Original Paper

Immunohistochemical characterization of tumoral vessels in oral squamous cell carcinoma

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Abstract

Tumors require a blood supply for growth and hematogenous dissemination. Angiogenesis is one of the mechanism by which tumors acquire their microcirculation. Structurally and functionally, these newborn vessels are abnormal, showing increased permeability, delayed maturation, and potential for rapid proliferation. Such vascular defects could be an explanation for the aggressivity of oral squamous cell carcinoma (OSCC). For these reason we studied the morphology of tumoral vessels in such tumors by using immunohistochemistry and immunofluorescence. Forty formalin-fixed, paraffin-embedded tissue blocks of OSCC were processed for double enzymatic and fluorescence immunohistochemistry. We were interested in analyzing the tumor vessel architecture, and their maturity and activity in such tumors. The tumor vessel architecture had a chaotic pattern, mostly of different sizes, aberrant morphology, tortuous, without clear lumen, and irregularly branches. Regarding pericytes recruitment, the immature and intermediate vessel types (both negative to smooth muscle actin-SMA) were the most numerous type of tumoral vessels. The mature ones (positive to SMA) were readily more numerous at the invasive front of OSCC (85.4 vessels/4 mm² ± 38.3), especially in poor differentiated tumoral type. Investigation of the tumor vessel basal membrane, as reactivity for collagen IV, revealed variability in thickness (2.59 µm ± 0.48), small surface projections, discontinuities and loose associations with endothelial cells; these abnormalities being more obviously at the tumor-host interface and in poor differentiated OSCC. The most active angiogenesis was noticed in poor differentiated OSCC (0.23 ± 0.04), at the tumor-host interface with the immature and intermediated vessel as the most active tumor vessel types. In conclusion, our study revealed some peculiar structurally and functionally defects of tumor vessels in OSCC, changes that could be selective targets for the new developing antiangiogenic drugs.

Keywords: basal membrane, endothelial cell, immunohistochemistry, oral squamous cell carcinoma, pericytes, tumor vessel.

Introduction

As it has been experimentally demonstrated, angiogenesis represents one of the crucial events in the process of tumorigenesis, and consists in the achievement of a wide nutrition sanguine network, based on the formation of new vessels from the existing microvasculature [1]. In a series of classically experimental setups, Folkman J et al. [2] showed that solid tumors do not grow more than 2–3 mm in diameter without auto-increasing their blood supply.

Oral squamous cell carcinoma (OSCC) represents an important pathology of the upper digestive tract, being the sixth common cancer in the world [3]. Although improvements have been achieved in surgical techniques, radiation therapy protocols, and chemotherapeutic regimes, the overall 5-year survival rate for this disease remains at about 50% and has not significantly improved for the past 30 years [4]. On the other hand, angiogenesis is one of the major factors implicated in the progression of OSCC [5–7].

The purpose of our study was to assess the morphology of tumoral vessels in OSCC at the tissue level, by utilizing enzymatic and fluorescent, simple and double immunohistochemistry.

Material and Methods

Tissues and clinical parameters

Forty formalin-fixed, paraffin-embedded OSCC tissue blocks from the archive of the Department of Pathology, No. 1 Emergency County Hospital of Craiova, were included in the current study. All these samples originated from complete resection material.

By reviewing all Hematoxylin-stained slides, we selected the best section from each block, as they included central and peripheral areas of the tumor, avoiding areas with necrosis.

The clinicopathological characteristics of these cases were reviewed from patients’ records. Staging was performed according to the IUCC system, and grading was performed according to WHO.
Antibodies raised against CD105, alpha smooth muscle actin (SMA), FVIII (von Willebrand Factor), collagen IV and Ki67 were utilized to label the endothelial cells, pericytes and the basement membranes (Table 1).

