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A quantitative investigation of immunocytochemically stained blood vessels in normal, benign, premalignant and malignant human oral cheek epithelium

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Abstract The present study was designed to determine whether increased vascularity occurs during malignant transformation of human oral cheek epithelium. Nine normal (N) samples were taken from the resection margins of benign lesions; the pathological lesions were classified as chronic inflammation (CI; n=11), fibrous hyperplasia (FH; n=12), lichen planus (LIP; n=8), dysplasia (DYS; n=5), squamous cell carcinoma (SCC; n=25; well differentiated [SCCWD]; n=10; moderately to poorly differentiated [SCCMPD]; n=15) and epithelium adjacent to carcinomas (EAC; n=6). Sections were stained with monoclonal antibody (mAb) against vimentin using an ABC immunoperoxidase technique. All blood vessels present within a depth of 0.9 mm of lamina propria were quantified irrespective of their morphology. The blood vessel parameters quantified were volume density ($V_{VBV,\ CT}$), number per unit area ($N_{ABV,\ CT}$), length per unit volume ($L_{VBV,\ CT}$) and mean transverse sectional area (A_{BV}). $V_{VBV,\ CT}$ increased significantly between normal and all pathological groups. Amongst the pathological groups, statistical differences were detected between CI and SCC, CI and EAC, FH and SCCWD, FH and EAC, LIP and SCC, LIP and EAC, DYS and SCCWD and DYS and EAC. The EAC group had the highest $V_{VBV,\ CT}$ and the values of $N_{ABV,\ CT}$ and $L_{VBV,\ CT}$ were significantly higher in all the pathological groups when compared with the normal group. No significant differences were detected between any of the pathological group. The parameter A_{BV} increased significantly between normal and DYS, FH, SCC, EAC, FH and EAC, FH and SCC, CI and EAC, CI and SCC, LIP and EAC and LIP and SCC. Spearman rank correlations detected a positive correlation between the severity of oral lesions and all of the blood vessel parameters. We conclude that a mAb against vimentin improved the identification of smaller blood vessels and the blood vessel data suggest that angiogenesis occurs in premalignant and malignant lesions of human oral cheek epithelium. Angiogenesis seems to play an essential role in sustaining the actively growing and transforming cells.

Key words Angiogenesis · Human · Morphometry · Oral cheek epithelium · Blood vessels

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Introduction

Angiogenesis sustains actively growing or transforming cells and occurs in neoplastic conditions [20] and in the normal process of wound healing [1]. Experimental evidence has shown that angiogenesis in neoplasms is mediated by a humoral factor known as tumour angiogenesis factor or TAF [5, 8]. Angiostatic effects can be produced by combinations of heparin and corticosteroids [3]and platelet factor 4 [22]. Furthermore, angiogenesis is modulated by factors that stimulate endothelial proliferation and migration and extracellular matrix degradation such as proteases [15].

Although angiogenesis has been documented qualitatively in a variety of tumours, no studies have yet quantified the morphological alterations occurring in human oral cheek epithelium using objective blood vessel parameters. The main aim of the present study was designed to assess whether angiogenesis occurs in carcinomas and in lesions with differing malignant potential from human oral cheek mucosa using combined immunohistochemical and morphometric methods.

Materials and methods

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. Samples which were all from the buccal mucosa, were fixed in

10% formalin for 24 h, routinely processed for histology and embedded in paraffin wax. Nine normal (N) samples were taken from the resection margins of benign cheek lesions which showed absence of cornified and inflammatory cells. The pathological lesions comprised non-specific chronic inflammation (CI; n=11), fibrous hyperplasia (FH; n=12), lichen planus (LIP; n=8), dysplasia (DYS; n=5), squamous cell carcinoma (SCC; n=25) 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. SCCs comprised well differentiated (SCCWD; n=10) and moderately to poorly differentiated (SCCMPD; n=15). Lesions were selected on the basis that they had minimal risk of developing malignancy (N, FH, and CI); that they were lesions with a high risk of malignant transformation (EAC, LIP and DYS) or that they were overtly malignant (SCCs). The subject groups were not sex and age-matched. The diagnoses were made independently by three authors (YJ; LY and FHW). The criteria for diagnosis were based on previous publications [2, 16, 29].

