Anatomical Site Variation in Oral Mucosal Vascularity

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Purpose: Normal oral mucosa is often used as a control material when studying angiogenesis. However, it is not known whether there is regional variation in vascularity within the oral cavity or indeed between keratinised and non-keratinised mucosa. Therefore our aim was to compare vascularity between anatomical sites to determine if such variation should be taken into account when using normal mucosa as a control for the investigation of pathological neoangiogenesis.

Materials and Methods: The angiogenic profile of frozen sections from four oral sites was assessed in 30 post mortem cadavers. Blood vessels were identified immunohistochemically using four antibodies and quantified by two methods.

Results: No difference was found between keratinised and non-keratinised oral mucosa. Use of alcohol was associated with an increase in vasculature and tobacco use was associated with a decrease (\(\alpha\)-MVD). vWF and CD-31 were consistently associated with higher values.

Conclusions: High risk sites for oral cancer in the United Kingdom did not display increased angiogenic profiles. Neither age nor gender affected the vascular count. Whilst there was no difference between keratinised and non-keratinised sites, due to regional variation in vasculature, it is recommended that controls should be taken from the same oral site as the lesional material.

Key words: angiogenesis, oral mucosa, vasculature, site variation

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INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing vessels and has been shown to precede overt tumour formation (Folkman et al, 1989). Although angiogenesis cannot be measured directly, it can be inferred by quantification of the vasculature (here after referred to as vascularity), thus providing an index of angiogenesis (Weidner et al, 1991). A number of studies have investigated the expression of various putative markers of angiogenesis, by comparing normal tissue with benign pathological lesions, dysplasias, and carcinomas. These studies reported an increase in vascularity with the transition from normal to dysplasia, and from normal to carcinoma regardless of the marker or methodology used (Jin et al, 1995; Tipoe et al, 1996; Ravi et al, 1998; Sauter et al, 1999; Pazouki et al, 1997).

In normal oral mucosa blood flow is greatest in the gingivae and tongue compared to that of the hard palate and the buccal mucosa but in all regions of the oral mucosa it is greater than in the skin at normal body temperatures (Ten Cate, 1994).

It is not known to what extent regional differences in vascularisation influence measurements (Collins, 1994) when comparing vascularity of normal mucosa to that of pathological processes. In order to determine the extent of such variation multiple biopsies from the same individual are necessary. However, acquiring such tissue from patients is not ethically acceptable. Our previous work demonstrated that oral tissue could be used for immunohistochemical analysis for up to 14 days post mortem (Ogden et al, 1993).

Therefore the primary aim of this study was to determine the anatomical site variation in the vascular pro-
file in post mortem oral specimens. Furthermore skin specimens were used for comparison.

**MATERIALS AND METHODS**

Ethics committee approval for the acquisition of post mortem tissue had been obtained. Post mortem samples of clinically disease-free oral mucosa were obtained by 5 mm punch biopsy from four oral sites: dorsal tongue (DT, 30); ventral tongue (VT, 29); hard palate (HP, 22); and buccal mucosa (BM, 24) from 30 cases (total of 105 specimens). Sub-auxilliary skin was harvested from 19 of the 30 cases for comparison. All samples were taken within 14 days post mortem. Clinical details (age, gender etc) were recorded from the notes of the Forensic Medicine Department, Dundee Royal Infirmary. In some cases details about smoking and alcohol consumption were also available (Table 1). Smoking was defined as anyone who smoked regularly (minimum of 5 cigarettes/day). Alcohol was defined as an intake of greater than 21 units per week for men and 14 units per week for women.

