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Modulation of mammary tumor vascularization by mast cells: Ultrasonographic and histopathological approaches

Ana I. Faustino-Rocha [a,b,*], Adelina Gama [c,d], Paula A. Oliveira [b,c], Katrien Vanderperren [e], Jimmy H. Saunders [e], Maria J. Pires [b,c], Rita Ferreira [f], Mário Ginja [b,c]

[a] Faculty of Veterinary Medicine, Lusophone University of Humanities and Technologies, Lisbon, Portugal
[b] Center for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal
[c] Department of Veterinary Sciences, School of Agrarian and Veterinary Sciences, UTAD, 5001-911 Vila Real, Portugal
[d] Animal and Veterinary Research Center (CECAV), School of Agrarian and Veterinary Sciences, UTAD, 5001-911 Vila Real, Portugal
[e] Department of Veterinary Medical Imaging and Small Animal Orthopedics, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[f] Organic Chemistry, Natural Products and Foodstuffs (QOPNA), Mass Spectrometry Center, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

Aims: The inhibition of mast cells' degranulation may be an approach to prevent the formation of new vessels during the mammary carcinogenesis.
Materials and methods: Female Sprague-Dawley rats were randomly divided into five experimental groups. Mammary tumors were induced by intraperitoneal injection of *N*-methyl-*N*-nitrosourea (MNU). Animals from group II were treated with ketotifen for 18 weeks immediately after the MNU administration, while animals from group III only received the ketotifen after the development of the first mammary tumor. Mammary tumors vascularization was assessed by ultrasonography (Doppler, B Flow and contrast-enhanced ultrasound) and immunohistochemistry (vascular endothelial growth factor-A).
Key findings and significance: Similar to what occurs in women with breast cancer, the majority of MNU-induced mammary tumors exhibited a centripetal enhancement order of the contrast agent, clear margin and heterogeneous enhancement. Ultrasonographic and immunohistochemical data suggest that the inhibition of mast cells' degranulation did not change the mammary tumors vascularization.

1. Introduction

Breast cancer remains as one of the most frequently diagnosed cancers among female population worldwide [1]. Tumors require the development of new vessels to growth > 1–2 mm^3 and metastasize to distant organs [2,3]. In this way, the assessment of tumor angiogenesis is important in order to predict the prognosis of several tumors, including breast tumors [4].

Ultrasonography is one of the most frequently used imaging modalities in clinical practice. Its use in order to evaluate tumor angiogenesis has gained increasing interest in cancer research [2,3]. Color and Power Doppler have been frequently used to evaluate tumors' vascularization. However, these ultrasound methods are not sensitive to the detection of slow flow and small volume blood flows in capillaries within the tumor parenchyma [5,6]. Pulsed Doppler allows to better characterize the vascularization through the calculation of pulsatility index and resistive index. These indexes are calculated from the blood flow velocities in vessels during cardiac cycle and are indicators of downstream resistance in arteries [7,8]. Increased pulsatility index and resistive index have been associated with malignancy of human mammary tumors [9,10]. B Flow is a non-Doppler technology that improves the visualization of blood vessels. This method uses a morphological approach, similarly to angiography, allowing the real-time visualization of hemodynamic flow in relation to stationary tissue [11]. Beyond these methods, the use of contrast media is a possible approach to improve the detection of tumors vessels by ultrasonography [12,13]. Contrast-enhanced ultrasound (CEUS) constitutes an excellent method to assess the structural and functional features of tumor angiogenesis by measuring tumor flow and vascular volume [12,13]. In the last years, the sensitivity and specificity of CEUS have greatly improved due to the development of more sophisticated ultrasound equipment, the introduction of the second-generation contrast agents, and the development of software able to perform quantitative analysis [14]. The microbubbles of the second-generation contrast agent SonoVue® have high reflectivity, enhancing the detectable ultrasound signal from the blood pool by as much as 40 dB (decibels). Its use allows the identification of slow and low-volume blood vessels inside the tumors with 20 to 39 μm in diameter [13,15].

* Corresponding author at: CITAB, Faculty of Veterinary Medicine, Lusophone University of Humanities and Technologies, Lisbon, Portugal.
E-mail address: anafaustino.faustino@sapo.pt (A.I. Faustino-Rocha).