Five-micrometer-thick serial sections were cut from each paraffin-embedded block. The sections were deparaffinized in xylene and rehydrated through graded concentrations of alcohol. These sections were subjected to double immunohistochemistry for different pairs of these antibodies as it is described below. All the experimental procedures have been carried on in the facility of the Research Center for Microscopic Morphology and Immunology (University of Medicine and Pharmacy of Craiova).

### Table 1 – Antibodies utilized in the study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species/clone/producer</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD105</td>
<td>Ms, SN6h, Abcam</td>
<td>1:50</td>
<td>Proteinase K digestion</td>
</tr>
<tr>
<td>CD105</td>
<td>Ms, SN6h, Dako</td>
<td>1:1000</td>
<td>–</td>
</tr>
<tr>
<td>SMA</td>
<td>Rbb, E184, Abcam</td>
<td>1:500</td>
<td>–</td>
</tr>
<tr>
<td>FVIII</td>
<td>Ms, F8/86, Dako</td>
<td>1:25</td>
<td>10 mM citrate buffer, pH 6</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Ms, CIV22, Dako</td>
<td>1:25</td>
<td>–</td>
</tr>
<tr>
<td>Ki67</td>
<td>Ms, MIB-1, Dako</td>
<td>1:50</td>
<td>–</td>
</tr>
</tbody>
</table>

**Enzymatic double immunohistochemistry**

For CD105 (Abcam, Ms)–SMA (Abcam, Rbb) pairs, enzymatic double detection was performed utilizing a sequential procedure and the EnVision G|2 Doublestain System, (DAB+/Permanent Red) visualization system (Dako). After the first antigen retrieval procedure, sections were incubated in 3% hydrogen peroxide in PBS for 15 minutes to block endogenous peroxidase activity.

Sections were next processed accordingly to the EnVision G|2 staining protocol. First, the unspecific antibody-binding sites were blocked utilizing the blocking reagent provided in the kit. The sections were then incubated with the first antibody (CD105) overnight at 4°C. Next day, the sections were washed thoroughly, incubated with the secondary antibody-enzyme system and the color was developed with DAB+ (Dako EnVision G2). After washing, the second antigen retrieval step was performed and then the slides were incubated with the second primary antibody overnight at 4°C (SMA).

In the third day, the signal was detected with the complementary secondary antibody-enzyme system and the signal was developed with Permanent Red (Dako EnVision G2). All antibody dilutions were made in 1% bovine serum albumin and 0.05% Tween.

All sections were finally counterstained with Hematoxylin and mounted in a glycerol-based mounting medium (Dako).

Internal negative controls were obtained by omitting the primary antibodies and external positive controls consisted in tonsil specimens.

**Fluorescent double immunohistochemistry**

Due to its increased sensibility, we have also performed double immunofluorescence on selected slides (five best cases from each OSCC histological type, and on at least five sections from each case), utilizing also a sequential approach.

The maturity of tumoral vessels was investigated on CD105/FVIII and CD105/collagen IV double stained sections, while the activity of tumoral vessels was assessed on CD105/Ki67 double stained sections.

Antigen retrieval was performed prior each antibody incubation as already described. For each pair of reactions, the first antibody was amplified utilizing a tyramide amplification step, while the second antibody was detected utilizing a fluorescent labeled secondary antibody.

Briefly, after antigen retrieval and blocking steps, the sections were incubated with the first antibody over night at 4°C. Biotinylated goat anti-mouse secondaries (Dako) were added for one hour, and then the biotin was amplified with a streptavidin–HRP complex (NEL700 tyramide kit, NEN Life Science Products). Finally, HRP was amplified with a biotinyl tyramide step (NEL700), and this biotin was detected with streptavidin–fluorescein (NEL720) for 30 minutes.

Because all the pairs utilized antibodies raised in the same species (mouse), the reactions were separated by an antibody elution step performed in a blocking solution of a low pH buffer at 50°C for 30 minutes (authors unpublished data).

