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Morphological Characterization of Sprouting and Intussusceptive Angiogenesis by SEM in Oral Squamous Cell Carcinoma

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Summary: The word angiogenesis indicates the formation of new vascular segments from existing vessels such as capillaries and venules. Blood vessel formation in tumors is the result of rapid, disorganized vascular growth through two distinct mechanisms: sprouting and intussusceptive angiogenesis. The objective of this study was to elucidate the morphological aspects of these two vascular growth mechanisms in oral squamous cell carcinoma induced in hamster buccal pouch. Eight Syrian golden hamsters had their right buccal pouch treated with DMBA 0.5% and 10% carbamide peroxide for 90 days in order to produce squamous cell carcinoma in this site. Next, buccal pouches of the animals were submitted to the vascular corrosion technique and then analyzed by scanning electron microscopy. The vascular figures of sprouts were observed in the entire vascular network of the buccal pouches, as opposed to the intussusceptive angiogenesis that was predominantly observed in the sub-epithelial network. It was possible to differentiate the figures of sprouts from artifacts by the analysis of the blind ending of these structures. Intussusceptive angiogenesis was identified by the presence of holes trespassing the lumen of the capillaries. Vascular expansion occurred through intussusceptive angiogenesis in two ways: by the fusion of the pillars to form a new capillary and, by increasing the girth of the pillar to form meshes. The method of corrosion associated with scanning electron microscopy proved to be an excellent tool to study the two types of angiogenesis in oral squamous cell carcinoma in the hamster buccal pouch. SCANNING 36:293–300, 2014. © 2013 Wiley Periodicals, Inc.

Key words: oral cancer, corrosion casting, sprouting angiogenesis, intussusceptive angiogenesis, scanning electron microscopy

Introduction

The word angiogenesis indicates the formation of new vascular segments from existing vessels such as capillaries and venules (Risau, '97). Its regulation acts as a balance between the promoters and the inhibitors. The greater or lesser stimulus for vascular proliferation is the result of this balance, suiting the blood supply to the needs for oxygen and nutrients (Bergers and Benjamin, 2003).

This phenomenon is present in physiological and pathological processes, and it is considered essential for the progression of malignant neoplasm (Folkman, '90). As opposed to physiological angiogenesis, the formation of blood vessels in tumors results from the loss of the regulatory balance between positive and negative angiogenesis signaling, which leads to rapid and disorganized vascular growth. This increased vascular network has vessels with different characteristics from those considered normal (Nagy *et al*., 2010).

The ischemic environment induced by tumor growth is considered one of the reasons for the loss of balance between angiogenesis promoters and inhibitors, significantly increasing the expression of pro-angiogenic factors. This is probably due to an inadequate supply of nutrients and oxygen in an environment where there is a rising metabolic demand (Bergers and Benjamin, 2003).

Two distinct angiogenesis mechanisms can be described: sprouting (Ribatti and Crivellato, 2012) and intussusceptive angiogenesis (IA) (Ribatti and Djonov, 2012). The first refers to the proliferation of endothelial cells and the subsequent increase in vessel size, following the

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migration of these cells into the lumen. The mother vessel is then subdivided into several initial smaller vessels (Risau, '97). This type of angiogenesis has the advantage of being invasive and therefore being able to fill vascular gaps, such as in wound healing. However, it is a relatively slow process, characterized by the need for extensive cell proliferation and increased vascular permeability (Ribatti and Djonov, 2012).

On the other hand, vascular intussusception is a process by which a single capillary is divided longitudinally into two by the formation of a transluminal septum. In this case, the endothelial cells on opposite sides of the vessel extend intraluminally to establish contact, in order to form a pillar. This pillar subsequently increases in girth, originating a new capillary network. It occurs when the capillary system expands "within itself" enhancing their complexity, initially without any need for cell proliferation.

The IA is fast, occurring in hours or even in minutes (Burri *et al*., 2004). The intussusceptive pillars can be seen in capillaries and small arteries and veins. Pericytes, smooth muscle cells and myofibroblasts act as contractile elements and produce the collagen fibrils necessary to form the pillars (Burri *et al*., 2004; Hlushchuk *et al*., 2008; Ribatti and Djonov, 2012).

The use of high-resolution imaging methods is of utmost importance for the direct morphological study of different types of angiogenesis. Three-dimensional imaging, such as magnetic resonance and microtomography, currently do not have the necessary definition for these studies, since it is only possible to visualize intussusceptive pillars with equipment that has a resolution of at least 1 μm (Clauss and Breier, 2005).

Analysis of vascular corrosion casts by scanning electron microscopy (SEM) has been considered one of the definitive methods for unequivocal identification of sprouts and intussusceptive pillars (Motta *et al*., '92). The analysis of these casts by SEM allows evaluating the angioarchitecture of the tissue in high-resolution power and with depth of focus. Additionally, applied to the study of tumor vascularization, the resin casts provide not only significant information for morphologists, but also can be useful as a tool to guide the development of new cancer therapies.

