Intra-epithelially entrapped blood vessels in ameloblastoma

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BACKGROUND: The ameloblastoma is a benign but locally aggressive odontogenic neoplasm with a high recurrence rate. While significant progress has been made in our understanding regarding the role of tumoral vasculature relative to the diverse behavioral characteristics of this tumor, no attention has been paid to a distinct subset of blood vessels entrapped within its epithelial compartment. As vascular niches are known to influence tumoral growth, clarification of these vessels is important. The objectives of this study were to investigate the morphologic characteristics of intra-epithelially entrapped blood vessels (IEBVs) in ameloblastoma and to speculate on their relevance.

MATERIALS AND METHOD: Here, we evaluated the frequency, microvessel density (MVD), morphology, and distribution pattern of IEBVs in 77 ameloblastoma of different subtypes based on their immunoreactivity for endothelial markers (CD34, CD31, CD105), vascular tight junction protein (claudin-5), pericyte [α-smooth muscle actin (α-sma)], and vascular basement membrane (collagen IV).

RESULTS: IEBVs were heterogeneously detected in ameloblastoma. Their mean MVD (CD34 = 15.46 ± 7.25; CD31 = 15.8 ± 5.04; CD105 = 0.82 ± 0.51) showed no significant correlation with different subtypes, and between primary and recurrent tumors (P > 0.05). These microvessels may occur as single/clusters of capillary sprouts, or formed compressed branching/non-branching slits entrapped within the epithelial compartment, and in direct apposition with polyhedral/granular neoplastic epithelial cells. They expressed proteins for endothelial tight junctions (claudin-5-positive) and pericytes (α-sma-positive) but had deficient basement membrane (collagen IV weak to absent). Aberrant expression for CD34, CD31, and CD105 in tumor epithelium was variably observed.

CONCLUSIONS: Although rare in occurrence, identification of IEBVs in ameloblastoma could potentially represent a new paradigm for vascular assessment of this neoplasm.

Keywords: ameloblastoma; endothelium; pericyte; tight junction; vascular basement membrane

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for a wide spectrum of physiological and pathological processes including the growth and progression of most solid tumors (1). Tumor-associated blood vessels, like normal blood vessels, consist of endothelial cells, vascular smooth muscle cells/pericytes, and an investing basement membrane (2). However, unlike normal blood vessels, they may display structural and functional abnormalities including thin walls, with defective and leaky endothelium (3), underdeveloped endothelial cell-to-cell junctions (4), and detachment of the smooth muscle and pericytes (5–7). Tumor angiogenesis is regulated by a variety of molecules including CD34, CD31, and CD105 (8, 9). CD34 and CD31 are pan-endothelial markers. CD105/endoglin is a transforming growth factor-β (TGF-β) that preferentially binds to activated endothelial cells (9). As there are no pericyte-specific markers, alpha-smooth muscle actin (α-sma) is used to identify these contractile cells that encircle the abluminal endothelial wall (6). Collagen IV is a major component of vascular basement membrane (10). To measure angiogenesis, quantitation of microvessel density (MVD) is widely used (10).

Ameloblastoma, the most common and clinically significant odontogenic epithelial neoplasm of the jaws, is a benign but locally invasive tumor with a high recurrence rate (11). Recent advances on the behavioral characteristics of this neoplasm have emphasized on the importance of vasculature on its tumor biology (12–23). Several lines of evidence indicated that MVD and distribution could have a role in the diverse biologic conduct of this odontogenic tumor (22, 23). However, to date, no attention has been paid to a distinct subset of blood vessels entrapped within the epithelial compartment of this neoplasm. The objectives of
this study were to investigate the morphologic characteristics of these intra-epithelially entrapped blood vessels (IEBVs) and to speculate on their relevance.

**Materials and method**

**Sample**

Formalin-fixed paraffin-embedded tissues of 77 ameloblastoma cases [26 unicystic (UA), 32 solid/multicystic (SMA), 3 desmoplastic (DA), and 16 recurrent ameloblastoma (RA)] were obtained from the archives of the Oral Pathology Diagnostic and Research Laboratory, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia. These cases were reviewed and selected according to established criteria (11). Patients’ characteristics were recorded.