A second antigen retrieval step was performed and then the sections were incubated over night at 4°C with the second primary antibodies. The next day, the signal was detected with an Alexa 594 labeled goat-anti-mouse antibody (Invitrogen), diluted as 1:300. All antibody dilutions were made in 1% bovine serum albumin and 0.05% Tween.

The sections were counterstained with DAPI for 10 minutes, differentiated in 400 mM copper sulfate solution for 15 minutes and coverslipped with anti-fade mounting medium (Invitrogen).

**Image acquisition and analysis**

The sections were imaged with a Nikon Eclipse 90i microscope equipped with a 5-megapixel cooled CCD camera and with narrow-band fluorescent filters centered for Alexa 594, Alexa 488 and DAPI excitation and emission wavelengths; 20× and 40× images were acquired utilizing a Nikon frame grabber and the Nikon NIS-Elements software. All images were acquired and processed in TIFF format.

For the light microscopy interpretation of the CD105/SMA double stained sections, two observers (PD and SA), without the knowledge of the clinical data, evaluated independently the slides recording the number of immature (CD105+/SMA-) vessels, intermediate (CD105+/SMA+) and mature vessels (CD105-/SMA+), both in the tumors as well as in the invasion fronts. This quantitative analysis aimed to assess the percentage of these three vessel types in OSCC.
Ten most vascular areas (hot spots) within a section were captured at 20× magnification and RGB masks were generated for each stain (DAB for CD105 and Permanent Red for SMA) utilizing the NIS-Elements software.

The investigators quantified the presence of SMA and CD105 on the vessels, as number of vessels/investigated area. Only the signals that corresponded to vessels large enough to have a lumen were included in the study.

For colocalization studies on fluorescent slides, images were obtained by sequential scanning each channel with the specific pair of filters to eliminate the “cross-talk” of chromophores and to ensure a reliable quantification. To ensure that the colocalization was done on signals coming from the same optical planes, the fluorescent images were subjected to a blind deconvolution algorithm based on a multi-pass, adaptive point spread function (PSF) subtraction of diffracted light (AutoQuantX2 demo version, Media Cybernetics).

On the fluorescent slides, the investigations followed three directions, and the assessments were again done as number of vessels/area.

On CD105/FVIII double stained sections, we recorded the number of vessels positive only for each of the markers and for both of them.

On CD105/Collagen IV double stained sections, we recorded the number of new forming vessels (CD105+ only), the number of double stained vessels (thus having a basal membrane) and the thickness of their walls (to differentiate between still immature vessels with a thin and discontinuous basal membrane, and the mature ones with a thicker basal membrane).

The CD105/Ki67 double stained sections were utilized to estimate the number of active tumoral vessel (that are proliferating). The proliferating capillary index (PCI) was determined by calculating the ratio of the number of microvessels with proliferating endothelial cells related to the total number of microvessels on the studied areas.

All records were made or transferred to Excel worksheets and the data were analyzed either in Excel (Microsoft) or in SPSS (SPSS Inc.).

Results

Clinicopathological data

The major clinicopathological characteristics of the patients included in the study are schematized in Table 2.

The mean age of the patients’ group was 66 (range 40–84). Related to gender, the majority of patients were male (29; 72.5%). Topographically, the most common location was on the lips with 21 cases (52.5%), followed by tongue with 14 cases (35%).

The investigation of risk factors revealed that 77.5% of the patients had a history of excessive alcohol consumption, 60% of the patients were heavy smokers and 37.5% of them had both alcohol and tobacco as risk factors. According to the clinical staging, a total of 32 patients (80%) had advanced (III and IV) clinical stages. There were 21 patients (52.5%) who had histological well-differentiated tumors, 11 patients (27.5%) who had tumors with moderate differentiation and eight patients (20%) with poorly differentiated tumors (Table 2).