For immunohistochemistry 4 μm sections were cut and stained with a monoclonal antibody against vimentin (Clone 9; Sigma Co.) using standard ABC immunohistochemical techniques (Vectastain, Vector Laboratories). Sections were mounted on slides which were coated with poly-L-lysine (Sigma Co.). 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% hydrogen peroxide for 20 min. After rehydration and repeated rinsing with 0.01 M phosphate buffered saline (PBS) solution, 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 counterstained with Harris' haematoxylin. Negative control sections were processed using similar steps to those described above but without incubating them with the primary antibody. Instead, sections were incubated with PBS solution.

Morphometric procedures

A total number of 76 patients were analysed in this study. Sixty-six were males and 10 were females with an age range between 16 to 75 (mean age=49.1 years) one 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 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 [19, 26]. Table 1 summarises the sampling strategy adopted in this study.

Point counting was used to obtain the primary morphometric data using a Zeiss VIDAS image analyser at a final magnification of ×420. A coherent quadratic lattice B100 [26] was superimposed on the image of the screen. The total number of points in the grid was 324. Using a stage micrometer, the actual distance between two points in the grid was obtained and was found to be 10 μm. The volume density of blood vessels in the connective tissue stroma ($V_{VBV, CT}$) was determined by the relationship [26]

$$V_V = P_P$$

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

Table 1 Summary of sampling procedure

Groups	No. patients	Blocks/ patient	Sections/ patient	Fields/ section
N	9	1	1	10
CI	11	1	1	10
FH	12	1	1	10
LIP	8	1	1	10
DYS	5	1	1	10
EAC	6	1	1	10
SCCWD	10	1	1	10
SCCMPD	15	1	1	10

where P_{BV} and P_{CT} are the number of points falling on the blood vessels and on the containing connective tissue stroma respectively. Points falling on all layers of the blood vessel wall and the lumen were counted. 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 [14]. The formula is

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

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

The numerical density of the blood vessel profiles per unit area (N_A) 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 containing connective tissue stroma.

The length density was derived by using the following formula [26]

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

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

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

Statistical analyses

All data used to calculate each morphometric parameter were pooled to obtain a single value for each patient. Values were pooled to obtain a single mean and standard error of mean for each group for that particular parameter. The normal distributions of the data were 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 [23]. One-way analysis of variance was performed followed by a multiple range test (Duncan test) in order to detect the specific differences between groups using SPSS software package. Spearman rank correlations were performed using the same software.

Results

All of the blood vessels were stained positively. The inner walls of the blood vessels were often stained in an intense annular pattern, particularly those of the capillaries. The strongly positive stain was evident in the cytoplasm of the endothelial cells as well as in the

