Pergamon

Oral Oncol, Eur J Cancer, Vol. 32B, No. 1, pp. 24–31, 1996

Printed in Great Britain
0964–1955/96 $15.00 + 0.00

0964–1955(95)00049–6

The Relationship Between Vascularity and Cell Proliferation in Human Normal and Pathological Lesions of the Oral Cheek Epithelium

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The present study investigates relationships between neovascularisation and PCNA cell proliferation markers in different pathological lesions of the oral cheek mucosa. All specimens were fixed in 10% formalin and routinely processed for histology. Six normal (N) samples were taken from resection margins of benign lesions. The pathological lesions consisted of chronic inflammation ($n=10$), lichen planus ($n=7$), fibrous hyperplasia ($n=11$), dysplasia ($n=5$), squamous cell carcinoma ($n=22$) and epithelium adjacent to carcinoma ($n=6$). Adjacent 5 μm sections were stained with monoclonal antibodies against vimentin (clone no. V9) for identification of stromal blood vessels and against proliferating nuclear antigen (PCNA/PC10) using ABC immunoperoxidase techniques. Point counting was used to obtain the primary morphometric data using a Zeiss VIDAS image analyser. No attempt was made to classify the different types of blood vessels. The morphometric blood vessel parameters estimated were volume density, number per unit area, length per unit volume and mean transverse sectional area. PCNA indices were determined by estimating the percentage frequency of PCNA positive nuclei in basal and spinous strata. Generally, there were significant increases in all PCNA indices and blood vessel parameters between the N group and the different pathological lesions. A highly positive correlation was detected between all PCNA indices and blood vessel parameters. These data suggest that increased vascularity and angiogenesis occur in support of actively proliferating and transforming oral epithelial cells in order to permit growth. PCNA indices and blood vessel parameters may have a potential application as diagnostic and prognostic indicators.

Keywords: PCNA, oral cancer, human, blood vessels, angiogenesis, cell proliferation, morphometry

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INTRODUCTION

Sustained tumour growth occurs only in the presence of new capillary formation from the established microvasculature during post-natal life and is known as angiogenesis [1, 2]. This process is induced by angiogenic substances which are released by tumour and inflammatory cells, such as macrophages [3, 4]. Angiogenesis can be augmented by a reduction in angiogenic inhibitors, such as thrombospondin, in malignant cellular transformation [5]. The degree of vascularity in tumours has been used to predict the malignant and metastatic potential of tumours [6, 7]. The use of different objective descriptors in evaluating neovascularity in the malignant transformation of neoplasms might greatly enhance prognostication. To date, no studies have been performed utilising objective blood vessel parameters in human oral neoplasms.

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Received 26 May 1995; provisionally accepted 14 June 1995; revised manuscript received 23 July 1995.

The assessment of cell proliferation has been made simple and reliable with the development of monoclonal antibodies (PC10 and 19A2) directed against proliferating cell nuclear antigen (PCNA) [8, 9]. In astrocytomas, non-Hodgkin's lymphomas, bronchial dysplasias and normal bone marrow, PCNA/PC10 have proved to be reliable indicators of cell proliferation and a low PCNA index is well correlated with patients' survival [10–13]. However, the relationship between PCNA expression and cell proliferation is unclear in other tumours, such as breast and gastric cancers [10]. The present study aims to investigate whether there is a correlation between neovascularisation and cell proliferation as assessed using the PCNA marker in different pathological lesions of the oral cheek mucosa.

MATERIALS AND METHODS

Collection of samples and histological processing procedures

All specimens were obtained from the archival files of the Department of Oral Pathology, Qin Du Stomatological

College, Fourth Military Medical University, Xian, People's Republic of China. Sixty-seven samples were fixed in 10% formalin for 24 h, routinely processed for histology and embedded in paraffin wax. Six normal (N) samples were taken from the resection margins of benign oral lesions which showed absence of keratinised and inflammatory cells. The pathological lesions comprised non-specific chronic inflammation (CI; $n=10$), fibrous hyperplasia (FH; $n=11$); lichen planus (LIP; $n=7$), dysplasia (DYS; $n=5$), squamous cell carcinoma (SCC; $n=22$) and epithelium immediately adjacent to the margins of squamous cell carcinoma (EAC; $n=6$). The DYS group were all mildly dysplastic. FHs demonstrated varying degrees of epithelial keratosis. Three of the six EACs demonstrated mild cellular atypia whereas the other three showed no abnormal cytological features. SCCs were of different grades of differentiation, including well differentiated (SCCWD; $n=10$) and moderately to poorly differentiated (SCCMPD; $n=12$) lesions. The diagnoses were made independently by two of the authors (YJ and FHW). The criteria for diagnosis were based on previous publications [14–16].