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29 (72.5)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (27.5)</td>
</tr>
<tr>
<td>Age [yrs.]</td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>15 (37.5)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>25 (62.5)</td>
</tr>
<tr>
<td>Topography</td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>14 (35.0)</td>
</tr>
<tr>
<td>Other localizations</td>
<td>5 (12.5)</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
</tr>
<tr>
<td>Smokers (Sm)</td>
<td>24 (60.0)</td>
</tr>
<tr>
<td>Drinkers (Drik)</td>
<td>31 (77.5)</td>
</tr>
<tr>
<td>Sm + Drik</td>
<td>15 (37.5)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>8 (20.0)</td>
</tr>
<tr>
<td>III</td>
<td>23 (57.5)</td>
</tr>
<tr>
<td>IV</td>
<td>9 (22.5)</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
</tr>
<tr>
<td>Poor differentiated</td>
<td>8 (20.0)</td>
</tr>
<tr>
<td>Moderate differentiated</td>
<td>11 (27.5)</td>
</tr>
<tr>
<td>Well-differentiated</td>
<td>21 (52.5)</td>
</tr>
</tbody>
</table>

Tumor vessel architecture

As it was previously showed [8], CD105-positive vascular endothelial cells were identified in normal oral mucosa and in the microvessels in the tumor resection borders. These vessels were mainly located immediately underneath the epithelium and were regularly distributed, and had regular trajectories and cross-sectional shapes. As a rule, the tumor vessel architectures had a chaotic pattern. The highest vessel density was near the carcinomatous proliferations and in the inflammatory tumor stromal areas. In some cases, the tumoral microvessel architecture was more complex as a vascular network with multiple vascular loops and increased vessel tortuosity [8].

Vessels from the invading tumoral front, where they are often distributed circumferentially, acquire a prominent mantle around islands of carcinomatous proliferations. The same vascular pattern was observed also deep into well-differentiated OSCC, surrounding the carcinomatous islands [8]. Branches from these vessels slink among carcinomatous cells of these tumoral islands.

Tumor vessels varied greatly in size, predominating microvessels of small-caliber (smaller than 15 μm diameter) [8]. The tumor vessels are mostly of aberrant morphology, tortuous, without clear lumen, and branch irregularly. In the inner parts of the OSCC, which typical was less vascularized than tumor host interface, we noticed the existence of individual CD105 positive cells-isolated EC [8].

The largest tumor vessels were observed at the invasion front of OSCC and they had irregular courses and elongated cross-sectional shapes. These aspects are more obvious in areas with inflammatory reaction.
Assessing of tumor vessel maturity

Quantification of pericytes recruitment of the newborn vessels

Based on their size and reactivity to SMA we separated tumor vessels into three categories:

- Immature type consisting of single EC and non-perfused EC sprouts, CD105-positive and SMA-negative (Figures 1–3);
- Intermediate type represented by small vessels, often with visible lumen, but still not covered by pericytes (CD105-positive and SMA-negative) (Figures 1–3);
- Mature type consisting of larger vessels, which were predominantly covered by pericytes (CD105- and SMA-positive) (Figures 1–3).

The quantitative analyze of enzymatic double immunohistochemistry for CD105 and SMA, as we could see from Figure 4, showed that regardless the histological degree of OSCC, there was a clear-cut low proportion of mature vessels (SMA+) among all the counted vessels (p<0.001, Student t-test).

Related to their topography, we concluded that the number of mature tumor vessels were readily more numerous at the invasive front of OSCC (front/tumor: 85.4 vessels/4 mm² ± 38.3 vs. 50 vessels/4 mm² ± 36.4) (p<0.05, Student t-test). This fact was more obvious especially for poor differentiated OSCC.

Quantification of abnormalities and basal membrane formation in newborn tumor vessels

The single EC do not presented type IV collagen immunoreactivity, being the most immature tumor vessels (Figures 5 and 6).

Almost all endothelial sprouts had type IV collagen immunoreactivity along their perimeter. All the other tumor vessels presented variability in thickness (average 2.58 µm; range 0.36 to 8.29 µm; SD 0.48), small surface projections, discontinuities and loose association with endothelial cells (Figures 5 and 6).