Post mortem tissue was transported in Carmichael’s transporting medium (Ogden et al, 1993) and placed in liquid nitrogen/isopentane before storage in liquid nitrogen. The frozen samples were cut into 5 μm sections, which were placed onto a 3-Aninopropyl Triethoxy-Silane coated slides followed by incubation of the sections in 3% hydrogen peroxide in phosphate buffered saline (PBS) for 10 minutes. Sections were incubated in normal goat serum (Diagnostics Scotland, Carluke, Scotland) at 37 °C for 20 minutes prior to incubation with the appropriate primary antibody at 4 °C overnight (Table 2). The sections were then incubated with the appropriate biotinylated secondary antibody (anti-rabbit for factor VIII or von Willebrand’s factor (vWF) and anti-mouse for CD31, alpha smooth muscle actin (αSMA) and alpha v beta 3 integrin (αvβ3), all from Vector Laboratories Ltd) followed by the avidin-biotin complex (ABC) (Elite Vectorstain Reagent Kit, Vector Labs, Peterborough, England). Visualisation was achieved by incubation with 3,3’-diaminobenzidine (DAB) (Sigma) for 5 minutes and counterstained with Mayers heamatoxylin and eosin. Sections were then rehydrated in graded alcohols and mounted in a xylene-based solvent DPX (Merck Ltd). The antibodies used identified vascular endothelial cells (CD-31, vWF and αvβ3) the latter marker purportedly identifies neoangiogenic vessels, and αSMA identifies perivascular cells (pericytes and smooth muscle cells) indicative of mature vessels. Therefore this combination of commonly used vascular markers should allow the staining of both immature and mature vessels with minimal if any lymphatic vessel identification.

**Quantification of Microvessels**

A microscope (Leitz Wetzlar) with an eyepiece grid (Mertz) containing 100 points was used to assess the sections (×200 magnification). Two methods of scoring the vascularity were used: the microvascular volume (MVV) from 10 fields (Pazouki et al, 1997); and the average microvascular density (a-MVD) (Schor et al, 1998a). The MVV was calculated by the conventional stereological method of point counting ten random fields (Pazouki et al, 1997). Any vessel that coincided with a point on the eyepiece graticule was counted and the vascularity expressed as a percentage volume. The a-MVD technique involved counting the total number of vessels within the grid in ten fields and expressing the mean vascularity as the number of vessels per mm².

**Statistical Analysis**

The data for each of the five sites were analysed using Minitab computer software (version 12.1). Compari-
sons between the methods of quantification for the five sites were compared using a one way analysis of variance (ANOVA). This was followed by post-ANOVA Fisher’s test.

RESULTS

Positive staining was found for each of the four anti-vascular antibodies in every oral tissue examined. Variation in the vascular profile between the five anatomical sites, with each of the four antibodies, using both the MVV method of quantification is shown in Fig. 1 and the results of the a-MVD method is shown in Fig. 2. The data for each of the markers was pooled in order to analyse the variation between anatomical sites with each quantification method.

The MVV was statistically significantly greater in the DT than in skin for all four vascular markers, and in the VT the MVV was significantly greater with \( \alpha \beta 3 \), \( \alpha SM \) and CD31 \( (p<0.05) \) compared with the skin. However, the HP and BM was significantly more vascular than skin for only one marker, \( \alpha \beta 3 \) \( (p<0.05) \). When comparing different oral sites the MVV was statistically significantly higher in DT than the following sites: VT \( (\alpha \beta 3) \); HP \( (\alpha SM \) and CD31 \( (p<0.05) \). The vascularity was greater in the VT than HP \( (\alpha SM) \) and also greater than BM \( (\alpha SM, CD31) \) \( (p<0.05) \).

Using a-MVD a significantly higher vascularity was found in oral sites compared to skin with all four markers in DT, VT and BM \( (p<0.05) \), although in HP no difference was found using vWF compared with the skin. Comparison between oral sites suggested that DT had a significantly greater a-MVD than HP when using vWF, \( \alpha SM \) and CD31 \( (p<0.05) \) and also significantly greater than BM when using vWF and \( \alpha SM \) \( (p<0.05) \). A significantly greater a-MVD was found in VT than HP only with CD31 \( (p<0.05) \). Although there was a slight increase in vessel counts for younger males, no significant difference was found between the vascular markers based on the patients’ gender or age (median or above and below median), regardless of the marker or method used. Alcohol consumption was associated with a greater MVV for vWF and \( \alpha \beta 3 \) than abstinence \( (p=0.041, p=0.014) \). When using the MVV method, no statistical difference was found between smokers and non-smokers. Quantification of vascularity using a-MVD and vWF found a significantly higher vascularity in patients that consumed alcohol than those that abstained \( (p=0.031) \). Tobacco use was associated with a significantly lower \( \alpha \beta 3 \) expression than in non-smokers \( (p=0.019) \).