In this work, we elucidate the morphological aspects, location and implications of sprouting and intussusceptive angiogenesis in oral squamous cell carcinoma induced in the hamster buccal pouch. We also demonstrate the potential of the method of vascular corrosion associated with SEM to study these two types of angiogenesis.

Materials and Methods

All the experimental procedures carried out in this study and listed below were approved by the ethics committee of the Pontifical Catholic University of Rio Grande do Sul (no. 055/08 CEUA-PUCRS).

Eight male Syrian golden hamsters (*Mesocricetus auratus*) were obtained from Animal House Facility at Federal University of Pelotas (UFPel). At the beginning of the experiment, they were 5 weeks old, weighing approximately 70 g. All hamsters were maintained in ventilated racks under controlled temperature (25°C) and a 12 h light/dark cycle.

The animals were treated with a 0.5% dimethylbenzanthracene (DMBA; Sigma Chemical Company, St. Louis, MO) solution diluted in acetone and, 10% carbamide peroxide gel (Opalescences 10%, Ultradent Products, Inc., South Jordan, UT), in their right buccal pouches for a period of 90 days. The applications occurred on alternate days, 3 days per week for DMBA, and 2 days per week for the carbamide peroxide. The untreated left pouch was considered a control.

Vascular Casting and Corrosion

After tumor induction, all animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (Dopamin®, Laboratório Agribrands, Sao Paulo, São Paulo, Brazil 0.1 mg g^{-1} of animal weight) and xylazine (Anasedan®, Laboratório Agribrands, 0.01 mg g^{-1} of animal weight). Next, a medial incision from the xiphoid process to the pubic symphysis was performed to expose the heart, the aorta and the vena cava. After this, heparin 5,000 IU mL^{-1} (Hepamax-S®, Bausiegel, Brazil, 0.001 mL g^{-1} of animal weight) was applied in the left ventricular cavity followed by incising the left atrium to introduce a 24-gauge cannula. The cannula was fixed at the origin of the ascending aorta and the inferior vena cava and the abdominal aorta were clamped. Another incision was completed in the right atrium to permit solution leakage.

The vascular system was rinsed with approximately 50 ml of saline solution, followed by 20 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and again 50 ml saline solution until the fluid escaping from the right atrium was clear. Then, the cranial blood vessels were manually injected with approximately 10–15 ml of Mercox CL-2RB resin (Ladd Research Industries, Burlington, VT) in a mixture containing 10 g of methyl-methacrylate and 0.25 g of the catalyst benzoyl peroxide, according to the recommendations of the manufacturer.

The resin-perfused animals were kept motionless at room temperature during 2 h for pre-polymerization. The heads were separated and tempered in a warm water bath at 40°C overnight, until the final resin polymerization. The pouches were gently dissected from the animal's head and macerated for 4 days in 7% NaOH solution at 45°C. During maceration, the

specimens were daily washed with warm (40˚C) running tap water, and soaked in a fresh NaOH solution. Once the vascular casts became completely visible with no surrounding tissues, the samples were washed in several changes of distilled water, cleaned in 5% formic acid for 24 h and, washed again in distilled water. The completely corroded specimens (vascular replicas) were dried in a laboratory oven (Odontobras EL 1.1, Odontobras, Brazil) at 37˚C. The corrosion cast technique described above is an adaptation of the method described previously by (Lametschwandtner *et al*., '90).

Scanning Electron Microscopy Analysis

Dry casts were mounted on aluminum stubs using double-faced carbon tape and sputtered with carbon (SCD 005 Sputter Coatter, Bal-Tec, Principality of Lichtenstein) and a final layer with gold (CEA 035 Sputter Coatter, Bal-Tec). The samples were examined with a scanning electron microscope (Phillips XL 30, Eindhoven, The Netherlands) at an accelerating voltage of 5–20 kV and magnifications of 15–5,000 times. The diameter of the electron beam (spot) ranged from three to five and working distance 30–10 mm, depending on the magnitude used to observe the image.

The study of electron micrographs was performed descriptively and comparatively according to (Konerding, '91), evaluating the pattern of organization of the vascular network, path of the vessels, type of blood vessel and angiogenesis figures. The occurrence of sprouting and intussusceptive angiogenesis was qualitatively determined, being considered as absent or present and, when present, rare or numerous.

Results

The SEM analysis of corrosion casts of the buccal pouches without a tumor revealed the existence of two different vascular networks. The pouch wall, from abluminal to luminal, consisted of a vascular system composed of vessels running along the longest axis of the pouch, followed by a delicate vascular network consisting of arterioles, venules, and capillaries, with no definite orientation (Fig. 1). No evidence of a vascular growth was identified in the control pouches. Images of vascular sprouting and intussusception, as well as any other figure of angiogenesis were not present in these casts.