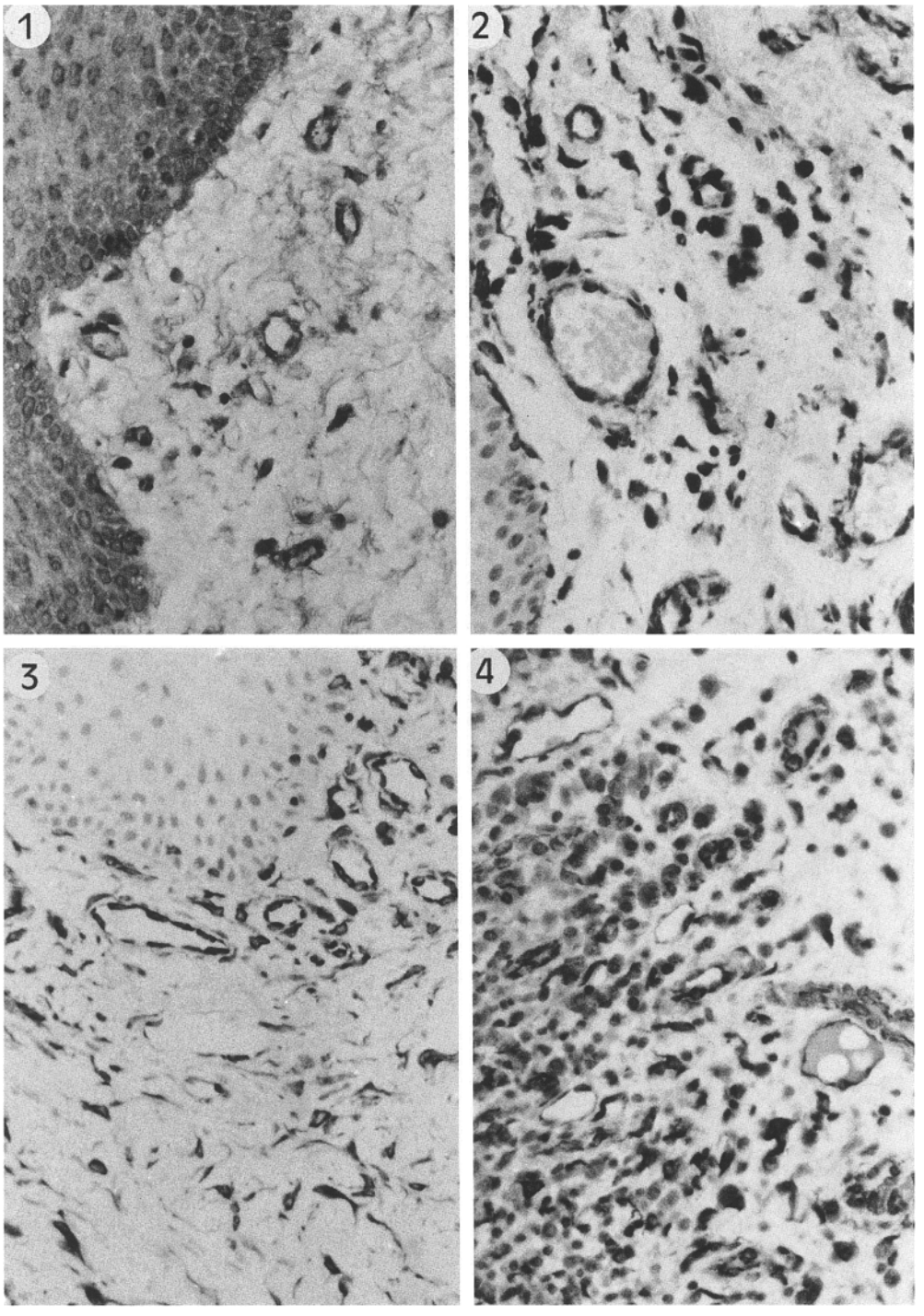

Fig. 1 Normal cheek mucosa. Positively stained capillaries are present in the upper portion of lamina propria. (×400)

Fig. 2 Chronic inflammation of cheek mucosa. An increased number of dilated blood vessels is present in the lamina propria. They are surrounded by numerous chronic inflammatory cells. (×400)

Fig. 3 Fibrous hyperplasia. Blood vessel density is increased in the upper portion of the lamina propria when compared with the normal counterpart. (×400)

Fig. 4 Lichen planus. The number of capillaries is increased in the superficial part of the lamina propria when compared with the normal cheek mucosa. Numerous lymphocytes are present seen between the blood vessels. (×400)

adjacent mesenchymal cells. Fibroblasts were also stained but there was no difficulty in distinguishing them from the blood vessels. In normal tissues the incidence of blood vessels scattered in the lamina propria was low (Fig. 1). The number of capillaries increased relatively near the connective tissue papillae when compared to other sites of the lamina propria. In the reticular region of the lamina propria, a few small diameter arteries with large lumens and thickened walls were observed.

In chronic inflammation there was an increased frequency of blood vessels when compared with the normal counterpart. The lumens of the capillaries located in the upper part of the lamina propria were enlarged. The blood vessels were surrounded by numerous inflammatory cells, particularly lymphocytes (Fig. 2).

