background cytoplasmic staining was taken as optimal in this study.

The histological grade of the tumour was evaluated using standard histopathological criteria. Nuclear atypia was determined by counting the number of atypical nuclei in 20 high power fields without overlap in the most active area of the tumour section (40× objective, 10× eye piece). The extent of PCNA PC10 positivity was evaluated by determining the positively stained nuclei present in at least 1000 malignant cells in the most active area of the specimen by using a Nikon microscope (20× objective and 10× eye piece) connected to a computer-assisted image analyser (Joyce Loebl, Magiscan 2A, 1986) using a minimum threshold of nuclear staining intensity, so that all cells were counted as positive regardless of the staining intensity. The significance of PCNA positivity and tumour grades and atypia was assessed using student's t-test and results were considered significant when $P<0.05$.

Immunohistochemical staining with PCNA PC10 (monoclonal mouse anti-PCNA) was compared with immunostaining of Dako-Ki-67 (monoclonal mouse anti-human Ki-67 antigen) and silver staining for nucleolar organizer regions (AgNOR), using 4 cases of squamous cell carcinoma (2 gingiva and 2 tongue). The specimens were also tested for immunostaining using frozen and paraffin sections, with different fixatives (Bouin, 10% formalin, Carnoy, alcohol and acetone). Times of tissue fixation were divided into 1, 3, 6, 12, 24 and 48 h for Bouin, 10% formalin and Carnoy solutions, and into 10, 20, 60, 120 min for alcohol and acetone. The immunostaining technique for Dako-Ki-67 was the same as that for Dako-PCNA PC10; dilution of Dako-Ki-67 was 1:20 for 1 h at room temperature. For the AgNOR method, paraffin and post-fixed frozen sections were stained for 30 min at room temperatures in the solution consisting of 30 ml of A solution (1% gelatin, 2% formic acid) and 60 ml of B solution (50% silver nitrite).

Table 1. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA), Ki-67 and AgNOR staining in squamous cell carcinoma

Frozen sections							
Bouin							
(h)	0	1	3	6	12	24	48
PCNA	−	−	−	−	−	−	−
Ki-67	−	−	−	−	−	−	−
AgNOR	+	+	+	+	−	−	−
10% formalin							
(h)		1	3	6	12	24	48
PCNA		−	−	−	−	−	−
Ki-67		−	−	−	−	−	−
AgNOR		+	+	+	+	+	+
Carnoy							
(h)		1	3	6	12	24	48
PCNA		−	−	−	−	−	−
Ki-67		−	−	−	−	−	−
AgNOR		+	+	+	+	+	+
Acetone							
(min)		10	20	60	120		
PCNA		−	−	−	−		
Ki-67		−	+	+	+		
AgNOR		+	+	+	+		
Alcohol							
(min)		10	20	60	120		
PCNA		−	−	−	−		
Ki-67		−	−	−	−		
AgNOR		+	+	+	+		
Paraffin section							
Bouin							
(h)		1	3	6	12	24	48
PCNA		−	−	−	−	−	−
Ki-67		−	−	−	−	−	−
AgNOR		+	+	+	+	+	+
10% formalin							
(h)		1	3	6	12	24	48
PCNA		−	−	+	+	+	+
Ki-67		−	−	−	−	−	−
AgNOR		+	+	+	+	+	+
Carnoy							
(h)		1	3	6	12	24	48
PCNA		+	+	+	+	+	+
Ki-67		−	−	−	−	−	−
AgNOR		+	+	+	+	+	+

−, Negative staining; +, positive staining; (h), hours; (min), minutes

Results

In fresh frozen sections, all sections post-fixed with five kinds of fixatives (Bouin, 10% formalin, Carnoy, alcohol, acetone) were negative for PCNA staining in nuclei, whereas they were positive in almost all cases for AgNOR staining (Table 1). AgNOR staining after Bouin fixation showed negative results only after longer fixation times (12, 24, 48 h). Ki-67 staining was only positive in acetone-fixed sections (20, 60, 120 min).

In paraffin-embedded sections, 10% formalin and Carnoy-fixed tissue showed a positive reaction for PCNA PC10 and AgNOR was also positive in Bouin-fixed tissue. Ki-67 reactivity was negative in all cases. AgNOR staining was positive in nuclear materials in both malignant cells and normal epithelial cells except for keratinized cells.