After 90 days of tumor induction, it was possible to notice the formation of new blood vessels by sprouting and intussusceptive angiogenesis. These angiogenesis figures occurred differently according to the vascular network found in the distinct histological layers of the pouch.

The vascular figures of sprouts were found as blind ending vessels with tapering tips, flat, and sharp-pointed. The sprout figures were differentiated from other structures through detailed analysis of the tip of the blind ending vessels. Vessels that ended in a sharp cut surface or smooth and rounded tips were considered as artifacts of the vascular corrosion technique (Fig. 2).

Vascular sprouts and intussusceptive angiogenesis were present in all vascular networks of the buccal pouch. The comparison between their occurrences showed that intussusceptive growth was rarely observed in the outer vascular layers of the pouch, being numerous in the sub-epithelial vascular network (Fig. 3). In this region, the formation of intussusceptive pillars was the dominant structural change.

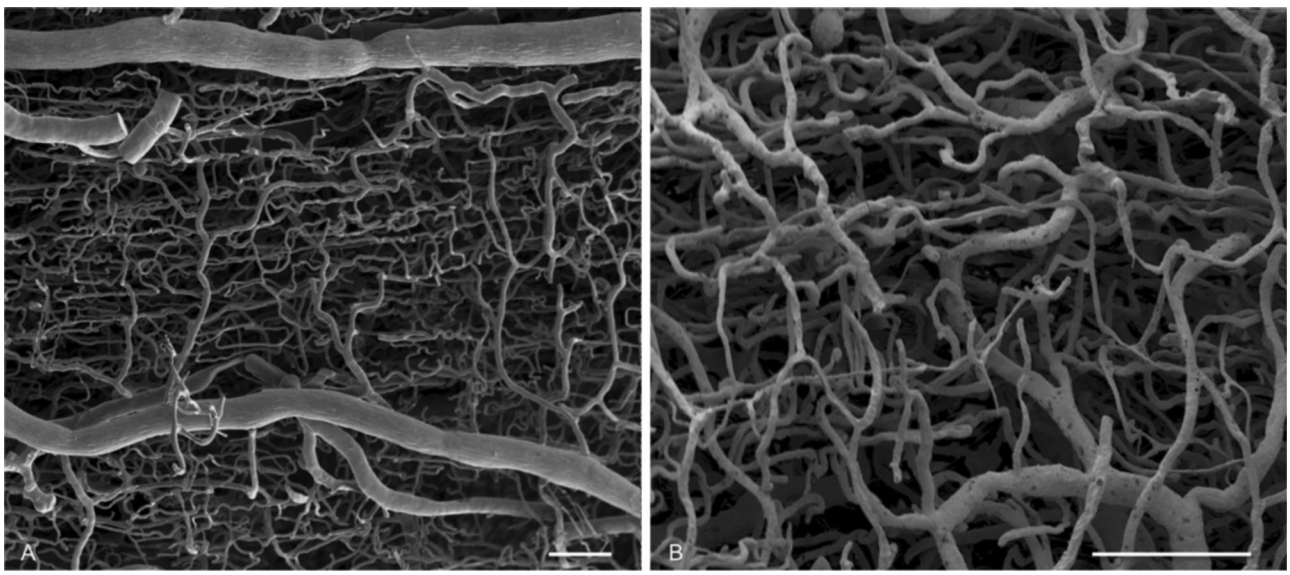

Fig 1. Angioarchitecture of a control pouch. In (**A**) note the vascular network of the outermost layer of the pouch that supplies skeletal muscle. In (**B**) observe the capillary bed of sub-epithelial vascular network found in the luminal surface this organ. Scale Bar: 100 μm.