In fibrous hyperplasia the distribution and pattern of the blood vessels were similar to those in the normal group except for an increase in frequency of blood vessels which also appeared more dilated (Fig. 3).

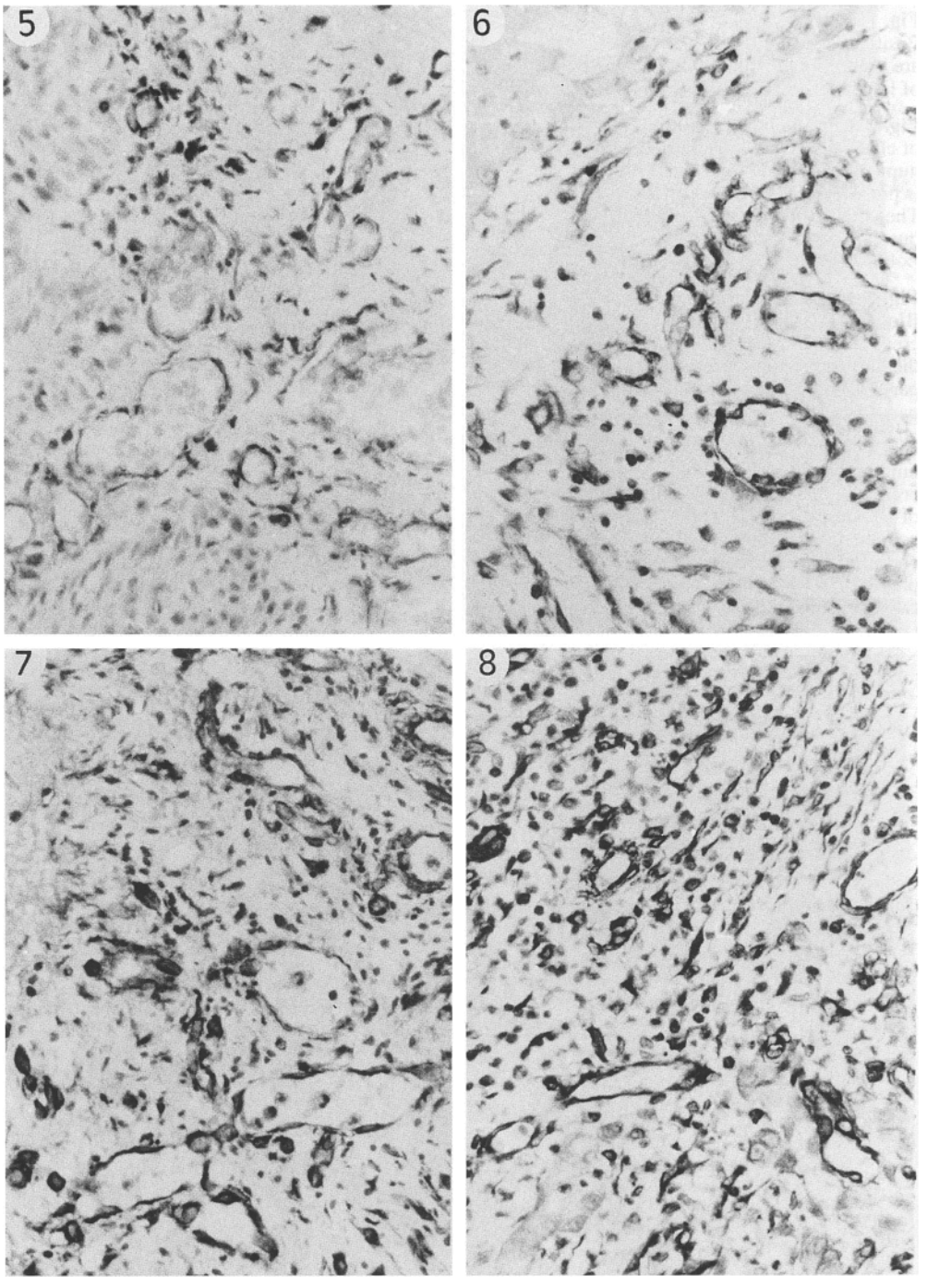

Fig. 5 Dysplasia. Numerous dilated blood vessels are seen in the lamina propria. (×400)