The integrin \( \alpha \beta 3 \) is thought only to be expressed on the endothelial cells of “new” angiogenic vessels therefore indicating truly angiogenic vessels (Drake et al, 1995). For this reason the data was expressed as a ratio of \( \alpha \beta 3:vWF \) and \( \alpha \beta 3:CD31 \) and also \( \alpha SM:vWF \) and \( \alpha SM:CD31 \) and anatomical variation assessed. These ratios were not statistically different between all four oral sites. However, these ratios were lower in the skin than in the oral tissues but only reached significant levels for the ratio of \( \alpha \beta 3:vWF \) \( (p<0.05) \). The sum of the values for \( \alpha \beta 3 \) and \( \alpha SM \) were compared with vWF and CD31 and a significantly lower MVV was

![Fig. 1 Variation in Microvascular Volume (MVV). The MVV was assessed in four intraoral sites (dorsal tongue (DT), ventral tongue (VT), hard palate (HP), and buccal mucosa (BM)) and armpit skin (APS) from post mortem specimens using four antibodies. The MVV is expressed as a percentage of the area positively stained with each of the vascular markers. (see text for details)](image1)

![Fig. 2 Variation in average Microvascular Density (a-MVD). The a-MVD was assessed in four intraoral sites (dorsal tongue (DT), ventral tongue (VT), hard palate (HP), and buccal mucosa (BM)) and armpit skin (APS) from post mortem specimens using four antibodies. The a-MVD is expressed as the number of vessels per mm³. (see text for details)](image2)
DISCUSSION

Tumour growth beyond 1-2 mm³ depends on the induction of a functional microcirculation from the surrounding host tissue (Folkman et al, 1990). In animal studies Grunt et al (1986) described three phases of tumour angiogenesis: initially sinusoidal dilated host capillaries form globular outgrowths; then radially arranged sprouts, which preferentially arise from venous host vessels, grow into the centre of the tumour. This is followed by necrosis of the central component of the tumour and the development of a central avascular cavity. Thus the tumour vascular system is organised like a hollow sphere, with a central cavity and a peripheral vascular ‘envelope’ with large vessels embracing the tumour and centrifugally growing vasculature sprouts, which arise from the venous component of the vascular ‘envelope’ and invade the surrounding host tissue. Later many vessels of the basket-like envelope obliterate so that the peripheral vascular density decreases continuously. Thus vascular sprouting and proliferation of viable tumour cells is confined to the basal regions of the tumour. These newly formed vessels have leaky and weak basement membranes that allow tumour cells to penetrate more readily than mature vessels. In fact Benjamin et al (1999) found that some tumour associated vessels lack peri-endothelial cells. In one study intratumoural arteriole density was found to be significantly associated with histological grade, proliferative activity, and patient survival in hepatocellular carcinoma (Morinaga et al, 2001). A study on disease progression in the development of laryngeal carcinoma found that vessel size, configuration and intensity of vWF staining increased with disease progression, maximal with advanced stage carcinoma (Laitakai et al, 2004). Hannen et al (2002) found that only vessels with a diameter greater than 10 μm could be considered functional and therefore involved in the process of metastasis. They concluded that the microvascular density of tongue squamous cell carcinoma was not truly reflective of the functional vascularity of these tumours as they found a significantly greater microvascular density in non-metastasised compared with metastatised tumours. This suggested that the greater vascularity was accounted for by small diameter vessels. A thorough review of the literature revealed no accounts of the investigation and contribution of the venous system to tumour prognosis.

The majority of oral studies have utilised endothelial specific antibodies such as factor VIII (vWF), CD-31 or CD-34 related antigens. However, no single antibody is sufficiently specific for staining endothelial cells (Vermeulen et al, 1996). CD-31 binds a 130-kDa transmembrane glycoprotein platelet endothelial cell adhesion molecule-1 (PECAM-1) of the immunoglobulin superfamily. It is expressed on the surface of circulating platelets, monocytes, neutrophils, and selected T-cell subsets and is a constituent of the endothelial intercellular junction. PECAM-1 is found in large amounts on endothelial cells and is less abundant on platelets and most leukocytes. It plays a major role in a number of cellular interactions, most notably in the adhesion cascade between endothelial cells (ECs) and polymorphonuclear leukocytes, monocytes, and lymphocytes in inflammatory processes and between adjacent endothelial cells during the process of angiogenesis (Sun et al, 1998; Nakada et al, 2000). Using CD-31 cross reactivity can occur with plasma cells, platelets and lymphocytes (Parums et al, 1990).