Immunohistochemistry

Four micrometre sections were cut and stained with a monoclonal antibody against vimentin (Clone 9; 1:120 dilution; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) and PCNA (PC10; 1:100 dilution; Dako, Denmark) on adjacent sections from the same patient using standard ABC immunohistochemical techniques (Vectastain, Vector Laboratories, California, U.S.A.). Sections were mounted on slides which were coated with poly-L-lysine (Sigma). Sections were deparaffinised and rehydrated by passing through xylene and decreasing grades of ethanol. Endogenous peroxidase was blocked by immersing the sections in 0.75% H_2O_2 for 20 min. After rehydration and repeated rinsing with 0.01 M phosphate buffered saline (PBS), the sections were incubated with normal horse serum for 20 min in order to block non-specific background staining, and subsequently incubated with the primary monoclonal antibody at 4°C overnight.

After thoroughly washing with PBS solution, the sections were incubated with biotinylated horse anti-mouse immunoglobulin for 30 min followed by horseradish peroxidase-labelled ABC complex (Vectastain) for 30 min. Diaminobenzidine (DAB)–hydrogen peroxidase was used as the chromogen. Sections were lightly counterstained with Harris' haematoxylin. Negative control sections were processed using similar steps as described above but were incubated only with PBS.

Morphometric procedures

Sampling procedures. A total of 67 patients were analysed in this study. 56 were males and 10 were females with an age range between 16 and 78 years (mean age 47.1). One out of the five serial sections was chosen for immunostaining and measurement. Each field quantified contained the basal layer of the epithelium and lamina propria. The maximum depth of the lamina propria evaluated was 0.9 mm at a final magnification of × 420. Each field consisted of blood vessels, connective tissue stroma and a part of the epithelium. For the purpose of our investigation, blood vessels were defined as those structures lined by endothelial cells which might contain erythrocytes. A stratified systematic random sampling procedure was adopted [17]. Table 1 summarises the sampling strategy adopted in this study.

Table 1. Summary of sampling procedure

Groups	No. patients	Blocks/ patient	Sections/ patient	Fields/ section
N	6	1	1	10
CI	10	1	1	10
FH	11	1	1	10
LIP	7	1	1	10
DYS	5	1	1	10
EAC	6	1	1	10
SCC	22	1	1	10

Morphometric techniques. Point counting was used to obtain the primary morphometric data using the Zeiss VIDAS image analyser at a final magnification of × 420. A coherent quadratic lattice was superimposed on the image on the screen. The total number of points in the grid was 324. Using a stage micrometer, the actual distance between two adjacent points in the grid was found to be 10 μm. The volume density of the blood vessel profiles in the connective tissue stroma ($V_{VBV,CT}$) was determined by the relationship [17]

$$V_V = P_P$$

$$\text{i.e. } V_{VBV,CT} = \frac{P_{BV}}{P_{CT}}$$

where P_{BV} and P_{CT} are the number of points falling on the blood vessel profiles, including both their walls and lumens and on the connective tissue stroma, respectively. The minimal sample size for $V_{VBV,CT}$ in terms of the number of points to be applied to each section was determined by calculating the relative standard error, RSE [18]. The formula is

$$\text{RSE} = \frac{\sqrt{1 - V_V}}{\sqrt{n}}$$

where n is the number of points applied to the blood vessel profiles to obtain a certain $V_{VBV,CT}$ with a specified RSE. In this study, an RSE of $<5\%$ was employed.