Fig. 6 Epithelium adjacent to the carcinoma. The whole thickness of the lamina propria is vascularised extensively with blood vessels of varying sizes. (×200)

Fig. 7 Squamous cell carcinoma (SCCWD). The size of the blood vessels varies, their frequency is increased and the majority are dilated. (×400)

Fig. 8 Squamous cell carcinoma (SCCMPD). Numerous blood vessels are seen within the carcinoma islands and also surrounding them. (×400)

In lichen planus there was an obvious increase in the number of capillaries and in the density of inflammatory cells in the lamina propria when compared with normal and chronic inflammation groups (Fig. 4).

Blood vessels were increased moderately in frequency in dysplasia and were evenly distributed throughout the whole layer of the lamina propria when compared with the benign and normal groups (Fig. 5). Large diameter capillaries were observed frequently.

In epithelium adjacent to carcinoma the whole thickness of the lamina propria was extensively vascularised, containing varying sizes of blood vessels and large diameter capillaries when compared with the normal counterpart (Fig. 6). The lamina propria was also infiltrated with a moderate amount of inflammatory cells, particularly lymphocytes. The frequency and size of the blood vessels appeared increased when compared with the normal and benign groups but there were no apparent differences in the frequency and size of these vessels between the papillary and reticular parts of the lamina propria.

In squamous cell carcinoma the number and size of the blood vessels were increased and were prominent in

Table 2 Quantitative data of blood vessel parameters (±=Standard Error of Mean)

Groups Parameters	N	CI	FH	LIP	DYS	EAC	SCCWD	SCCMPD
$V_{VBV, CT}$ ($\mu m^3/\mu m^3$)	0.027 (±0.001)	0.053 (±0.003)	0.068 (±0.005)	0.067 (±0.006)	0.076 (±0.010)	0.106 (±0.011)	0.099 (±0.007)	0.089 (±0.007)
$N_{ABV, CT}$ '($\times 10^{-4}$ μm^{-2})	2.54 ($\pm 1.3\times 10^{-5}$)	4.00 ($\pm 3.1\times 10^{-5}$)	4.77 ($\pm 1.95\times 10^{-5}$)	5.34 ($\pm 3.5\times 10^{-5}$)	4.45 ($\pm 4.96\times 10^{-5}$)	4.91 ($\pm 2.21\times 10^{-5}$)	5.07 ($\pm 3.74\times 10^{-5}$)	4.72 ($\pm 3.38\times 10^{-5}$)
$L_{VBV, CT}$ ($\times 10^{-4}$ μm^{-2})	5.00 ($\pm 2.5\times 10^{-5}$)	8.00 ($\pm 6.3\times 10^{-5}$)	10.00 ($\pm 3.92\times 10^{-5}$)	11.00 ($\pm 7.1\times 10^{-5}$)	9.00 ($\pm 9.92\times 10^{-5}$)	10.00 ($\pm 4.41\times 10^{-5}$)	10.00 ($\pm 7.48\times 10^{-5}$)	9.00 ($\pm 6.76\times 10^{-5}$)
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)	99.81 (±6.33)	96.74 (±7.99)

Table 3 Results of statistical analysis. One-way ANOVA followed by Multiple Range Test (LSD); Overlap of (*) down the column indicates that group comparisons are not statistically significant at 95% confidence interval

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

Table 4 Spearman rank correlations

Groups	$V_{VBV, CT}$	$N_{ABV, CT}$	$L_{VBV, CT}$	A_{BV}
r value	0.67	0.37	0.37	0.57
p value	0.00001	0.001	0.001	0.00001

the group when compared with the normal and benign groups (Figs. 7, 8). The increased vascularity seemed to be dependent on the degree of cellular differentiation of the carcinoma. The number of blood vessels appeared to be greater in SCCWD than in SCCMPD. Most of the vascular walls and lumens of vessels in SCCMPD appeared thinner and smaller when compared with SCCWD. The capillaries were usually found in close proximity to the carcinoma islands forming a vascular layer around them (Fig. 8).