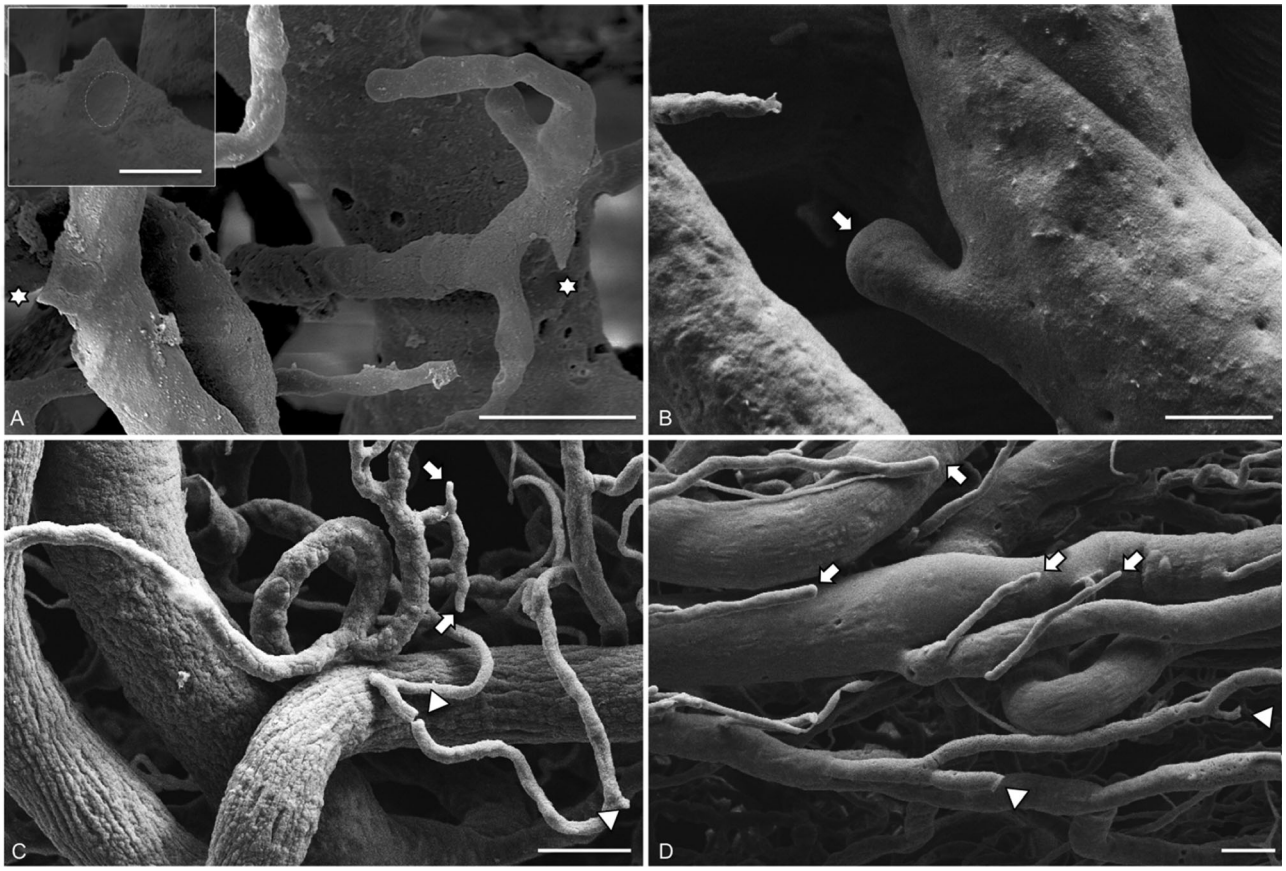

Fig 2. Sprouting angiogenesis and artifacts. In (**A**) observe real figures of sprouting angiogenesis (asterisks). At higher magnification (box; scale bar: 20 μm), it is possible to detect the presence of a rounded imprint (dashed line), suggesting a nuclear imprint of an endothelial cell. In (**B**), note a blind ending vessel with a rounded and smooth tip (arrow), suggesting incompletely filled vessel. In (**C**), observe fractured vessels (arrowheads) and incompletely filled vessels. In (**D**), see long blind ending capillaries with a smooth tip, representing retrogradely injected venules (arrows) and. The arrowheads indicate broken vessels. Scale bars: 50 μm.

The presence of IA in the sub-epithelial vascular network was seen in all samples, mainly in the capillaries. This type of angiogenesis has been identified by the presence of holes trespassing the lumen of the capillaries, representing the formation of transvascular pillars (Fig. 4).

In some areas, where there were numerous holes with a larger circumference in the sub-epithelial capillary network, it may be suggested that this new network was formed by an increase in the girth of the pillars. The result of this expansion was the formation of capillary meshes with many anastomoses, which considerably increased the size and complexity of the vasculature in this region (Fig. 5).

Furthermore, in certain areas were also observed where two or more holes were very close, which merged and split the original vessel into two new vessels. Consequently, vascular expansion occurred through IA in two ways: by the fusion of the pillars to form a new capillary (Fig. 4) and, by increasing the girth of the pillar to form meshes (Fig. 5). No pillar formation was observed at the bifurcations points of two blood vessels.

Discussion

In a previous study, we described the angioarchitecture of squamous cell carcinoma in the Syrian hamster cheek pouch (De Oliveira *et al*., 2009). Now we performed a detailed analysis of the structural characteristics of sprouting and IA after 90 days of tumor induction.

The distinct vascular patterns observed in the buccal pouch without a tumor were in accordance with the histological arrangement of this organ. The delicate blood vessels located at the luminal portion of the pouch correspond to the vascularization of the lamina propria, constituted by dense connective tissue. The second network belongs to the outermost layer of the pouch, showing a characteristic angioarchitecture of skeletal muscle blood supply. Figures of vascular growth were not found in any of these two networks.