The numerical density of the blood vessel profiles per unit area ($N_{ABV,CT}$) was determined by

$$N_{ABV,CT} = \frac{N_{BV}}{A_{CT}}$$

where N_{BV} is the total number of blood vessel profiles and A_{CT} is the total area of the connective tissue stroma.

The length density was derived by using the following formula [17]

$$L_{VBV,CT} = 2 \times N_{ABV,CT}.$$

The mean transverse sectional area (A) was obtained according to the following relationship

$$A_{BV} = \frac{V_{VBV,CT}}{L_{VBV,CT}}.$$

PCNA indices. Those epithelial cells possessing distinct

Table 2. PCNA sampling procedures per experimental group

Layers	No. nuclei/ field	No. fields/ section	Total no. nuclei
Basal (B)	20	10	200
Spinous (SP)	50	10	500
Basal and spinous (BS)	70	10	700

brown reaction products in their nuclei were considered to be PCNA positive regardless of the staining intensity. The PCNA positive nuclei were further classified into strongly or weakly positive. A strongly positive nucleus has an intense brown homogeneous reaction product, whereas a weakly positive nucleus has less intensity or may appear patchy when compared to the former. The counting was performed using an Olympus microscope fitted with an eyepiece meter disc graticule. In all groups, the epithelium was divided into basal (B) and spinous (S) layers. Epithelial cells in the basal layer were those cells seen in direct contact with the lamina propria. All epithelial cells above this region were considered to be in the spinous layer with the exception of markedly flattened keratinised cells. A systematic random sampling procedure was used (Table 2). The PCNA indices were determined by the following relationships:

(1) The percentage of PCNA positive cells in the basal (BCP) and spinous (SCP) layers was obtained by dividing the sum of the PCNA positive cells in the B or S layer by the total number of cells in the B or S layer, multiplied by 100.
(2) The percentage of strongly positive cells in the basal (BCSP) and spinous (SCSP) layers was calculated by dividing the sum of the PCNA strongly positive cells in the B or S layer by the total number of cells in the reference epithelium, multiplied by 100.
(3) The percentage of positive cells in both layers (BSP) was determined by dividing the PCNA positive cells in the B and S layers by the total number of cells in the B or S layers, multiplied by 100.

The rationale for using three methods of counting was to determine whether the PCNA indices varied between the two main layers of stratified epithelium, as well as to determine which amongst the PCNA indices were the most sensitive objective discriminators of cell proliferation.

Statistical analyses. All data used to calculate each morphometric parameter were pooled to obtain a single value for each patient. Values of each morphometric parameter were pooled to obtain a single mean and standard error of the mean for each group for that particular parameter. The normal distribution of the data was tested by chi-square goodness of fit. When necessary, logarithmic transformation of the data was performed in order to make the data suitable for statistical analysis. One-way ANOVA followed by a Duncan multiple range test [19] were performed between groups and blood vessel parameters. The Spearman rank correlation coefficient [20] was used to determine whether linear relationships exist between the blood vessel parameters and the PCNA indices, respectively. All statistical tests were calculated using Statgraphic Plus.

RESULTS

Qualitative results

The PCNA immunolabelled nuclei were clearly identifiable. In normal epithelium, PCNA positively stained nuclei were predominantly observed in the parabasal layers, approximately two to three cell layers from the basal layer. Few of the basal cells were positively stained (Fig. 1). No keratinised or granular cells were positive in any of the sections. The number of positively stained nuclei in basal and parabasal layers increased in the CI group. These findings were also seen in the FH group but showed more intense staining and higher numbers of labelled nuclei when compared with the N and CI groups (Fig. 2). PCNA positive nuclei significantly increased in both basal and parabasal layers of the EAC group when compared with the N group (Fig. 3). Some stained nuclei were also present in the superficial regions of the epithelium. Variable numbers of PCNA stained nuclei were observed in the tumour masses of the SCC group. PCNA immunoreactivity was more frequently detected in the peripheral part of the tumour islands and was almost absent in the central keratinised region of well-differentiated carcinomas (Fig. 4). Poorly-differentiated SCCs showed a diffuse pattern of staining with a significantly elevated number of PCNA immunoreactive cells when compared with the well- and moderately-differentiated SCCs.