Normal squamous epithelium of oral mucosa and skin ($n=10$) showed positive nuclear PCNA immunore-

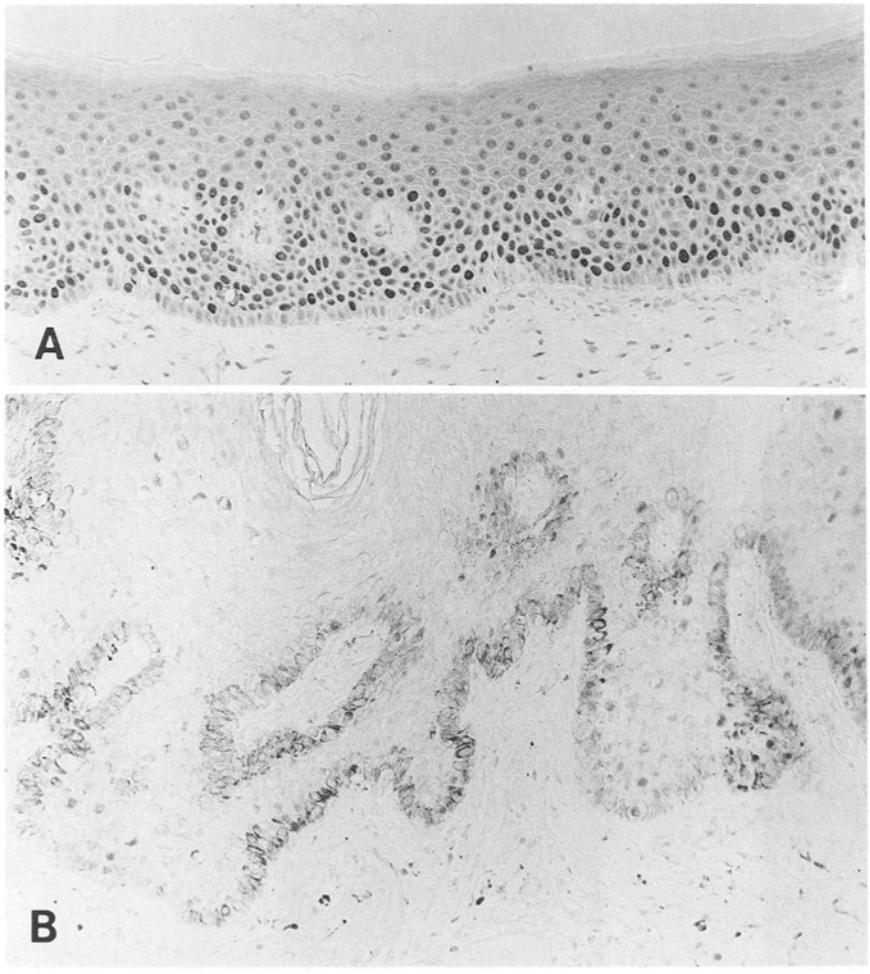

Fig. 1. A Leukoplakia: the PCNA-positive nuclei are seen in the basal and spinous layer. ×100. **B** Condyloma acuminatum: the positive nuclei are seen in the basal layer only. ×100

Table 2. Percentage of PCNA-positive cells in normal mucosa, basal cell carcinoma and pre-malignant lesions

	No.	% of PCNA-positive nuclei Mean (S.D.)*
Normal mucosa and skin	10	9.8 (4.9)
Basal cell carcinoma	20	30.7 (6.9)[a] 43.8 (6.0)[b]
Leukoplakia	14	22.3 (7.7)
Bowen's disease	12	42.2 (11.7)
Senile keratosis	12	41.2 (7.0)

* $P<0.001$
[a] positive cells in neoplastic basaloid cells
[b] positive cells in infiltrating tiny nests ($n=8$)

Table 3. Correlation of immunohistochemical staining for PCNA with tumour cell differentiation

Differentiation	No.	% PCNA-positive nuclei			%Total positive Mean (SD)
		<10	10–20	>20	
Well-differentiated	12	9	2	1	9.1 (5.7)*
Moderately differentiated	18	4	10	3	14.5 (5.3)*
Poorly differentiated	18	8	9	1	10.4 (6.3)