These abnormalities progressively decreased from inside to the tumor-host interface. Related to degree of differentiation we noticed that these abnormalities were more obviously in poor differentiated OSCC.

Quantification of so-called functional tumor vessels by estimating the presence of Weibel–Palade bodies in newborn tumor vessels

Almost all-single EC from investigated tumors do not presented Weibel–Palade bodies, missing their reactivity to FVIII (von Willebrand Factor) (Figure 7).
The smallest tumor vessels with clear visible lumen presented a variable immunoreactivity to these antibodies (Figure 7). The more mature tumor vessels, especially those from the invading front were constantly positive to this marker.

Related to degree of differentiation we noticed that immunoreactivity to von Willebrand Factor of the tumor vessels was obviously lower in poor differentiated OSCC comparative with the others (Figure 7).

Regardless the degree of differentiation, the FVIII positive-only tumor vessels were the most numerous at the tumor-host interface (FVIII/CD105+ only vessels: 50.8 vessels/4 mm² ± 36.3 vs. 7.3 vessels/4 mm² ± 4.5) (p<0.05, Student t-test) (Figure 8).

Inside the tumor, the double positive vessels (for FVIII and CD105) were the most numerous regardless the histological degree of differentiation (average 29.33 vessels/4 mm² ± 14; 85 vessels/4 mm² ± 7; 56 vessels/4 mm² ± 11.36 for poor, moderate and well-differentiated types) (Figure 8).

Assessing of so-called active tumor vessel (tumor vessels with proliferating endothelial cells)

Independently with the degree of maturation, we noticed the presence of proliferating endothelial cells in all types of tumor vessels (Figure 7). We identified significant differences in the degree of active angiogenesis between different types of OSCC as well as within one tumor type.

Related to the degree of tumor differentiation, the highest PCI value was noticed in poor differentiated OSCC (0.23 ± 0.04 and 0.12 ± 0.04; for poor, respective moderate histological degrees) (Figure 9).

Quantitatively, we also found that active angiogenesis was more obvious at the tumor-host interface. Even if we did not stained simultaneously the EC, pericytes and dividing nuclei of EC, we could conclude on seriated sections that the immature and intermediated tumor vessel types were the most active (Figure 7).

Discussion

For more than a century, tumors had been observed to be more vascular and comprised of vessels with a different organization, structure and function than normal tissues [9]. In general, it is believed that the patterns of angiogenesis and the types of new blood vessels induced by individual tumors differ strikingly from one another.
Figure 3 – Poor differentiated OSCC, (A–B) vessels from the tumor, (C–D) vessels from the tumor–host interface: immature (arrowheads), intermediate (arrows), and mature (empty arrowheads). CD105 (brown) and SMA (red). Bars represent 25 µm.

Figure 4 – Comparison between the total number of vessels and the mature vessels in the tumor versus the tumoral edges. The number of vessels has been assessed on a 4 mm² area on five slides from each case. Bars represent standard deviation.
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Figure 5 – OSCC vessels from the tumor, (A) vessel without a basal membrane (white arrowhead), (B) mature vessel with a basal membrane (white arrow) and single EC (white arrowhead), (C) vessels with discontinuities of the basal membrane (white arrows) and single ECs (white arrowheads), (D) vessel with endothelial sprout covered by basal membrane (white arrow) and single EC (white arrowhead). CD105 (green, Alexa 488), collagen IV (red, Alexa 594) and DAPI. Bars represent 25 µm.

Nevertheless, Warren BA [9] noticed that vascular morphology of a specific tumor is characteristic for that tumor but may not be unique to that tumor.

In contrast with normal microvessels, tumoral vasculature follows a chaotic pattern and is hierarchically disorganized [9].