Von Willebrand factor (vWF), a multimeric glycoprotein, is synthesized exclusively in ECs and megakaryocytes and stored in Weibel-Palade bodies in ECs and in platelet-granules (Mannucci, 1995). As carrier for coagulation factor VIII in the plasma, vWF may coordinate formation of fibrin and platelet-rich thrombi. Under physiological conditions, vWF is released from ECs into the plasma and to the abluminal cell surface, where it binds to the subendothelium. Here it mediates initial platelet adherence to the subendothelium by linking to specific platelet membrane receptors and to constituents of subendothelial connective tissue. In pathological conditions, ECs stimulation by agents such as thrombin, fibrin, histamine, epinephrine, vasopressin, endotoxin, cytokines, components of the complement systems, and shear stress is followed by a rapid release of vWF from the storage granules into the circulation (Lopes and Maeda, 1998; Meert et al, 2002; Zanetta et al, 2000). However, vWF is not expressed equally in all ECs with less vWF stored in the ECs of microvessels compared with macrovessels and greater expression on the venous side than the capillary side of the circulation. Cross reactivity can occur due to the presence of vWF within lymphatic endothelium, megakaryocytes and platelets reducing its specificity and the presence of vWF in plasma contributes to background staining (Wang et al, 1994).

De Jong et al (1995) demonstrated that CD-31 was more sensitive but less specific than vWF for staining.
endothelial cells. Miettinen et al (1994) showed that the endothelial markers CD-34, CD-31, vWF and BNH9 in vascular tumours all labelled normal vascular and lymphatic endothelial cells approximately equally with the exception of CD34 which showed inconsistent expression within the lymphatics. In addition, CD34 stained fibroblasts and aortic smooth muscle cells, and BNH9 reacted with many epithelial cells including squamous and gastrointestinal epithelia. Another comparison of CD-34, CD-31 and vWF in malignant B-cell lymphomas found a significantly larger number of vessels were stained with anti-CD34 than with anti-CD31 or anti-Factor VIII. Moreover, among the smallest capillary-sized vessel profiles in all lesion types, there was a selective relative loss of stainability of anti-CD31 and anti-Factor VIII, resulting in a substantial total loss of visualised capillary-sized vessels compared with anti-CD34 (Norrby and Ridell, 2003).

The monoclonal anti-α-smooth muscle actin is specific for the single isoform α-actin that is expressed by pericytes and smooth muscle cells. They are regarded as constituents of mature blood vessels (Hellstrom et al, 1999). However, Kurz et al (2002) found that αSMA was not a marker for pericytes. Kinouchi et al (2003) used the ratio of αSMA positive vessels to CD-31 positive vessels as an indicator of vessel maturity and found that high grade renal cell carcinomas had a significantly lower ratio than low grade carcinomas suggestive of vessel immaturity. Another study using such a ratio found poorer survival with Barrett’s carcinoma if there was greater CD-31 staining compared with αSMA (Möbius et al, 2004).

The integrin αβ3 is a heterodimeric cell membrane adhesion molecule that plays an important role in maturation of blood vessels of the stroma (Jones et al, 1997). This integrin is also expressed on certain activated leucocytes, macrophages and osteoclasts. However its expression appears most prominent on cytokine activated endothelial or smooth muscle cells and therefore is thought to play a critical role in the process of angiogenesis particularly in the mediation of cell-matrix interactions (Drake et al, 1995; Brooks et al, 1994; Varner, 1997).