* $P<0.02$

activity in the basal cells with a mean of 9.8% of positive nuclei 9.8 (SD 4.9). All cases of malignant and pre-malignant lesions showed positive nuclear staining of varying intensity. The pattern of staining was usually granular throughout the nucleoplasm. No staining of mitotic figures was observed. Leukoplakia, with minimal dysplastic changes, showed positive nuclei mostly in the basal, lower spinous and a few in the upper spinous layer (Fig. 1 A). Condyloma acuminatum, on the other hand, showed positive nuclei only in the basal layer (Fig. 1 B). Senile keratosis and Bowen's disease showed a very high number of positive nuclei (Fig. 2). There was a significant difference between lesional tissue and normal epithelium ($P<0.001$), as shown in Table 2. The positive nuclei in basal cell carcinoma and well-differentiated squamous cell carcinoma were confined to the basal, parabasal, lower spinous layer and peripheral cells of the epithelial nests. They were found scattered in the tumour infiltrate with characteristic immunoreactivity

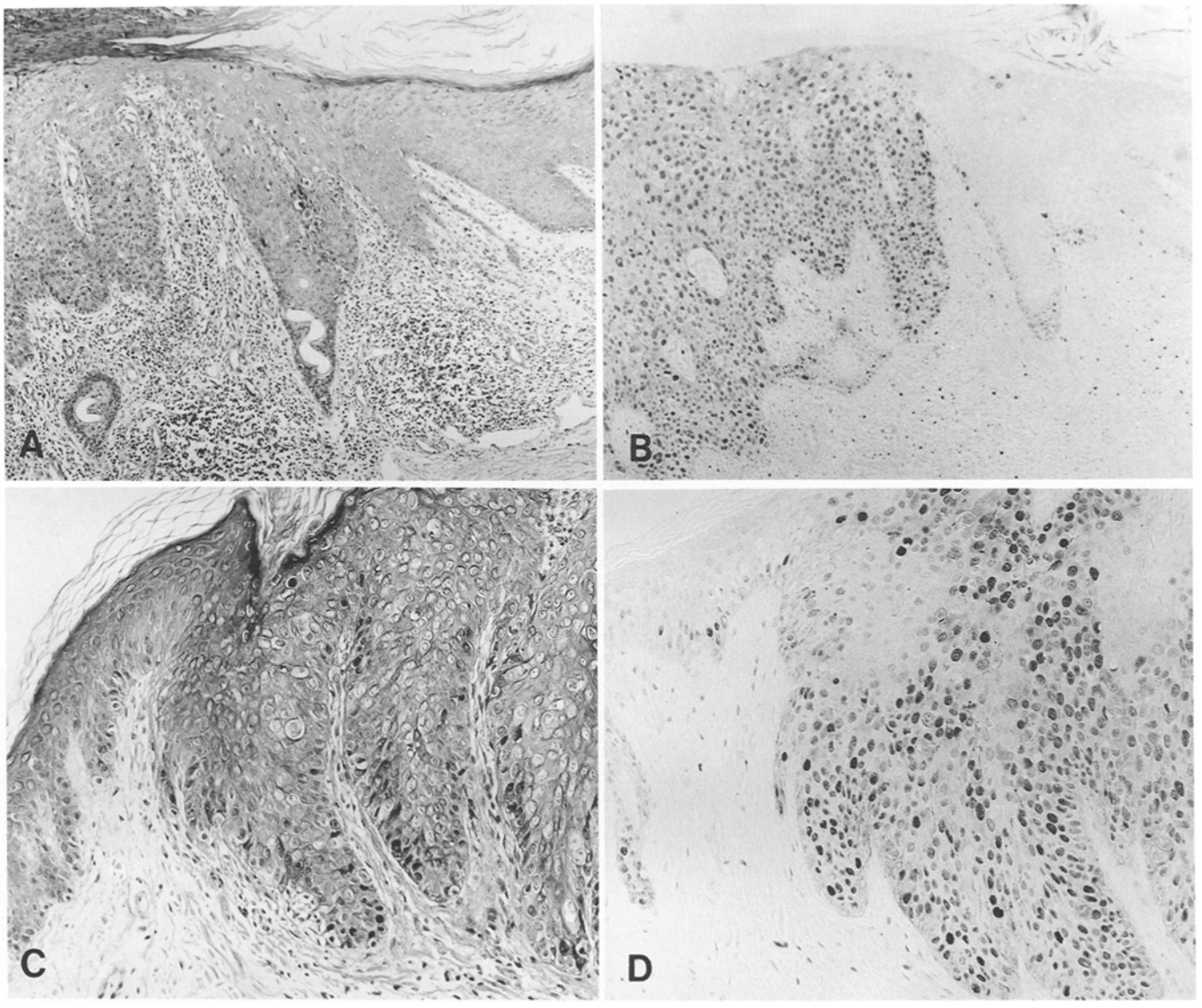

Fig. 2. **A** Bowen's disease of skin. ×100. **B** PCNA-positive nuclei in Bowen's intra-epithelial carcinoma are scattered throughout the epithelial layer; however, the normal skin shows positive nuclei only in the basal cells. ×100. **C** Senile keratosis of skin. ×100. **D** PCNA-positive nuclei in senile keratosis lesion are scattered throughout the epithelial layer. ×100

Table 4. Correlation of immunohistochemical staining of PCNA for degree of nuclear atypia

Degree of atypia	No.	%PCNA-positive nuclei			%Total positive Mean (SD)
		<10	10–20	>20	
Low	13	9	2	2	8.0 (3.7)*
Moderate	19	5	11	3	10.7 (6.5)
High	16	7	8	1	15.4 (4.9)*

* $P<0.001$

of tumour cells at the tumour invading front of connective tissues of poorly differentiated carcinoma (Fig. 3). A statistically significant difference of PCNA positivity was observed between well-differentiated and moderately differentiated carcinoma ($P<0.02$) (Table 3) and between a low and a high degree of nuclear atypia ($P<0.001$) (Table 4).

Clinical records were available for 35 cases of oral squamous cell carcinoma. No correlation between survival and PCNA index was observed (data not shown).

Discussion

Different methods have been used for studying cell kinetics in tumour specimens, and include tritiated thymidine incorporation, monoclonal antibody detection of 5′ bromodeoxyuridine (BrdU) incorporation and flow cytometry (Garcia et al. 1989; Coltrera and Gown 1991). Radiolabelling with tritiated thymidine has numerous drawbacks, including the requirement of fresh, viable tissues for thymidine incorporation, the necessity for tis-

