Scanning Electron Microscopy of vascular corrosion cast – a bench-to-bedside approach in cancer research

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On purpose of integrating the knowledge from basic researches with clinical studies, it can be stated that scanning electron microscopy of vascular corrosion casts is one of the most widely used methods nowadays to analyze the tridimensional microvasculature of solid tumors. This method permits the observation of changes and regressions in the vascular network, allowing the study of the effect of antiangiogenic therapies on the angioarchitecture of tumors. The present study aimed to analyze the effect of bevacizumab (anti-VEGF antibody) on the blood vessels of squamous cell carcinoma. Six five-week-old Syrian golden hamsters were randomized into two groups of three animals each. All animals had their right buccal pouches treated with the carcinogen dimethylbenzantracene (DMBA) and the carbamide peroxide for tumor induction during 55 days. After tumor induction, 3 animals were treated with bevacizumab for 30 days. Next, the animals had their vascular system casted with Mercox and prepared to scanning electron microscopy analysis. The qualitative analysis of the electron micrographs showed remodeling in the vascular network after the treatment with bevacizumab.

Keywords corrosion casting; scanning electron microscopy; cancer; angiogenesis; anti-angiogenic drugs; translational medicine research

1. Introduction

Efforts to clinical application of new scientific discoveries are as old as medicine. Currently, all drugs on the market have been successful in the process of transition from pre-clinical studies to clinical trials. The quest to improve the quality of patients' health has been the objective of researchers from the most remote phases of biomedical science [1].

Despite new discoveries in molecular and cellular biology and high investments done in research in the last decades, the development of new drugs has declined [1]. Several agencies and institutions, such as the National Congress of the United States, have expressed concern about the large disconnection between the promise of basic research on better therapeutic strategies and improvement in health indicators [2]. One of the reasons for this is the difficulty in the transition of knowledge from the pre-clinical research to practical application.

The translational research (bench-to-bedside) aims to bridge innovative tools of basic science with clinical studies according to the physicians’ needs for their patients [3]. The constant feedback between these two research fields accelerates the discovery of more sensitive and specific diagnostic methods as well as the implementation of high-efficiency cancer treatments [4].

The oral cancer is among the six most common types of cancer worldwide, presenting low survival rate [5]. The lack of specific early markers, insidious clinical manifestations and absence of effective therapies are some obstacles in the treatment of oral cancer [6].

The induction model of squamous cell carcinoma with dimethylbenzanthracene (DMBA) in the buccal pouch of the Syrian golden hamster (Mesocricetus auratus) has been used for over fifty years in carcinogenesis studies [7, 8]. The buccal pouch of the hamster is an excellent model to study progressive anatomical modifications and functional vascular alterations that occur in the mucosa during chemical carcinogenesis [9]. In addition, this animal model presents a close relationship between cell and molecular events that occurred in the development of pre-malignant and malignant lesions in human, such as mutations and changes in expression of oncogenes and cell proliferation markers [10].

In the early 1970's, the researcher Judah Folkman suggested the hypothesis that tumors could recruit its own blood supply through the production of pro-angiogenic molecules, allowing the tumor to obtain nutrients and oxygen required to increase in volume [11]. The Vascular Endothelial Growth Factor (VEGF) is one of the major factors involved in angiogenesis [12]. VEGF binds to receptors on cell membrane, signaling for cellular growth and formation of new blood vessels. Moreover, this factor is identified as responsible for causing alterations to the hematogenic barrier of the vessels allowing tumor metastasis [13].

The central importance of angiogenesis in cancer and understanding of how tumor blood vessels are formed have led to production of new therapies aimed to disrupt this process. The use of anti-VEGF antibody for the treatment of malignancies in experimental studies began in the 1990s [14]. In 1997, the efforts of preclinical studies culminated in...
the development of a drug for clinical use, bevacizumab (Avastin®, Roche, Switzerland), an anti-VEGF-A monoclonal antibody [15].

Fujita, Sano et al., 2007 [16] first studied the action of bevacizumab in a model of squamous cell carcinoma of head and neck in mice. The results showed that bevacizumab significantly reduced growth rate and microvessel density of this tumor. Since then, clinical phase I studies [17] and I / II [18] have been conducted suggesting that the agent bevacizumab has anti-tumor effect in cancers of the head and neck.

On purpose of integrating the knowledge from basic researches with clinical studies, it can be stated that scanning electron microscopy (SEM) of vascular corrosion casts is one of the most widely used methods nowadays to analyze the tridimensional microvasculature of solid tumors [19-21]. This method permits the observation of changes and regressions in the vascular network, allowing the study of the effect of antiangiogenic therapies on the microangioarchitecture of tumors.