The blood supply of the oral mucosa is extremely rich, consisting of a deep venous plexus of large vessels in the submucosa which gives rise to a secondary plexus in the papillary layer of the lamina propria. The epithelium itself is avascular and therefore its metabolic needs must come via the vessels of the lamina propria (Avery, 1988). Anatomical variations occur such that blood flow through the mucosa is greatest at the gingivae and the tongue compared with the hard palate and the buccal mucosa (Ten Cate, 1994). Given that angiogenesis is essential for tumour growth, our study hypothesised that tumours arise more frequently in the UK in sites with a higher vascular component. In the UK the sites most predisposed to carcinogenesis are the ventral aspect of the tongue and the floor of the mouth. Could it be that there is a relationship between vascularity and cancer predilection? In order to investigate anatomical variations, multiple biopsies from four sites in the same individual were required. As no ethical grounds for such multiple biopsies from patients could be found, post mortem tissue was used. Our previous work on such tissue showed that vascular markers remained reliable for at least 3 days post mortem; in fact little change occurred over a 14 day period (unpublished data). One advantage of using post mortem tissue was that the cases could be selected to match age and risk factors for oral cancer.

The results showed that vascularity in the oral mucosa was significantly greater than the skin. The significance of this difference depended upon not only the quantification method, but also the marker used. For example the dorsum of the tongue had a greater vascularity than skin with all four markers using MVV and a-MVD, however, the hard palate was only greater than skin when using αβ3 (MVV method) and when using αβ3, CD-31 and α-SMA (a-MVD method). Comparisons between oral sites found that the dorsum of the tongue had the greatest vascularity with both MVV and a-MVD. Again the significance of the result differed, depending upon the marker used. However, no significant difference was found between keratinised and non-keratinised mucosa.

The vascularity measured using vWF (MVV and a-MVD) and αβ3 (a-MVD) was greater in patients that consumed alcohol than non-drinkers. As regards exposure to tobacco, αβ3 (a-MVD) was greater in non-smokers than smokers. However the caveat to these findings is that not all the information regarding smoking and alcohol was available for the total samples, therefore the data was only analysed for 20 and 21 patients respectively. Our findings suggest that alcohol may potentially stimulate angiogenesis although the mechanism for this is unknown. Also, constituents of tobacco smoke may inhibit new blood vessel formation, which might help explain, in part, why oral mucosal healing is delayed in smokers. In fact, although smoking has been shown to inhibit angiogenesis in vitro (Melkonian et al, 2000) nicotine has been shown to stimulate angiogenesis (Heeschen et al, 2001).

The higher values for vascularity achieved with vWF and CD-31 could be because both markers stain
lymphocytes and fibroblasts leading to an over-estimation of the vascularity. In agreement with Chandrachud et al (1997) CD-31 produced greater values than vWF. Also the method of quantification affected the vascularity when using α-SMA as this purportedly recognises mature vessels, which being of a larger diameter would give higher values with MVV than a-MVD.

Although it is commonly stated that vascularity is higher in tumours than in the corresponding normal tissue, this clearly depends on the location and type of the tumour (unpublished work). Schor et al (1998b) reported that vascularity values in lung tumours were not significantly higher than those recorded in the normal bronchus. This suggests that the site under investigation is a critical factor when considering vascularity.

The markers αβ3 and αSMA identify antigens associated with both new and established vessels respectively. Therefore the sum of the data from these two markers may provide an index of the overall vascularity of the tissues. However, compared with the sum of those two markers vWF consistently underestimates the vascularity regardless of the method of quantification (p=0.009 for MVV). C D31 is not significantly different than the combined value with both methods, although there was a tendency to overestimation with a-MVD. However, since αβ3 positive staining was found throughout the oral cavity and the skin it may not be unique to neovascularisation.

CONCLUSIONS

Our findings suggest that differences exist between vWF and CD-31 in oral tissues, with CD-31 producing higher vascularity values which may indicate a predisposition towards false positives. Both αSMA and CD-31 give higher values for vascularity than αβ3. The latter marker is not unique to new vessels. Oral vascularity is significantly higher than that of skin, and although differences exist between intra-oral sites with dorsal tongue being the most vascular (which should be considered when comparing normal with tumours), there appears to be no association between site predilection and oral cancer in this population. Whilst unlikely, it is possible that in other ethnic groups where oral cancer commonly arises in other oral sites e.g. buccal mucosa in Asia, that this site has a higher vascular count. No association was found with increasing age or gender, but dependent upon the vascular marker used there was an association with alcohol consumption and tobacco use. The fact that αβ3 levels are reduced in smokers may help explain in part why wound healing is delayed in such an environment. Further studies are required to evaluate the level of tobacco exposure and alcohol consumption on stimulating a vascular response.

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