Tumor vessels often exhibit a serpentine course, branch irregularly, and form arterio-venous shunts. As we showed here, vessels are most abundant at the invading tumoral front, where they are often distributed circumferentially, forming a prominent mantle around islands of carcinomatous proliferations [8].

The same vascular pattern was also observed deep into well-differentiated OSCC, surrounding the carcinomatous islands. Branches of these vessels slink among carcinomatous cells of these tumoral islands. Overall, the inner parts of the OSCC are typical less vascularized than tumor-host interface.

A previous study [8] proves that quantification of microvessel density with CD105 in OSCC reveals that the highest density was present at the invading tumoral front, followed by intratumoral localization. This confirms the previous observations that CD105 expression declines as we move away from the invasive front of the tumor [10].

These results could set up the basis of an antiangiogenic therapy based on anti-endoglin monoclonal antibodies (mAbs), termed SN6 series mAbs, which in one study provided to inhibited angiogenesis, tumor growth and metastasis in mice in a dose-dependent manner [11].

The first attempt of tumor vessels classification belongs to Warren BA [9] in the late 1970s, which described nine distinct types of tumor vessel. Thus, according to Warren, in tumor microcirculation can be described variances as follows:

- arteries and arterioles resembling those of normal tissues, but with reduced innervation and vasomotion;
- capillaries resembling normal capillaries;
- capillary sprouts consisting of blind-ended, growing branches of existing capillaries;
- sinusoidal vessels representing large vessels with nonparallel EC lining and occasional pericytes;
blood channels with no or incomplete EC lining, consisting in blood percolates in channels lined wholly or in part by tumor cells founded in melanomas and some sarcomas;
- giant capillaries consisting in large vessels (up to 50 µm diameter), lined only by EC with some fibrous supporting tissue, commonly tortuous and describing arcs around the expanding tumor;
- capillary with fenestrated endothelium, commonly found in endocrine tumors but also found in many other types of tumor;
- arterio-venous anastomoses similar to those found in normal tissues where arterioles drain directly into venules or small veins.

This classification is more of an historical interest, the identified vessels types belonging to a large number of different human and animal tumor types.

Recently, by introduction of an adenovirus expressing VEGF-A164 (Ad-VEGF-A164) into mouse hosts, allowed to replicate at least six different types of blood vessels, all of them founded in human and animal tumors [12–14]. These include:
- feeder vessels – large, tortuous arteries and veins whose;
- mother vessels – large, thin-walled, hyper-permeable, light fenestrated pericytes-poor sinusoids that are engorged with red blood cells;
- bridged mother vessels – mother vessels in which EC processes extend into and across the lumen;
- capillaries – resembling normal capillaries;
- glomeruloid vascular proliferation (glomeruloid bodies) – poorly organized vascular structures that macroscopically resemble renal glomeruli; they are comprised of EC and pericytes with minimal vascular lumens and reduplicated basement membranes;
- arterio-venous malformation (stabilized mother vessels) – mother vessels that have acquired an asymmetric coat of smooth muscle cells or, alternatively, fibrous connective tissue, resembling with other arterio-venous malformations founded in other settings.

**Figure 6 – OSCC vessels from the tumor, (A) vessel from within a carcinomatous island without a basal membrane (white arrowhead) and an endothelial sprout (white arrowhead), (B) vessel with a thin, discontinue basal membrane (white arrow) and endothelial sprouts (white arrowheads), (C) vessel with a relative thick and continue basal membrane, but detached from endothelium (white arrow) and single EC (white arrowhead), (D) multiple vessels in stroma of a well-differentiated OSCC with variable basal membrane thickness, with discontinuities and separation from the endothelium (white arrows) and single EC inside carcinomatous islands (white arrowheads). CD105 (green, Alexa 488), collagen IV (red, Alexa 594) and DAPI. Bars represent 25 µm**
Figure 7 – OSCC vessels, (A) small vessels with variable immunoreactivity for FVIII (white arrowheads), (B) an elongated vessels with visible lumen and obvious reactivity for FVIII (white arrow), (C) single ECs without reactivity for FVIII (white arrowheads) and small vessels with variable immunoreactivity for the same marker (white arrows), (D) active vessels with proliferating endothelial cells (arrowheads) together with non active vessel (white arrow) and single EC with proliferate activity (*), (E–F) active vessels (white arrows) with different proliferate activity and scattered single ECs with (*) or without (arrowheads) mitotic activity in a poor differentiated OSCC. (A–F): CD105 (green, Alexa 488), FVIII (red, Alexa 594) and DAPI. (D–F): CD105 (green, Alexa 488), Ki-67 (red, Alexa 594) and DAPI.