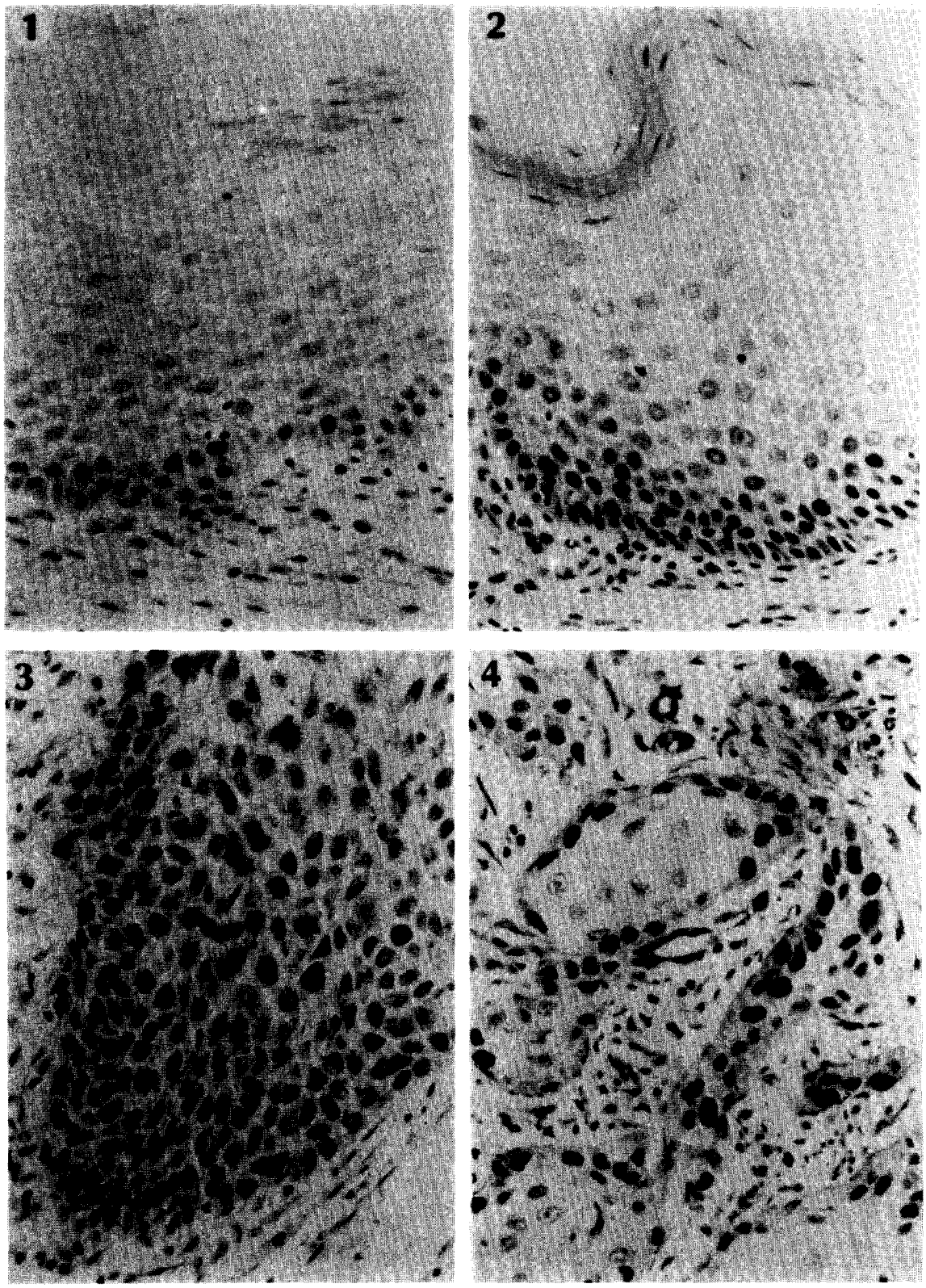
The frequency of blood vessel profiles was low in the N group (Fig. 5) when compared with any of the pathological lesions, although the number of blood vessel profiles increased relatively near the connective tissue papillae when compared to the rest of the connective tissue stroma. The benign groups (CI, FH and LIP) showed an increased number of blood vessel profiles accompanied by inflammatory cells, predominantly lymphocytes, in the lamina propria (Fig. 6). In the DYS group, blood vessel profiles increased moderately in number and were evenly scattered in the lamina propria when compared to the N and benign groups. The lamina propria of the EAC group showed extensive vascularisation with varying sizes of blood vessels when compared with the N group. Moderate lymphocytic infiltration was observed in the lamina propria (Fig. 7). The frequency and size of the blood vessels were markedly increased in the SCC group when compared with the benign and N groups (Fig. 8) and vascularity was highly dependent on the degree of cellular differentiation.