The quantitative results are presented in Table 2. Summaries of the statistical analyses between normal and the different pathological groups are shown in Tables 3 and 4 but in detail, the following changes were found:

Volume density

The $V_{VBV, CT}$ increased significantly between normal and SCC groups with the highest value being in the EAC group. The values of $V_{VBV, CT}$ in the benign (CI and FH) and potentially premalignant (LIP, DYS and EAC) groups increased significantly (more than two fold) when compared with the normal group. The values of $V_{VBV, CT}$ in the EAC group increased significantly and were doubled when compared with benign and LIP groups and increased more than three times when compared with the normal group. No significant differences in $V_{VBV, CT}$ were detected between SCCWD and SCCMPD but $V_{VBV, CT}$ was significantly different between SCCWD and CI, between SCCWD and FH, between SCC and LIP, between SCCWD and DYS, between EAC and FH, between EAC and LIP, between EAC and CI and between EAC and DYS.

Numerical density

The parameter $N_{ABV, CT}$ increased significantly between normal and SCC groups. The values of $N_{ABV, CT}$ in the all of the pathological groups was significantly increased almost two fold when compared with the normal group. The value of $N_{ABV, CT}$ was highest in the LIP group. No significant differences in $N_{ABV, CT}$ were detected in comparisons between any of the pathological groups.

Length density

The values of $L_{VBV, CT}$ almost doubled between normal and in all of the pathological groups. The change was statistically significant, with the highest value being in the LIP group. No significant differences in $L_{VBV, CT}$ were detected between any of the pathological groups.

Mean transverse sectional area

The values of A_{BV} increased significantly between normal and SCC groups with the highest value being the EAC group. There were significantly differences in A_{BV} between normal and FH, DYS, SCC, EAC, FH and EAC, FH and SCC, CI and EAC, CI and SCC, LIP and EAC and LIP and SCC groups.

Sperman rank correlations detected a highly positive correlation between the increasing severity of groups lesions and increases in all of the blood vessel parameters.

Discussion

Several studies have shown that neovasclarisation or angiogenesis is an important requirement for continuous growth of tumour cells [7, 13]. This process can be modulated by several angiogenic factors including tumour TAF, endothelial cell growth factors, basic fibroblast growth factors [FGF], transforming growth factors and angiogenin as well as by the extracellular matrix surrounding the blood vessels [6, 9, 10, 18]. However, angiogenesis is not observed exclusively in pathological conditions but occurs also in some physiological events such as wound healing and embryogenesis [21]. In a physiological environment, there seems to be a balance between angiogenic and angiostatic factors but in carcinogenesis, increased activity of angiogenic factors and a decrease in angiostatic factors is the predominant response [17].

Quantitative blood vessel parameters describing the degree of neovascularisation have been used as prognostic indices in different types of tumour. Weidner et al. [27] have found that all patients with high microvessel density count (MDC) had a higher recurrence rate within 33 months when compared with those patients with low MDC. They believed that MDC is a good prognostic index and also a measure of angiogenesis associated with metastasis. Wakui et al. [25] have shown that the $V_{VBV, CT}$ of low and intermediate grades of prostatic carcinoma without bone metastases was similar to that observed in the normal prostate. Values of $V_{VBV, CT}$ of high grade primary prostatic carcinomas were higher when compared with the normal but were similar to those of prostatic carcinoma with bone metastases. They speculated that high grade prostatic carcinoma possessed the potential for tumour metastasis as blood capillary growth might facilitate entry of the tumour cells into the blood vessels, increasing the chance of metastasis. Gasparini et al. [12] have shown that the degree of angiogenesis correlates positively with the prognosis of head and neck carcinomas.