Ackermann *et al*. (2013) observed in vascular corrosion casts the presence of intussusceptive pillars in the normal mucosa of intestine, suggesting that IA may be related to the maintenance and repair of the

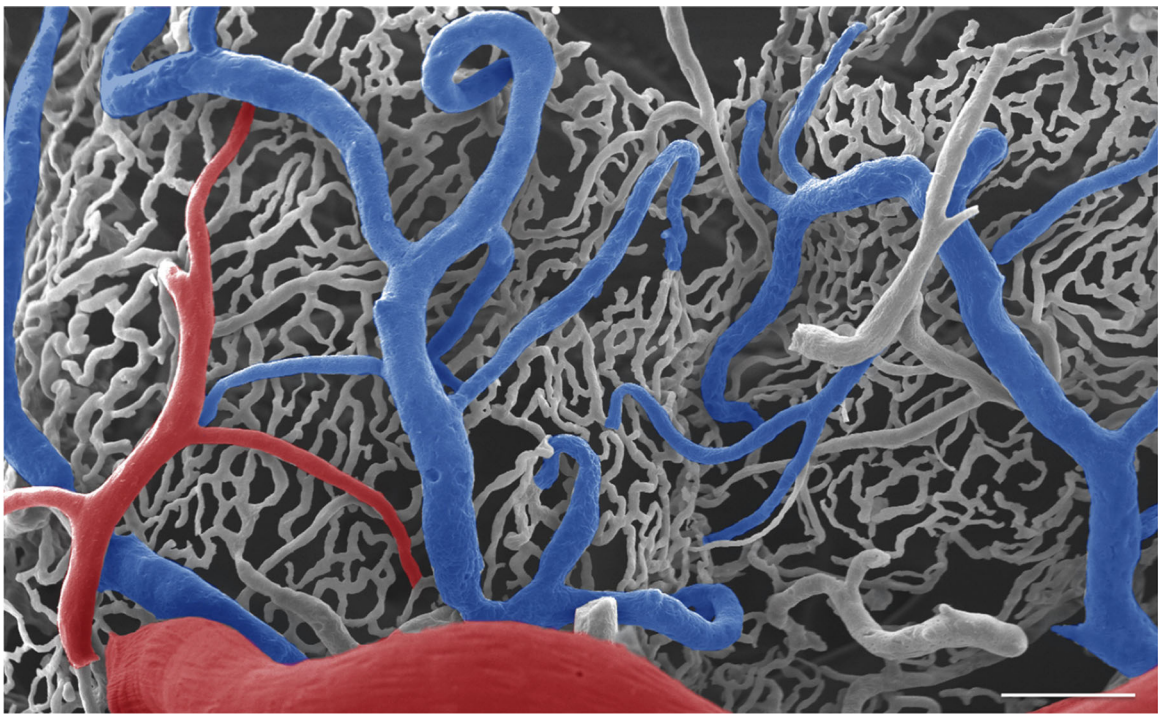

Fig 3. Angioarchitecture of the sub-epithelial vascular network of the buccal pouch after tumor induction. In this image, the sub-epithelial capillaries are situated below the main supplying and draining blood vessels The larger arterial and venous blood vessels were colored respectively in red and blue. Scale bar: 100 μm.

morphostasis in epithelia with high cell turnover. In contrast, these pillars were not found in the vascular network of the buccal pouch, supporting the fact that this organ is essentially related to food storage, performing no intense metabolic activity such as digestion or other enzymatic processes.

Angiogenesis plays a critical role in the growth and expansion of the vasculature (Burri *et al.*, 2004) in response to both physiological and pathological processes, such as inflammation and cancer (Dvorak, 2005). The study of tumor vascular microanatomy is essential to understand tumor hemodynamics and the manner in