Bars represent 25 μm
Figure 8 – Repartition of FVIII-positive tumoral vessels according to the histological degree and region (tumor/edge). Bars represent standard deviation

Figure 9 – PCI values increases with the decrease of the differentiation, for both tumor and edge regions. Bars represent standard deviation

In 2003, Gee MS et al. [15] segregated tumor vessels based on size, perfusion, EC proliferation and the presence of pericytes into three categories:

- immature type consisting in non-perfused EC sprouts, highly proliferative, emanating from functional vessels;
- intermediate type represented by small, perfused vessels which, like the angiogenic sprouts, were not covered by pericytes;
- mature type consisting in larger vessels, which were predominantly pericytes covered with quiescent ECs and few associated sprouts.

As previously described [8], the tumor vessels from all forty studied cases of OSCC are mostly of aberrant morphology, tortuous, without clear lumen and with large gaps between endothelial cells. In addition, they varied greatly in size, predominating microvessels of small-caliber (smaller than 15-µm diameter).

According to Gee MS et al. classification in our casuistry, the immature and intermediate tumoral vessel type prevailed. Deep into the tumor we observed many isolated EC, CD105 positive, SMA and collagen IV negative, and with a variable reactivity for FVIII. These are the most immature vessels, without pericytes and basal membrane and often lacking Weibel–Palade bodies. They have the highest proliferative rate among all other types of tumoral vessels encountered in OSCC. Gee MS et al. [15] found that these single ECs were positive to FVIII and other endothelial markers (CD31 and Tie2), but not for melanocyte-specific antigen S100. In addition, they are non-functional vessels, as proved by the loss of affinity of their nuclei for Hoechst 33342 reagent. Dual staining for FVIII and PCNA revealed that a much greater percentage (54 ± 15%) of single ECs were proliferating. The same authors proved that larger tumors tend to have a lower fraction of nascent vessels and a higher fraction of mature vessels, suggesting that the spectrum of tumor vessels shifts as tumors enlarge [15].

The most encountered tumor vessel type in our study was the intermediate one. These vessels were CD105 and collagen IV positive, SMA negative and with variable reactivity for FVIII. Morphologically these vessels do not present pericytes, but they have a basal membrane with variable thickness and often with discontinuity and with Weibel–Palade bodies. These results are in concordance with those obtained by Gee MS et al. [15]. In their paper, the authors proved that the incidence of intermediate tumor vessels varied between 33–43%, 90% of their EC stains for Hoechst 33342 reagent. Therefore, these vessels were of small-caliber (under 15 µm diameter), perfused without pericytes and smooth muscle cells. The same authors established that the decrease in vessel density with therapy is attributable primarily to loss of pericyte-negative vessels; due both to angiogenesis inhibition and regression of existing vessels. In addition, it is possible that also acquisition of pericytes during therapy is impaired.