The present study has quantified blood vessel parameters in a variety of oral cheek lesions. These comprised purely benign lesions with no increased capability of malignant transformation [CI and FH groups), potentially premalignant lesions (LIP, DYS and EAC) and overt carcinoma of low and moderate to poor gades. These lesions were chosen in order to document whether subtle changes in the blood vessels in the whole spectrum of the disease process from normal to malignant transformation could be detected by using objective morphometric techniques. The control group was taken from margins of benign oral lesions without cornified or inflammatory cells. This as an important aspect of the study since most studies have used control tissue obtained from the resection margins of malignant lesions. Such specimens may have undergone subtle morphological changes undetectable by visual observation [30].

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 [4, 11, 24]. Monoclonal antibodies against vimentin stains smooth muscle of arterioles and venules but these vessels were readily identified because of their differences in blood vessel wall thickness and luminal size. However, vimentin staining was of great value in identification of tangentially sectioned vessels where morphological identification of blood vessels is difficult. Vimentin antibodies stained endothelial cells of blood capillaries distinctly during tumour angiogenesis [25]. 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. [25] in the study of tumour angiogensis of prostatic carcinoma and by White et al. [28] in hamster cheek pouch carcinogenesis. The length density parameter was determined by assuming that the blood vessels were linear structures rather than being tubular in shape. The calculation of mean transectional area may be affected by the inherent assumption in length density estimation. Furthermore, the numerical density ($N_{ABV, CT}$) 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 section may be due to vessel tortuosity.

Our data implies that neovascularisation occurs in all the pathological human oral cheek lesions investigated, as there was a significant increase in the numerical density of blood vessels (Table 2). $V_{VBV, CT}$, $N_{ABV, CT}$ and $L_{VBV, CT}$ were distinctly different between the N and all of the pathological oral cheek lesions. $V_{VBV, CT}$ increased approximately fourfold between N and SCC groups which could be attributed to significant increases in numerical density and mean transectional area of blood vessels. These data suggest that the increase in relative vascular volume in the stroma of premalignant and malignant oral cheek lesions is accompanied by both angiogenesis and vasodilatation of the blood vessels which may reflect the increasing nutrient requirements of actively growing and dividing cells. It is also possible that increases in A_{BV} might represent a shift to larger diameter vessels rather than the occurrence of vasodilatation in smaller vessels. It could be due also to the effects of the vasoactive substances secreted by inflammatory cells which are present in abundance in the stroma of all pathological lesions except FH. Although the numerical and length densities were significantly different between the normal and all the pathological groups, these parameters were not significantly different between any of the group lesions. These data suggest that the increase in volume density and frequency of the blood vessels is an early event in malignant tranformation of oral cheek lesions and that the increased nutritional requirements are accomplished by vasodilatation. The highly positive correlations between the different groups lesions and all of

the blood vessels parameters (Table 4) support the concept that angiogenesis is correlated with the malignant and metastastic potential of human oral cheek lesions. Similar findings to those observed in the present study were also described in cheek pouch mucosa experimentally treated with DMBA. The volume, numerical and length densities as well as the mean transverse sectional area of blood vessels significantly increased in chemically induced oral neoplasia when compared with the untreated mucosa. The blood vessel parameters in these lesions were characterised by increases in vascular volume density as a result of increased in both vessel profile frequency and increased individual size [28].

The EAC group had the highest value of $V_{VBV, CT}$ and A_{BV} amongst the pathological lesions. Furthermore, $N_{ABV, CT}$ and $L_{VBV, CT}$ had values that were similar to those of the SCCWD. Three of the six cases of EAC showed evidence of cellular atypia and Wright & Shear [30] described early changes of dysplasia in epithelium immediately adjacent to oral squamous cell carcinomas. The LIP group also showed the highest value of $N_{ABV, CT}$ and $L_{VBV, CT}$. DYS, EAC and LIP are considered to have an increased risk of developing malignancy.

In conclusion, the present study suggests that angiogenesis occurs in premalignant and malignant lesions of human oral cheek epithelium and that the blood vessel parameters may have diagnostic and prognostic potential.

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