Quantitative results

Generally, there was a significant increase in all blood vessel parameters (Tables 3, 4) and PCNA indices (Tables 5, 6) between the N group and the different pathological cheek lesions.

$V_{VBV,CT}$ was increased significantly between N and benign groups (CI, FH and LIP), between N and DYS, between N and EAC and between N and SCC. $V_{VBV,CT}$ was also significantly elevated between benign groups and EAC and between benign and SCC groups. $N_{ABV,CT}$ and $L_{VBV,CT}$ were significantly higher in all pathological groups when compared with N. No statistical differences were detected between any of the pathological groups. A_{BV} was increased significantly between N and CI, between N and EAC, between N and DYS and between N and SCC.

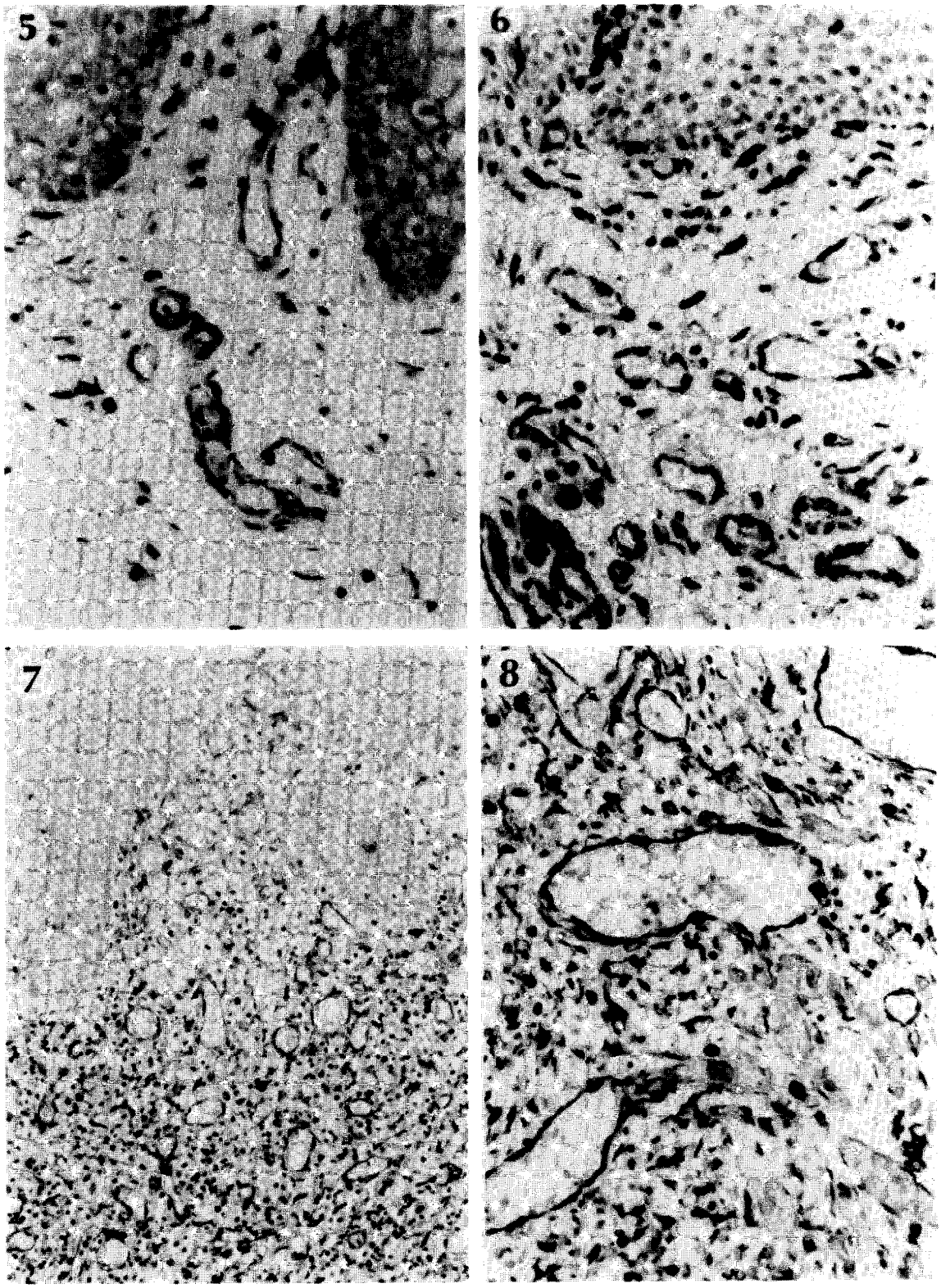
BCP was statistically different between N and benign groups (CI, FH and LIP), between N and EAC and between N and SCC but BCP was not significantly different between N