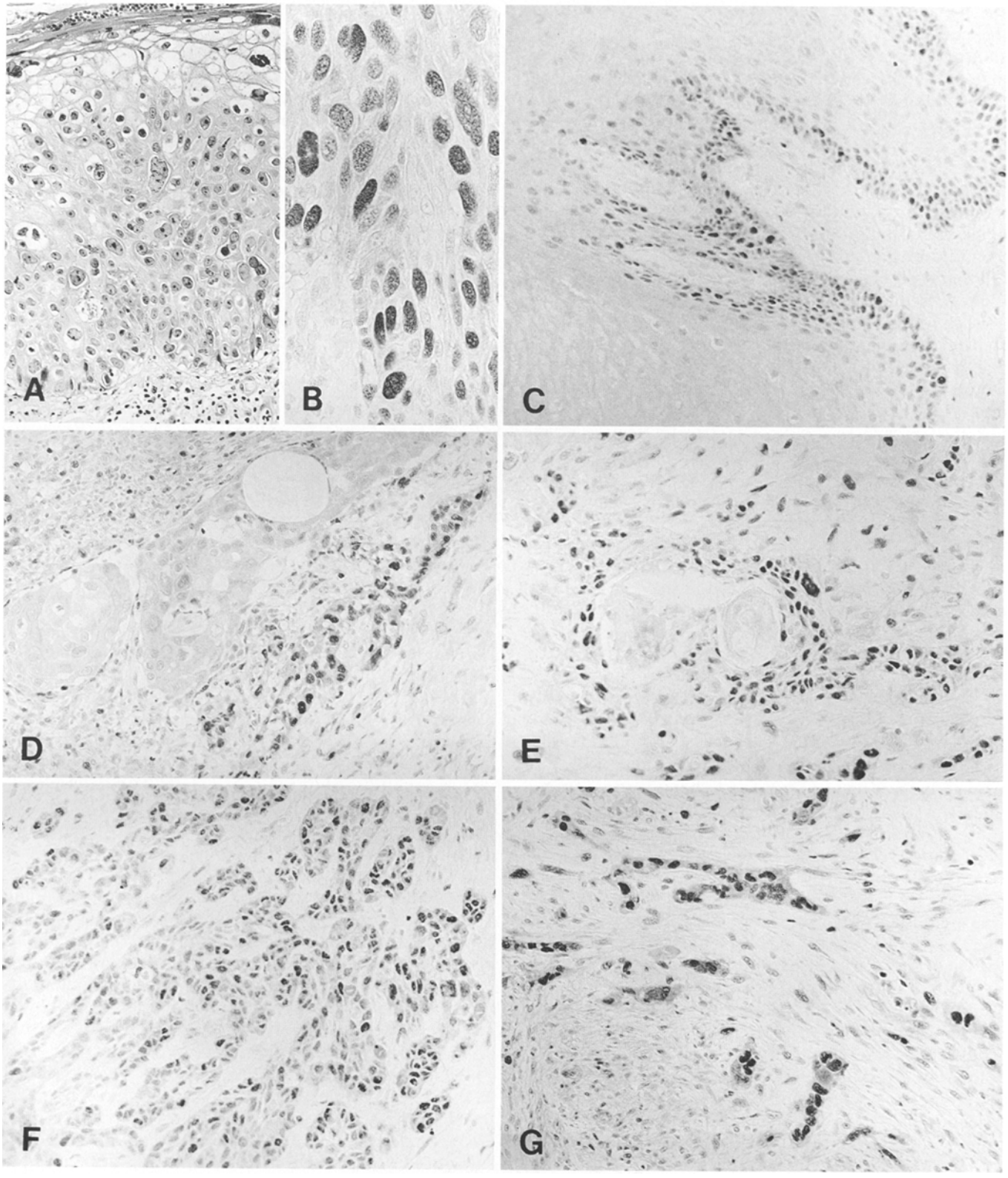

Fig. 3. **A** Bowen's intra-epithelial carcinoma. ×100. **B** Nuclear PCNA staining pattern is granular throughout the nucleoplasm. ×200. **C** Well-differentiated squamous cell carcinoma. PCNA-positive cells are seen in the basal layer. ×100. **D** Squamous cell carcinoma cells in a massive and infiltrating growth. PCNA-positive cells are scattered in the infiltrating tumour cells only. ×100. **E** Tumour cells in epithelial pearls. Only the peripheral cells are positive for PCNA staining; keratinized cells are negative. ×100. **F, G** PCNA-positive tumour cells in poorly differentiated squamous cell carcinoma. All the infiltrating invasive tumour cells stain positively for PCNA in tumour cell nuclei. ×100

sue incubation with nucleotide and altered uptake of the label, as well as hazards of handling radioactive substances. The use of BrdU and anti-BrdU antibodies eliminates some of the disadvantages, but still needs fresh and viable tissues. Although flow cytometry has many advantages, it is a degradative technique which results in the loss of tissue architecture; thus it cannot be used for the simultaneous analysis of cell proliferation features and histopathology. The use of immunocytological methods can potentially simplify and expand the clinical analysis of proliferating cells, as it preserves tissue architecture and cell to cell relationship, and viable tumour cell areas may be evaluated and separated from necrotic areas with a simultaneous evaluation of tumour cell kinetics and histology.