**Immunohistochemistry**

New 5-μm-thick sections from each selected tissue block underwent immunostaining with various antibodies against blood vessel wall components namely CD34, CD31, CD105, α-sma, collagen IV, and claudin-5 (Table 1). Briefly, antigen retrieval was carried out by microwave treatment (99°C) of deparaffinized sections in 10 mM of citrate buffer (pH 6, 20 min). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min, and sections rinsed in 0.05 M Tris-buffered saline (TBS) (5 min, two times) before immersing in blocking solution (Dako Corporation, Carpinteria, CA, USA) for 20 min at room temperature. The tissue sections were then incubated with optimally diluted primary antibodies (Table 1) for 30 min at room temperature. Immunoreactions were performed using the Envision Kit (Dako). The distribution patterns and immunoreactivity for all markers were evaluated by descriptive and quantitative methods. Digitized images (Olyvia DotSlide Virtual Slide System, Olympus Imaging Inc., Tokyo, Japan) of all slides in each case were assessed by two investigators, and representative sections were selected. For analysis, blood vessels in ameloblastoma were subclassified into two main categories: (i) IEBVs were those present entirely within the tumor epithelial compartment and in direct apposition with the neoplastic polyhedral epithelial cells and (ii) non-IEBVs were those present in (intratumoral) and around (peritumoral) the tumor (within 2 mm² area from tumor invasive front) but located within the connective tissue compartment. The angiogenic index of ameloblastoma was determined based on the mean number of CD34—, CD31—, or CD105-positive blood vessels in five hot spots identified for each marker in the tumoral areas and examined at low magnification (×40 and ×100) (24). Single or clusters of endothelial cells with or without lumen, in similar locations, were considered to be individual vessels. The total number of positive vessels located in the hot spots was assessed under ×200 magnification by two pathologists. The MVD was calculated as the average count from the five hot spot fields of view. These observations were confirmed by visual and computational counts. The measured values were expressed as mean ± standard deviation.

Immunoreactivity for claudin-5, α-sma, and collagen IV in IEBVs was semiquantitatively analyzed for staining intensity and quantity and categorized as 0 = negative, + = weakly, ++ = moderately, and +++ = strongly positive.

**Statistical analysis**

Statistical test was performed using SPSS version 15.0 for Windows. Differences in MVD between groups were compared with Student’s t-test or ANOVA where appropriate. Comparisons for categorical variables were conducted using chi-square test or Fisher’s exact test. A P value of <0.05 was considered to be significant.

**Results**

**Patients’ characteristics**

The study sample was from 39 (50.7%) male and 38 (49.3%) female patients with an overall mean age of 30.6 ± 16.4 years (age range: 10–75 years). Sixty-seven (87.0%) tumors were from the mandible, seven (9.1%) from the maxilla, and a case each from the buccal mucosa (1.3%), submental (1.3%), and pre-auricular (1.3%) region.

**Immunohistochemical findings**

Immunohistochemical results are shown in Table 2 and illustrated in Figs 1–6.

**Microvasculature histomorphology**

Intra-epithelially entrapped blood vessels were identified as small vessels entrapped within the tumor epithelial border.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clonality</th>
<th>Catalogue ID</th>
<th>Manufacturer</th>
<th>Specificity</th>
<th>Dilutions</th>
<th>Positive controls</th>
</tr>
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<tbody>
<tr>
<td>CD34</td>
<td>Mouse monoclonal</td>
<td>M7165</td>
<td>Dako Corporation</td>
<td>Human</td>
<td>1:1000</td>
<td>Hemangioma</td>
</tr>
<tr>
<td>CD31</td>
<td>Mouse monoclonal</td>
<td>M0823</td>
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<td>Hemangioma</td>
</tr>
<tr>
<td>CD105</td>
<td>Mouse monoclonal</td>
<td>M3527</td>
<td>Dako Corporation</td>
<td>Human</td>
<td>1:200</td>
<td>Hemangioma</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>Rabbit polyclonal</td>
<td>Ab53765</td>
<td>Abcam Inc, Cambridge, MA, USA</td>
<td>Cow, human, mouse, pig</td>
<td>1:1000</td>
<td>Salivary glands</td>
</tr>
<tr>
<td>α-sma</td>
<td>Mouse monoclonal</td>
<td>M0851</td>
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<td>Skin</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Mouse monoclonal</td>
<td>M0785</td>
<td>Dako Corporation</td>
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compartment and in direct apposition with the neoplastic polyhedral/granular epithelial cells. These microvessels exhibited a varied morphology and heterogeneous distribution pattern. IEBVs may present as single or clusters of capillary sprouts or compressed slits with or without tortuous short branchings (Figs 1A–E, 2A,B and 3A–C). They stained universally positive for CD34 and CD31, whereas CD105 identified newly formed vessels (Fig. 3A–C). IEBVs exhibited a deficient basement membrane (collagen IV weak to absent) but were heterogeneously positive for endothelial tight junctions (claudin-5-positive) and pericytes (α-sma-positive) (Figs 4A–C and 5A,B). Aberrant expressions for endothelial markers, claudin-5, and α-sma were occasionally observed in the tumor epithelial cells (Figs 1B, 2B, 3A Inset and 5A,B).