Figs 1–4. Paraffin sections stained with PCNA/PC10 monoclonal antibody. The frequency of PCNA stained nuclei increases between normal and carcinoma (×400). Normal=1; chronic inflammation=2; epithelium adjacent to carcinoma=3; carcinoma=4.

and DYS. SCP was significantly different between N and the different pathological groups, except between N and LIP. BCSP was significantly different only between N and some of the pathological lesions (FH, LIP, EAC and SCC). SCSP was statistically different between N and FH, between N and EAC, between N and SCC and between FH and SCC. BSP was significantly different between N and all pathological lesions.

The Spearman rank correlation coefficient showed a highly



Figs 5–8. Paraffin sections stained with anti-vimentin monoclonal antibody. The frequency and size of the blood vessels increases between normal and carcinoma. Normal = 5 (× 400); chronic inflammation = 6 (× 400); epithelium adjacent to carcinoma = 7 (× 200); carcinoma = 8 (× 400).

positive correlation between PCNA indices and blood vessel parameters except for SCSP which showed borderline statistical significance with $N_{ABV,CT}$ and $L_{VBV,CT}$ (Table 7).

DISCUSSION

PCNA is a cell proliferation marker which has shown significant correlations with Ki67, uptake of bromodeoxy-

Table 3. Quantitative data of blood vessel parameters

Groups	N	CI	FH	LIP	DYS	EAC	SCC
Parameters							
$V_{VBV,CT}$ ($\mu m^3/\mu m^3$)	0.026 (±0.002)	0.054 (±0.003)	0.065 (±0.005)	0.067 (±0.007)	0.076 (±0.010)	0.106 (±0.011)	0.092 (±0.006)
$N_{ABV,CT}$ ($\times 10^{-4}\ \mu m^{-2}$)	2.54 (±0.13)	4.00 (±0.31)	4.77 (±0.20)	5.34 (±0.35)	4.45 (±0.50)	4.91 (±0.22)	4.86 (±0.25)
$L_{VBV,CT}$ ($\times 10^{-4}\ \mu m^{-2}$)	5.29 (±0.25)	7.83 (±0.63)	9.4 (±0.39)	10.2 (±0.71)	8.90 (±0.99)	9.82 (±0.44)	9.54 (±0.50)
A_{BV} (μm^2)	55.06 (±4.49)	70.41 (±6.27)	70.25 (±3.41)	63.21 (±4.88)	89.31 (±15.00)	108.76 (±12.24)	96.96 (±5.33)

± = Standard Error of Mean.