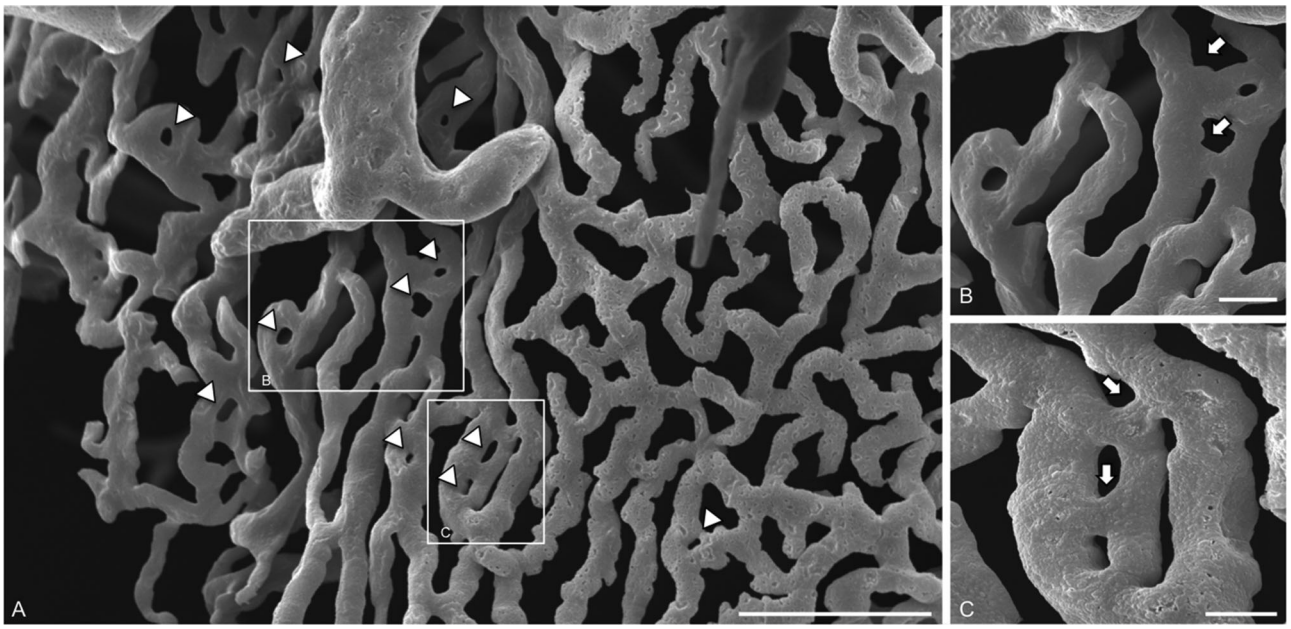

Fig 4. Vascular intussusception in the sub-epithelial capillary network of the buccal pouch. In (**A**), observe the intussusceptive pillars (arrowheads) represented by holes in vascular corrosion casts. Scale bar: 50 μm. Images (**B**) and (**C**) are higher magnifications of (A), showing pillars closely situated, suggesting their fusion to form new vessels. Scale Bars: 10 μm.