### Table 2  Mean microvessel density (MVD) scores for intra-epithelially entrapped blood vessel (IEBV) and non-IEBV in ameloblastoma subtypes

<table>
<thead>
<tr>
<th>Ameloblastoma subtypes</th>
<th>IEBV mean MVD ± SD</th>
<th>Non-IEBV mean MVD ± SD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD34</td>
<td>CD31</td>
</tr>
<tr>
<td>SMA (n = 32)</td>
<td>11.75 ± 16.75</td>
<td>13.59 ± 17.79</td>
</tr>
<tr>
<td>UA (n = 26)</td>
<td>12.07 ± 12.83</td>
<td>10.53 ± 12.72</td>
</tr>
<tr>
<td>DA (n = 3)</td>
<td>26.33 ± 36.96</td>
<td>22.33 ± 35.28</td>
</tr>
<tr>
<td>RA (n = 16)</td>
<td>11.70 ± 17.52</td>
<td>16.75 ± 28.31</td>
</tr>
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</table>

SMA, solid/multicystic ameloblastoma; UA, unicystic ameloblastoma; DA, desmoplastic ameloblastoma; RA, recurrent ameloblastoma.

**Figure 1** Morphologic and distribution characteristics of CD34-positive intra-epithelially entrapped blood vessels (IEBVs) (A–E) and non-IEBVs (F–G) in solid/multicystic (SMA) and desmoplastic ameloblastoma. Note aberrant diffuse expression of CD34 in tumor epithelial cells adjacent to IEBV (B). (F ×40; C, G, H ×100; A, D, E, F Inset ×200; B ×400).
Non-IEBVs, on the other hand, were randomly distributed in and around the tumor epithelium but located entirely within the connective tissue compartment (Figs 1F–H, 2C–E and 4D,E). These microvessels may occur as single or clusters of capillaries with/without lumen or formed a plexus of mature blood vessels. They were stained positive for CD34, CD31, CD105, claudin-5, and $\alpha$-sma. Non-IEBVs consistently demonstrated a continuous linear collagen IV positivity along the basement membrane (Fig. 4D, E).

**Microvasculature histomorphometry**

Positive expression rates in ameloblastoma sample for IEBVs and non-IEBVs as detected by CD34, CD31, and CD105 are shown in Fig. 5. Their differences were statistically significant for each marker assessed. Mean MVD scores for IEBVs and non-IEBVs are detailed in Table 2. For all ameloblastoma subtypes evaluated, mean MVD scores for IEBVs were significantly lower than for non-IEBVs ($P < 0.05$). Mean MVD for IEBV among the different subsets of ameloblastoma were not significantly different ($P > 0.05$).

**Discussion**

In the present study, we provide morphologic evidence for the existence of a distinct subset of blood vessels that are sequestered within the neoplastic epithelial precinct of ameloblastoma. To define the morphologic characteristics of these novel vessels [designated herein as intra-epithelially entrapped blood vessels/IEBVs a term adapted from Funayama et al.’s first description of these vessels in oral carcinoma in situ (CIS) (25)], a sample of 77 ameloblastoma of different subtypes was examined specifically for their immunoreactivity against endothelial markers (CD34, CD31, and CD105), and markers for vascular tight junction protein (claudin-5), pericytes ($\alpha$-sma), and vascular basement membrane (collagen IV). From our analysis, we were able to demonstrate that IEBVs represented a small percentage of the tumoral microvasculature of ameloblastoma and that their frequency and density distribution patterns were heterogeneous and uninfluenced by tumor status (primary vs. RA), clinicopathologic subtype (unicystic vs. solid/multicystic variant), and cellular growth pattern (follicular vs. plex-
In addition, we were able to clarify that IEBVs were highly abnormal in morphology. Similar to blood vessels in other tumors and oral CIS, IEBVs were irregular in size, shape, and branching pattern, showed lack of normal hierarchy and did not display the recognizable features of arterioles, capillaries, or venules (2, 25, 26). It is likely that this haphazard vessel distribution would contribute to the non-uniform perfusion of tumor cells (2). Moreover, evidence indicates that the tumoral vasculature creates a protective ‘microvascular niche’, within which tumor-initiating cells can resist therapy (26).