The mature type of tumor vessels in our OSCC casuistry was the least encountered type. These vessels were CD105, SMA, collagen IV and FVIII positive, presenting pericytes, continuous basal membrane and Weibel–Palade bodies. According to Gee MS et al. [15] these vessels pericyte covered represents 38 ± 5% of all vessels from 10 untreated K1735 tumors ranging in size from 4 to 13 mm in diameter. These vessels tended to be bigger than pericyte-negative vessels, the mean vessel cross-sectional area being 430 ± 215 µm² vs. 110 ± 75 µm². On confocal imaging of thick tumor sections, it was noted that that larger, pericyte-covered vessels branch off frequently into smaller, pericyte-negative vessels. In these vessels the proliferative index of EC was the lowermost one (14.7 ± 3.9%) from all tumor vessel types. Moreover, Gee MS et al. [15] proved a marked increase in the proportion of pericyte-covered vessels in treated tumors (62% in treated vs. 38% in untreated tumors). This fact may be explained by pericyte production of EC survival factors [16].
or by cell contacts formed with ECs providing survival
signals.

Eberhard A et al. [17] assessed proliferating capillary index (PCI as ratio of the number of microvessels with proliferating endothelial cells versus the total number of microvessels) in six different types of human malignant tumors, namely, glioblastomas, renal cell carcinomas, colon carcinomas, mammary carcinomas, lung carcinomas, and prostate carcinomas. Glioblastomas and renal cell carcinomas had significantly higher PCIs than mammary carcinomas, lung carcinomas, and prostate carcinomas. Glioblastomas and renal cell carcinomas had intermediate PCI values. The same authors quantify the microvessel pericyte coverage index (MPI) as percentage of capillaries associated with a SMA-positive pericytes in those malignant tumors. The lowest MPI values were found in glioblastomas and renal cell carcinomas, whereas mammary and colon carcinomas had the highest MPI values. Lung and prostate carcinomas had intermediate MPI values. The results indicate differences in the functional status of the tumor vasculature in different human malignancy reflecting varying degrees of maturation of the tumor vascular bed.

More recently, Lu C et al. [18] showed that pharmacologic targeting of both endothelial cells (with AEE788–VEGFR inhibitor) and pericytes (with ST1571–PDGF receptor inhibitor) substantially affects in vivo tumor growth and regression and significantly improves survival in an orthotopic ovarian cancer mouse model, particularly in combination with taxane chemotherapy. This antiangiogenic effect may be partially due to decreased pericyte coverage, thus increasing the sensitivity of tumor vasculature to therapy.

Our investigation on collagen IV expression in tumor vessels of OSCC showed variability in thickness, small surface projections, discontinuities and loose association with endothelial cells. Data from the literature specify the existence of discontinuity in basement membrane of tumor vessels [19–21]. On the contrary, Baluk P et al. [22] proved that almost the entire surface of tumor blood vessels was covered with a type IV collagen-immunoreactive layer. These authors showed that only 0.03% of the endothelial surface was devoid of type IV collagen immunoreactivity in the tumor models examined. The presence of instability in the relationship between some components of the basement membrane and EC or pericytes can be explained by the dynamic nature of the tumor vasculature. Baluk P et al. [22] found a complete coverage of most endothelial sprouts by basement membranes. Some reports showed that endothelial sprouts are coated by basement membrane at the outset [22, 23]. This fact was explain by pericytes that cover the sprouts, which contribute to the synthesis of basement membrane [24] at the tip of sprouts [22], ahead of advancing endothelial cell processes, guiding the growth of endothelial sprouts [25].

All these basal membrane modifications of tumor vessels are important because they can be a target for diagnostic or therapeutic agents [26]. Disruption of their contact with basement membrane can lead to endothelial cells’ apoptosis and tumor regression [27–29].

Conclusions

Our study proves that vessels from OSCC are mostly of aberrant morphology, tortuous, without clear lumen, especially of intermediate type with small-caliber, without pericytes and smooth muscle cells and variability in thickness, discontinuities and loose association with endothelial cells on their basal membranes. These morphological features of tumor vessels in OSCC are becoming more and more important as their structural components (endothelial CD105 receptors, pericytes and basal membranes) are becoming targets for the new developing antiangiogenic therapeutic strategies in human malignancies.

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