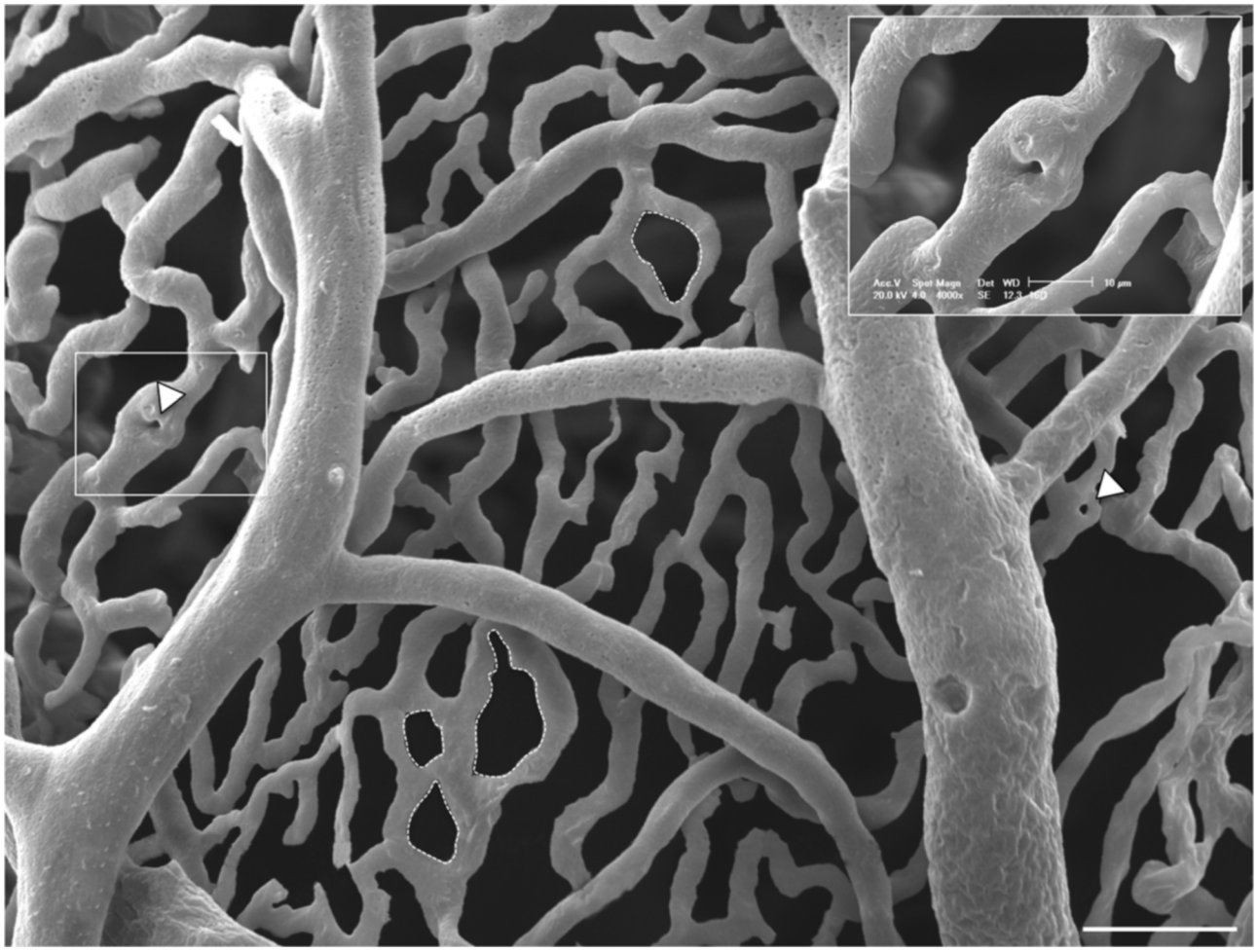

Fig 5. Capillary meshes (dotted line) in the sub-epithelial network of the buccal pouch. These meshes may be formed by the increased girth of the intussusceptive pillars (arrow), enhancing the frequency of anastomosis and the complexity of this vascular network. Scale bar: 50 μm.

which adaptations occur due to vascular changes of the tumor environment during its growth (Belien *et al*., '99; El Emir *et al*., 2007).

It was possible to obtain a comprehensive and detailed view of the vascular architecture of the oral squamous cell carcinoma in the buccal pouch through the vascular corrosion technique associated with SEM. In contrast to the traditional histological techniques of light and intravital microscopy, the vascular corrosion technique allows analyzing blood vessels without the presence of surrounding tissues, or specific cuts, or only on flat surfaces (Motta *et al*., '92). These advantages of the corrosion technique allowed us to identify the two different types of angiogenesis in vascular casts of the buccal pouch by their specific morphological characteristics.

However, the differentiation of sprouts from structures formed by defects during vascular casting may make it difficult to analyze tumor vascular casts. According to (Lametschwandtner *et al*., 2012) sprouts can be identified and discriminated from preexisting vessels or artifacts through a careful analysis of these structures. Sprouts are typically seen as elongated and tapered extensions in a blind ending from parental vessels. Furthermore, the presence of nuclear imprints of endothelial cells at the site of sprout, as observed in the present study (Fig. 2A), reduces the probability of it being an incompletely filled capillary.

Nevertheless, blind ending vessels showing rounded or sharp endings may represent respectively incomplete filling or fractures during specimen handling. The significant changes in blood flow (Fukumura *et al*., 2010) prevent proper vascular casting, increasing the possibility of the presence of artifacts. In our study, the presence of vessels incompletely filled by the resin may have occurred mainly due to these hemodynamic alterations in tumors.

The insertion of the intraluminal pillar, an event that characterizes the onset of intussusceptive angiogenesis (Patan *et al*., 2001), and its expansion may be

observed in vascular corrosion models (Ribatti and Djonov, 2012) through transluminal holes observed in the casts, like those found in our study. Besides the vascular corrosion technique, the three-dimensional reconstruction from serial sections examined by transmission electron microscopy (TEM) and light microscopy (Burri *et al*., 2004) enables the observation of such events. However, only the vascular corrosion technique allows direct three-dimensional observation of the hole.

Vascular intussusception is an alternative for sprouting angiogenesis, being an important mechanism of vascular growth (formation of small arteries, veins and capillaries), arborization of the vascular network, as well vascular pruning and branch remodeling (Clauss and Breier, 2005).

In our study, analysis of the micrographs suggested that the IA that occurred in oral squamous cell carcinoma induced for 90 days may be related to vascular expansion of the sub-epithelial capillary network. The vascular expansion was observed by the formation of a sub-epithelial capillary network with small holes, which represent the pillars. Larger holes corresponded to the expansion of the girth of the pillars or the union of parallel pillars, forming new capillaries.

Hlushchuk *et al*. (2011) observed in vascular corrosion casts that diminution of vascular endothelial growth factor (VEGF) levels could induce vascular regression by vascular pruning. In their study, intussusceptive pillars were located at the bifurcation points of two vessels and their subsequent fusions lead, finally, to a "cutting of" of the corresponding vessels branches. The lack of intussusceptive pillars close to bifurcations in the corrosion casts of the oral squamous cell carcinoma may be considered as a morphologic indicative that IA was fundamentally associated with vascular growth.

Such adaptations during tumor growth may have occurred in order to expand the capillary network in size and complexity within itself, since this process allows rapid vascular growth without any compromise in the vascular physiology or function of the blood vessels. Vascular intussusception then enables an improvement in the hemodynamic conditions by adjusting blood flow to metabolic demands of tumor in development (Burri *et al*., 2004).

The vascular corrosion method, associated with SEM analysis, provides a detailed view of the microvascular architecture of the tissue and organs. However, changes in blood flow (Goel *et al*., 2011) and the lack of studies that evaluate the performance of casting media in replicating tumor vessels may increase the chance of failures during the vascular casting and, consequently, the interpretation of micrographs from these vascular territories. This drawback may have caused the main limitation of our study, since four buccal pouches with tumor presented problems during vascular casting, two being severely affected.