Modern methods of corrosion are based on studies of Schwammerdam Jan that at the end of the 17th century macerated the surrounding tissues after injecting wax into the arteries, veins and ducts [22]. In 1971, a great progress was made when Takuro Murakami introduced prepolymerized methyl methacrylate resin to the SEM study of corrosion casts [23]. This resin has been considered as the appropriate medium for the study of microvascular beds in SEM due to their low viscosity, which allows the injection of small vessels, including capillaries [24].

Improved methods for casting blood vessels in association with high resolution imaging technologies allow the obtainment of qualitative and quantitative data from the heterogeneous angioarchitecture of tumors, providing important information not only for anatomist, but also for pathologist and clinicians.

The importance of the study of carcinoma of oral squamous cell angioarchitecture, is to reveal, through electron micrographs of corrosion casts, the actions of bevacizumab on tumor vascular network in order to better understand the action of these drugs in tumor vessels. The drug bevacizumab blocks VEGF-A, having as its main objectives the reduction of vascularization and the normalization of the vascular network of tumors, restoring hemodynamic balance in the tumor environment for more efficient cancer treatment [25].

2. Objectives

This study aimed to evaluate the effect of the antiangiogenic therapy with bevacizumab (anti-VEGF antibody) on the angioarchitecture of oral squamous cell carcinoma chemically induced in hamster buccal pouch.

3. Materials and Methods

All the procedures performed in this study were approved and carried out according to the ethics committee from Pontifical Catholic University of Rio Grande do Sul (CEUA-PUCRS n. 036/11).

Six male Syrian golden hamsters (Mesocricetus auratus), obtained from stock of a colony maintained in the Animal House at Federal University of Pelotas (UFPel), were used in this study. At the beginning of the experiment, they were 5 weeks old weighing approximately 70 g. The animals were divided randomly into two experimental groups each consisting of three hamsters.

All the animals were treated with the carcinogen DMBA (Sigma Chemical Company, St. Louis, MO) 0.5% diluted in acetone and carbamide peroxide in the form of 10% gel (Opalescences 10%, Ultradent Products, Inc. South Jordan, UT) used jointly as a tumor promoter, in their right jugal pouches for periods of 55 days. The applications occurred on alternate days, three days per week for DMBA and two days per week for the promoting agent. The untreated left pouch was used as a negative control.

Following tumor induction, 3 animals were treated with bevacizumab (Avastin®, Roche, Switzerland) (5 mg/kg/day i.p.) twice a week for 30 days. The remaining animals were not treated and their right pouches were considered as a control.

After the treatment, all animals were submitted to anesthesia using intraperitoneal injection of ketamine hydrochloride (0.1 mg/g) and xylazine (0.01mg/g). Next, a thoracic incision was performed and heparin was applied in the left ventricular cavity (5000 IU at a concentration of 0.001ml/g of animal weight) followed by incising the left atrium to introduce a cannula. The cannula was fixed at the origin of the ascending aorta, and another incision was completed in the right atrium to permit solutions leakage.

Thus prepared, the vascular system was injected with 50 ml of saline solution, followed by 20 ml of 2% paraformaldehyde and again another 20 ml of saline solution, before a final manual injection with approximately 10ml of the acrylic resin methyl-methacrylate (Mercoxs CL–2RB, Ladd Research Industries, Burlington, VT, USA), until leakage was visible.

The resin-perfused animals were kept motionless at room temperature for 2 h for pre-polymerization. The heads were separated and tempered in water bath (40°C, overnight) until the final resin polymerization. The pouches were dissected from the animal’s head and were macerated by a solution of 4% NaOH at 45°C. The NaOH solution was changed daily.
until the vascular casts became completely visible with no surrounding tissues. The completely corroded specimens (vascular replicas) were dried in a laboratory oven at 37°C.

Dry casts were mounted on aluminum stubs using double-faced carbon tape and sputtered with two layers of carbon (BAL-TEC model SCD 005) and a final layer of gold (BAL-TEC CEA 035), and examined with a scanning electron microscope (Phillips XL 30, Eindhoven, The Netherlands) at an accelerating voltage of 5-20 kV.

The study of electron micrographs was performed descriptively according to Konerding, 1991 [26], and the following aspects were evaluated: pattern of organization of the vascular network, path of the vessels, identifying the type of blood vessel branches and frequency of vascular and angiogenesis figures.

4. Results

The analysis of the vascular casts by SEM showed vascular changes in tumor microarchitecture after treatment with bevacizumab. I was observed a vessel remodeling in the specimens treated with anti-angiogenic drug (Figure 1).