Table 4. Results of statistical analysis of blood vessel parameters

Groups	$V_{VBV,CT}$	$N_{ABV,CT}$	$L_{VBV,CT}$	A_{BV}
N	*	*	*	*
CI	*	*	*	*
FH	*	* *	* *	* *
LIP	*	*	*	* *
EAC	*	* *	* *	*
DYS	* *	* *	* *	* *
SCC	* *	*	*	*

One-way ANOVA followed by multiple range test (Duncan); overlap of * down the column indicates that group comparisons are not statistically significant at 95% confidence interval.

uridine, flow cytometry and titrated thymidine incorporation [13, 21, 22]. PC10 clone (Dako) has the main advantage of staining formalin-fixed paraffin sections but the reliability for assessing cell growth has not been ascertained in some tumours because the PCNA half-life persists beyond the cell cycle or after the termination of mitosis, and the antigen may be expressed in non-cycling cells undergoing repair [23, 24]. For most PCNA indices, mean values for basal cells were generally higher than suprabasal cells. Inexplicably, values in basal and suprabasal cells for strongly positive indices were similar. Our data have shown significant increases in all of the PCNA indices between N and EAC and between N and SCC. Values of all PCNA indices were highest in the SCC, followed by the EAC. Three of the six EACs demonstrated cellular atypia and Wright and Shear [25] have also shown evidence of dysplasia in epithelium adjacent to squamous carcinoma. The DYS group had PCNA indices two to three times higher than the N group, but significant differences were only detected in SCP and BSP indices. Amongst the PCNA indices, BSP demonstrated a significant difference between N and all of the pathological cheek lesions. The PCNA indices showed varying discrimination between the pathological groups. Despite progressive increases between N and SCC, two of the PCNA indices had low values in the LIP and DYS groups but these groups still had values which were about two times greater than the N group. Our data also revealed that the percentage of PCNA strongly positive nuclei (BCSP and SCSP) did not discriminate between the normal and the pathological cheek lesions, whereas the percentage of all PCNA positive nuclei (BSP) discriminated between these groups. We have no explanation for such findings since other studies have shown that intensely stained nuclei correlated well with actively proliferating nuclei [11, 26]. Further work using the MIB 1 monoclonal antibody directed against the recombinant part of the Ki67 antigen may help to clarify this issue. The MIB 1 monoclonal antibody works in formalin-fixed paraffin-embedded tissues and reacts selectively with proliferating nuclei in all phases of the cell cycle, except the G_0 phase [27].

The blood vessel parameters suggest that angiogenesis occurs in the different pathological cheek lesions. The use of immunostaining (anti-vimentin) directed towards the endothelial cells of the blood vessels was used to improve the identification of the blood vessels, particularly the capillaries in the connective tissue stroma which are the main type of blood vessel affected during angiogenesis. Vimentin is a cytoskeletal intermediate filament which is present in mesenchymal cells, pericytes and endothelial cells lining blood capillaries [28–30]. Monoclonal antibodies against vimentin stain smooth muscle of the arterioles and venules but these vessels were readily identified because of the differences in vessel wall thickness and luminal size. However, vimentin staining was of great value in the identification of tangentially sectioned vessels where morphological identification of blood vessels is fraught with difficulty. Vimentin antibodies distinctly stained endothelial cells of blood capillaries during tumour angiogenesis [31]. No attempt was made to classify the different types of blood vessel as we found it labour intensive without much derived benefit. A similar approach has been performed by Wakui *et al.* [31] in the study of tumour angiogenesis of prostatic carcinoma and by White and Al-Azzawi [32] in hamster cheek pouch carcinogenesis. The volume, numerical and length densities showed a progressive increase between N and all pathological groups. A_{BV} was statistically different between N and all of the pathological lesions, except between N and LIP and between N and FH. $V_{VBV,CT}$ significantly increased approximately four-fold between N and SCC and the volumetric increases are attributable to significant increases in the frequency ($N_{ABV,CT}$) and profile dimensions of the vasculature (A_{BV}). These findings suggest that the relative volume of blood vessels in the connective tissue in the premalignant and malignant oral cheek lesions is accompanied by both vasodilatation and neovascularisation. These two processes may reflect the increasing requirement of nutrients in actively growing and transforming cells and could also be due to the vasoactive substances

Table 5. Quantitative results of PCNA indices (%)