The relationship between the distinct microenvironments of the tumors and the blood vessels with specific morphological peculiarities also could not be established, once the removal of tissues during the maceration process precludes the observation of the tissues surrounding the vessels. Furthermore, this study was designed under a comparative paradigm, generating only qualitative data of the vascular casts.

Even if there are some limitations, corrosion casting still is one of the most valid methods for obtaining a deep morphological 3D description of sprouting and intussusceptive angiogenesis. The authors believe that the results achieved here will contribute to develop future studies using corrosion casts to obtain quantitative data, since there are no previous experiments in the literature assessing these two types of angiogenesis in oral squamous cell carcinoma.

Conclusions

SEM analysis of the vascular network of the hamster buccal pouch after tumor induction allowed the identification and characterization of sprout and intussusceptive angiogenesis. We were also able to identify a difference in the occurrence of these types of angiogenesis in the vascular layers identified in this organ and, two distinct facets of the intussusceptive pillar formation in oral squamous cell carcinoma.

References

Ackermann M, Tsuda A, Secomb TW, Mentzer SJ, Konerding MA. 2013. Intussusceptive remodeling of vascular branch angles in chemically-induced murine colitis. Microvasc Res 87:75–82.

Belien JA, van Diest PJ, Baak JP. 1999. Relationships between vascularization and proliferation in invasive breast cancer. J Pathol 189:309–318.

Bergers G, Benjamin LE. 2003. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3:401–410.

Burri PH, Hlushchuk R, Djonov V. 2004. Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. Dev Dyn 231:474–488.

Clauss M, Breier G. 2005. Mechanisms of angiogenesis, 1st Edition Basel, Switzerland: Birkhäuser Verlag.

De Oliveira LB, Bampi VF, Gomes CF, De Souza MA. 2009. Angioarchitecture of squamous cell carcinoma from hamster buccal pouch: a scanning electron microscopy study. Scanning 31:188–194.

Dvorak HF. 2005. Angiogenesis: update 2005. Thromb Haemost 3:1835–1842.

El Emir E, Qureshi U, Dearling JL, *et al*. 2007. Predicting response to radioimmunotherapy from the tumor microenvironment of colorectal carcinomas. Cancer Res 67:11896–11905.

Folkman J. 1990. Endothelial cells and angiogenic growth factors in cancer growth and metastasis. Introduction. Cancer Metastasis Rev 9:171–174.

Fukumura D, Duda DG, Munn LL, Jain RK. 2010. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. Microcirculation 17:206–225.

Goel S, Duda DG, Xu L, *et al*. 2011. Normalization of the vasculature for treatment of cancer and other diseases. Physiol Rev 91:1071–1121.

Hlushchuk R, Riesterer O, Baum O, *et al*. 2008. Tumor recovery by angiogenic switch from sprouting to intussusceptive angiogenesis after treatment with PTK787/ZK222584 or ionizing radiation. Am J Pathol 173:1173–1185.

Hlushchuk R, Ehrbar M, Reichmuth P, *et al*. 2011. Decrease in VEGF expression induces intussusceptive vascular pruning. Arterioscler Thromb Vasc Biol 31:2836–2844.

Konerding MA. 1991. Scanning electron microscopy of corrosion casting in medicine. Scanning Microsc 5:851–865.

Lametschwandtner A, Lametschwandtner U, Weiger T. 1990. Scanning electron microscopy of vascular corrosion casts–technique and applications: updated review. Scanning Microsc 4:889–940; discussion 941.

Lametschwandtner A, Holl M, Bartel H, Anupunpisit V, Minnich B. 2012. Maturation of the gastric microvasculature in *Xenopus laevis* (Lissamphibia, Anura) occurs at the transition from the herbivorous to the carnivorous lifestyle, predominantly by intussuceptive microvascular growth (IMG): a scanning electron microscope study of microvascular corrosion casts and correlative light microscopy. Anat Sci Int 87:88–100.

Motta PM, Murakami T, Fujita H. 1992. Scanning electron microscopy of vascular casts: methods and applications. vol. xi. Boston: Kluwer Academic Publishers. 390 p.

Nagy JA, Chang SH, Shih SC, Dvorak AM, Dvorak HF. 2010. Heterogeneity of the tumor vasculature. Semin Thromb Hemost 36:321–331.

Patan S, Tanda S, Roberge S, *et al*. 2001. Vascular morphogenesis and remodeling in a human tumor xenograft: blood vessel formation and growth after ovariectomy and tumor implantation. Circ Res 89:732–739.

Ribatti D, Crivellato E. 2012. "Sprouting angiogenesis", a reappraisal. Dev Biol 372:157–165.

Ribatti D, Djonov V. 2012. Intussusceptive microvascular growth in tumors. Cancer Lett 316:126–131.

Risau W. 1997. Mechanisms of angiogenesis. Nature 386:671–674.