In this study, the vascular support components were also evaluated. We provide morphologic evidence that IEBVs demonstrated intact vascular tight junctions (claudin-5-positive) and complete pericyte coverage (α-sma-positive). However, the basement membrane identified by collagen IV was absent or incomplete around IEBVs of ameloblastoma. In tumor blood vessels from other organ systems, the basement membrane has been reported as absent, incomplete, or present, but with morphologic or functional abnormalities (7, 27, 28). The vascular basement membrane is a known source of both angiogenic and anti-angiogenic factors (29). Accordingly, during angiogenesis, the vascular basement membrane undergoes continuous remodeling to enable endothelial sprouts to form and new vessels to grow. It is therefore likely that the area containing IEBVs might represent a site of angiogenic activity and that the deficient basement membrane around these microvessels might be indicative of this ongoing remodeling process.

Another notable observation was the aberrant expression of CD34, CD31, CD105, and α-sma in the tumor epithelial cells. A large number of studies have shown that tumor cells secrete angiogenic growth factors to stimulate endothelial cell proliferation and induce angiogenesis (2, 30, 31). Angiogenic growth factors secreted by the tumor cells can directly bind to their receptors on endothelial cells and stimulate angiogenesis to promote endothelial sprouting, branching, differentiation, and survival (2). Our observations have led us to propose that IEBVs identified in ameloblastoma might be histogenetically distinct and may...
possibly be linked to a different mechanism of angiogenic recruitment. Previous studies indicate that in addition to angiogenic-dependent mechanism, non-angiogenic mechanisms such as vasculogenic mimicry and mosaic vessels may exist in certain tumors (30–32). However, unlike these tumor-lined vessels, the IEBVs identified here demonstrated complete endothelial lining and coverage by pericytes. A CD34- and CD31-assessed higher MVD (although not significant) for IEBVs was scored in DA compared to the other subtypes. In contrast, an earlier study reported scantiness of CD34-positive microvessels in tumor areas of DA (21). A plausible explanation for these contradictory results is the vascular heterogeneity exhibited by this subtype. Several lines of evidence indicate that neoplastic angioarchitectural networks exhibit an inherent variability with significant heterogeneity in their structure and distribution (2, 4–6). Other reasons for this discrepancy in findings include sensitivity of different endothelial markers used, selection of hot spot area, and presence of non-angiogenic mechanisms (30). As only a small sample of DA [five cases in Guzman-Medrano et al.’s study (21) and three cases in the present study] was evaluated, this calls for a larger series analysis to resolve the conflicting observations.

Most studies determined the clinical significance of tumor angiogenesis based on MVD scores from intratumoral and peritumoral locations (21, 30, 33, 34). In contrast, little is known about the clinical relevance of tumor angiogenesis based on IEBV vs. non-IEBV densities. Funayama et al. concluded that the recognition of IEBV was helpful in the differential diagnosis of oral CIS and that their data will provide a frame of reference for detecting oral CIS areas using narrow-band imaging (NBI) optical devices (25). In the present study, collectively, the observed significant differences between IEBV and non-IEBV density scores in ameloblastoma, the heterogeneous distribution pattern, and morphologic eccentricities of these vessels led us to suggest that IEBV might be a useful parameter in the vascular assessment of this neoplasm.

In conclusion, this study is the first to demonstrate the existence of a distinct subset of microvessels (IEBVs) enclaved within the tumoral epithelial compartment of ameloblastoma.
ameloblastoma. We highlight its abnormal morphology and density heterogeneity in the different ameloblastoma subsets. Of clinical relevance is that IEBV recruitment in ameloblastoma could potentially represent a new paradigm for future vascular assessment of this neoplasm.

References


**Funding source**

This study was supported by the Ministry of Higher Education Malaysia Fundamental Research Grant Scheme FP038-2013A.