![Fig.1 Lateral view of vascular cast of two hamster buccal pouches after 55 of tumor induction with no treatment (A) and after 30 days of treatment with bevacizumab (B).](image)

The vessels of control tumors showed mostly a tortuous course. These vessels were compressed, with flattened appearance. Moreover, the analysis of tumors treated with bevacizumab showed a repair on the course of the vessels, which have gained an aspect close to straight (Figure 2). The blood vessels of the buccal pouches treated with bevacizumab showed a branching pattern similar to those found in the pouches without tumor, opposing the trifurcations found in untreated tumors.

![Fig.2. Differences in the course of the blood vessels after the treatment with bevacizumab. In (A) observe the tortuosity of the blood vessels (arrowheads) found in control tumors. In (B), note that the blood vessels (arrowhead) became more elongated after anti-angiogenic treatment.](image)
The capillary bed observed in treated tumors showed more delicate and less sinuous vessels, contrasting to the capillaries of untreated tumors that showed outpouchings in their walls. The intussusceptive and sprouting angiogenesis, often found in untreated specimens, were uncommon in specimens treated with bevacizumab.

It was also observed several vascular structures with blind ending vessels in the microvascular network of buccal pouches with untreated tumors, which were less frequent in the specimens treated with bevacizumab (Figure 3). The study of the angioarchitecture of the control tumors showed changes in the vessels caliber during its course and resin leakage. These changes were rarely observed in the tumors treated with bevacizumab (Figure 3).

Fig. 3 Alterations in the microangioarchitecture of blood vessels after the treatment with bevacizumab. In (A), observe an intense change in caliber of the blood vessels (arrowheads), the large number of vessels in blind ending (circles), and tortuosity of capillaries. In (B), note the vascular network of a tumor treated with bevacizumab, where these changes were uncommon.

The vessels of control tumor casted by the Mercox® polymer represented a microvascular network with a profusion of microvessels arranged chaotically contrasted by large avascular zones, suggesting that certain areas of the tumor had no blood supply. In contrast, the tumors treated with bevacizumab presented a less heterogeneous vascular network (Figure 4).

Fig. 4 Alterations in the pattern of arrangement of the vascular network after the treatment with bevacizumab. In (A), note the heterogeneity of vascular network of control tumors (A) when compared with the tumors treated with bevacizumab (B). Asterisks indicate avascular zones.

5. Discussion

The understanding of the morphology and microcirculation plays an important role in understanding the phenomena that occur in different microenvironments of tumors [27].

The tumor blood vessels are not only disorganized in their morphology – lost of hierarchy, but they are highly aberrant in their structure and hence in their function [28, 29]. Thus, blood flow occurs rapidly in some vessels, while in
other regions it flows slowly. This leads to a sudden change in direction of blood flow. This altered pattern of flow creates an obstacle for uniform delivery anti-neoplastic drugs – one of the major hurdles for the treatment of this pathology [25].

In the present study, the vessels of untreated tumors showed variations of caliber along their course. Most of this change may have been caused by dilatation of the destabilized vessel walls, mainly by the action of VEGF in the endothelium. In addition, the excess of this factor may turn the blood vessels leaky, which was observed in this study by the frequent presence of resin leakage in untreated tumors.

Most of the vessels of control tumors showed flattened appearance. The presence of compressed structures is related to the addition of tissue during tumor growth resulting in an increased interstitial pressure on the vessels. Thus, these modifications may result in hemodynamic alterations such as low rate of blood flow and vascular occlusions.

In order to normalize the vascularization of tumors and ultimately improve the treatment of cancer, it is necessary to repair the structure and function of blood vessels, reestablishing a hemodynamic balance in the tumor environment. The anti-angiogenic therapies such as bevacizumab, are intended to reduce the vascularization and reorganize of the vascular network of tumors [25].

After the treatment with bevacizumab, it was possible to observe the maintenance of the calibers along the course of the tumor blood vessels as well as a reduction of the tortuosity. Furthermore, the bifurcations of these vessels were similar with the vessels of pouches without tumor. These findings suggest that this anti-angiogenic drug may have acted on restoring a less altered vascularity in such tumors.

The analysis of the electron micrographs of the tumor treated with bevacizumab, exhibited a homogeneous vascularization of pouch, suggesting an improvement in the tumor microenvironment that could result in an improvement in the delivery of other therapies used concomitantly in cancer treatment.

5. Conclusion

The effect of the antiangiogenic drug bevacizumab on the angioarchitecture squamous cell carcinoma may be demonstrated using the technique of vascular corrosion followed by SEM. The analysis of electron micrographs showed a reorganization of the vascular network after the treatment of this neoplasm with bevacizumab.

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References