Groups / Parameters	N	CI	FH	LIP	DYS	EAC	SCC
BCP	12.8±2.9	40.7±2.4	51.0±1.9	52.0±4.5	25.0±4.3	65.7±7.7	65.1±3.9
SCP	10.3±1.6	34.2±3.9	36.9±3.7	26.5±2.7	35.1±11.7	47.8±5.1	52.7±3.8
BCSP	4.0±1.0	18.1±2.3	28.7±2.6	26.1±4.7	10.6±3.1	42.3±6.5	47.0±4.2
SCSP	4.3±1.2	16.3±2.9	20.7±2.7	15.3±2.0	17.6±5.8	23.7±5.7	35.2±3.8
BSP	10.9±0.8	36.1±3.4	42.4±2.4	31.2±1.8	28.9±5.6	53.7±4.3	57.0±3.4

±standard error of mean; data expressed as percentage.

Table 6. Results of statistical analysis of PCNA indices

Groups	BCP	SCP	BCSP	SCSP	BSP
N	*	*	*	*	*
CI	*	**	***	**	**
FH	*	**	**	*	**
LIP	**	**	**	**	**
EAC	**	**	**	**	**
DYS	*	**	**	**	*
SCC	*	*	*	*	*

One-way ANOVA followed by multiple range test (Duncan); overlap of * down the column indicates that group comparisons are not statistically significant at 95% confidence interval.

Table 7. Spearman rank correlation coefficient between PCNA indices and blood vessel parameters

	$V_{VBV,CT}$ ($\mu m^3/\mu m^3$)	$N_{ABV,CT}$ ($\times 10^{-4}\ \mu m^{-2}$)	$L_{VBV,CT}$ ($\times 10^{-4}\ \mu m^{-2}$)	A_{BV} (μm^2)
BCP	r 0.6328	r 0.4276	r 0.4276	r 0.5193
	P 0.0001	P 0.0005	P 0.0005	P 0.0001
SCP	r 0.4758	r 0.2579	r 0.2579	r 0.4588
	P 0.0001	P 0.0392	P 0.0392	P 0.0002
BCSP	r 0.6257	r 0.3873	r 0.3873	r 0.5263
	P 0.0001	P 0.0017	P 0.0017	P 0.0001
SCSP	r 0.4261	r 0.2358	r 0.2358	r 0.4025
	P 0.0005	P 0.0554	P 0.0554	P 0.0011
BSP	r 0.6017	r 0.3185	r 0.3185	r 0.5514
	P 0.0001	P 0.0097	P 0.0097	P 0.0001

secreted by inflammatory cells which were observed in abundance in the stroma of all pathological lesions except FH.

The blood vessel data were obtained with some assumptions. The length density parameter was determined by assuming that the blood vessels were linear structures rather than being tubular in shape. The accuracy of mean transectional area may be affected by the inherent assumption in this length density estimation. Furthermore, $N_{ABV,CT}$ may not truly reflect an increase in the number of blood vessels because the increase in the frequency of blood vessel profiles seen in tissue sections may be due to increased vessel convolution.

Several studies have qualitatively documented the existence of angiogenesis in premalignant and malignant transformation [33, 34] but no studies have yet been carried out to quantify the extent of angiogenesis in human oral epithelium. In summary, our data suggest that increased vascularity and angiogenesis occur in actively proliferating and transforming oral epithelial cells in order to support growth.

The main objective of the present investigation was to determine whether there is a correlation between angiogenesis and the PCNA cell proliferation marker in the different pathological lesions of the cheek mucosa. The Spearman rank correlation coefficient showed a highly positive correlation between all PCNA indices and the blood vessel parameters except for SCSP which showed borderline statistical significance with $N_{ABV,CT}$ and $L_{VBV,CT}$. PCNA indices used in conjunction with the blood vessel parameters may have a potential application as diagnostic and prognostic indicators

and future studies using this approach should be directed towards analysis of larger sample sizes, particularly in the evaluation of epithelial dysplasias of different grades.

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Acknowledgements—We would like to thank Ms Carman Leung for her technical assistance and Mr Johnny Leung for his help with photography. Part of this work was supported by the Committee on Research and Conference Grants, The University of Hong Kong.