Tumours are considered to contain three groups of cells in kinetic terms: proliferating cells that are involved in the cell cycle (S, G_2, M, G_1), non-proliferating G_0 cells that may enter the cell cycle and cells that have lost the capacity to divide. The growth fraction of the tumour comprises the proliferating or cycling cells of the total cell population. In the present study, Ki-67 showed weakly positive immunostaining reaction only in acetone-fixed frozen sections and no reaction product in fixed paraffin sections. The reaction product with Ki-67 recognized an epitope associated with DNA-binding nuclear protein which is destroyed in fixed and paraffin embedding procedures. Ki-67 staining was then used in frozen sections; however, quantification of Ki-67 staining is not simple to standardize (Hitchcock 1991). Ag-NORs did not demonstrate specificity of reaction and were weakly positive using all fixatives, in both frozen and paraffin embedded tissues. PCNA PC10 antibody reactivity was specific in the range of 6 to 48 h in 10% formalin and Carnoy-fixed paraffin sections. However, tissue preservation was observed to be superior in formalin to Carnoy fixation. Most surgically removed tumours routinely fixed in 10% formalin provided excellent tissue preservation for immunohistochemical staining. Post-fixation procedures did not provide reasonable tissue preservation when PCNA PC10 monoclonal antibody was used. Following comparisons of immunostaining for PCNA PC10, Ki-67 and AgNOR staining, we have used the streptavidin biotin complex peroxidase technique using PCNA PC10, allowing the retrospective evaluation of tumour cell growth fraction in routinely processed and paraffin-embedded tissues.

Recently, van Dierendonck et al. (1991) described comparative results of expression with PCNA (polyclonal and monoclonal antibody), Ki-67 staining, and BrdU labelling in MCF-7 human breast carcinoma cells and found that PCNA-negative cells were Ki-67 negative; they concluded that anti-PCNA antibody may be an excellent marker to detect cells with S-phase DNA content in situ.

To our knowledge, this study is the first quantitative study of tumour cell growth fraction by immunohistochemical methods in malignant and pre-malignant lesions occurring in the oral and maxillofacial region. A semi-quantitative analysis of PCNA staining in tumours has shown a high correlation with flow cytometrically determined S-phase data on the same tumours (Garcia et al. 1989; Dawson et al. 1990; Hall et al. 1990). Our study showed a PCNA-positive cell index correlated with the differentiated and cellular atypia of oral squamous cell carcinoma. However, the values which were not significant in poorly differentiated carcinoma may be evidence of a loss of relationship between PCNA expression and differentiation. An increased expression of PCNA has been shown to correlate with blast transformation in chronic myelogenous leukaemia (Takasaki et al. 1984).

In our study, normal mucosa showed the proliferating cells only in the basal layers but in pre-malignant lesions the proliferating cells were scattered and significantly more numerous throughout the epithelial layers, suggesting disorganized cellular proliferation. Moreover, the index of proliferating cells was greater in pre-malignant lesions than in the normal specimens. The PCNA index in senile keratosis and Bowen's disease was considerably higher than in leukoplakia. This may be attributed to degree of dysplasia, as in our case minimal dysplastic changes were seen in leukoplakia. Thus this finding suggests that the pre-malignant lesions are proliferative disorders and the proliferating cell growth fractions correlate with the degree of dysplasia. However, in malignant tumours, although loss of cellular differentiation and cell growth fractions were correlated, it may be that differentiation is rather more important than proliferation in determining biological behaviour.

The basal cell carcinoma showed a higher index of proliferating cells in tiny infiltrating nests in connective tissue stroma than in the nests and cords of basaloid cells arising from the region of epidermal basal cells. This may be related to an aggressive growth pattern and more invasive clinical behaviour of basal cell carcinoma with tiny infiltrating nests in the stromal tissue.

Earlier authors employing PCNA in cell cycle studies have suggested differences in the sensitivity of the antibodies and methods to determine positive reaction of cells. In different studies, there is non-uniformity of tissue handling and scoring method of positive nuclei and therefore varied PCNA scores have been observed. Before the production of monoclonal antibodies Robbins et al. (1987) applied human serum containing anti-PCNA to formalin-fixed tissue sections of 64 human solid malignancies and demonstrated immunoreactivity in only 46 of the malignant tumours. The majority of reactive tumours showed a proliferating fraction of less than 1%. However, scores of 20–50% in tissue culture and 11–36% in haemopoietic malignancies have been reported (Miyachi et al. 1978; Smetana et al. 1983). Even using monoclonal antibodies, variable data on the fraction of PCNA-positive nuclei in tissue sections have been reported (Kurki et al. 1988; Garcia et al. 1989; Battersby and Anderson 1990). In this study, we have taken measures to standardize the tissue fixation and preservation. All nuclei were scored as positive regardless of staining intensity by computer-assisted image analysis. Morris and Mathews (1989) Landberg et al. (1990) and Kurki et al. (1988) have reported variations in the level of PCNA during the cell cycle. However, tumour cells

showing minimal PCNA positivity may occur as a result of the loss of antigenicity of the protein during tissue processing. In addition, over-expression of the antigen in certain tumours may represent an autocrine or paracrine growth factor influence on PCNA gene function, and this aspect needs further investigation.

DNA synthesis and rate of cellular proliferation have been found to be important factors in human malignancy. This study shows that monoclonal antibody PCNA PC10 is a valuable marker to label proliferating cells in formalin-fixed, paraffin-embedded tissues. We believe further investigations on prognosis and response to various treatment regimens of cancer should be carried out on the basis of cell cycle analysis using PCNA immunohistochemistry.

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