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Mast cells have been associated with the tumors' angiogenesis by release of several angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, interleukin (IL)-8, tryptase, chymase, heparin, nerve growth factor (NGF), and transforming growth factor (TGF)-β [16–18]. Ketotifen is a not only a second-generation histamine 1 receptor antagonist, but also an inhibitor of mast cell degranulation by the blocking of calcium channels essential for mast cell degranulation [19–21]. Once the inhibition of angiogenesis may represent a new therapeutic opportunity in cancer, we hypothesized that the inhibition of the mast cells' degranulation may be an approach to prevent the formation of new vessels during the carcinogenesis. So, the present study intended to assess the effects of mast cells´ inhibition on the vascularization of chemically-induced mammary tumors in a rat model.

2. Material and methods

2.1. Animals

Thirty-four female Sprague-Dawley rats with four weeks of age were used (Harlan Interfauna, Barcelona, Spain). Animals were placed in the facilities of the University of Trás-os-Montes and Alto Douro (UTAD) under controlled conditions of temperature (23 ± 2 °C), humidity (50 ± 10%), air system filtration (10–20 ventilations/h) and on a 12 h:12 h light:dark cycle. Animals were fed with a standard laboratory diet (4RF21, Mucedola, Italy) and tap water *ad libitum*. All procedures followed the European and National legislation on the protection of animals used for scientific purposes (European Directive 2010/63/EU and National Decree-Law 113/2013), and were approved by the Ethics Committee of UTAD (CE_12-2013) and by the Portuguese Ethics Committee for Animal Experimentation (Approval no. 008961).

2.2. Experimental protocol

After one week of quarantine and two weeks of acclimatization to the lab conditions, animals were randomly divided into five experimental groups as follows: group I (MNU, animals that only received the carcinogen MNU; n = 10), group II (MNU + ketotifen-1, animals that received the ketotifen immediately after the MNU administration; n = 10), group III (MNU + ketotifen-2, animals that only received the ketotifen after the development of the first mammary tumor; n = 10), group IV (ketotifen, animals not exposed to the carcinogen agent that were treated with the ketotifen; n = 2) and group V (control, animals not exposed to any drug; n = 2). At seven weeks of age, all animals from groups I, II and III received an intraperitoneal injection of the carcinogen agent *N*-methyl-*N*-nitrosourea (MNU) (Isopac®, Sigma Chemical Co., Madrid, Spain) at a dose of 50 mg/kg. Animals from groups IV and V received a single intraperitoneal injection of the vehicle (saline solution 0.9%). The first day of the experimental protocol was defined as the day of the MNU administration. On the day after the MNU or saline administration, animals from groups II and IV received the mast cell stabilizer ketotifen (Zaditen®, Defiante Farmacêutica S.A., Portugal) in drinking water, at a concentration of 1 mg/kg, 7 days/week for 18 weeks. Each animal from group III only received the ketotifen after the detection of the development of the first mammary tumor by palpation. Animals from groups I and V received only water during the protocol. Animals were observed twice a day in order to monitor their health status during the experiment. Mammary chains from all animals were palpated once a week to detect the development of mammary tumors.

2.3. Ultrasonographic evaluation

Eighteen weeks after MNU administration, immediately before the sacrifice, all animals were anesthetized by intraperitoneal injection of ketamine (75 mg/kg, Imalgene® 1000, Merial S.A.S., Lyon, France) and xylazine (10 mg/kg, Rompun® 2%, Bayer Healthcare S.A., Kiel, Germany). The vascularization of mammary tumors previously detected by palpation in MNU-exposed animals (groups I, II and III) was evaluated by ultrasonography using the following modes: Power Doppler, Pulsed Doppler, B Flow and CEUS. Mammary tumors were evaluated by two experienced examiners. For this, the animals were placed in supine position. The skin overlying each mammary tumor was shaved using a machine clipper (Aesculap GT 420 Isis, Aesculap Inc, USA) and acoustic gel (Aquasonic, Parker Laboratories, USA) was applied. Ultrasonographic evaluation was performed using the real-time scanner Logiq P6 (General Electric Healthcare, Milwaukee, USA) and a 10 MHz linear transducer. A standoff pad (Sonokit, MIUS Ltd, Gloucestershire, England) made of extremely soft polyvinylchloride especially created for skin-contact sonography was used. A sagittal view of each mammary tumor was obtained. Ultrasonographic exams were recorded in clip format. Then the color pixels density (CPD) was determined in Power Doppler and B Flow images following the methodology previously described [22]. The pulsatility index and resistive index were determined in Pulsed Doppler images by means of the equipment's software using the autotrace function.

2.4. CEUS

System settings were optimized for the contrast study with a mechanical index of 0.09, the gain compensation was adjusted for each mammary tumor. The position of the probe was maintained during the examination. The contrast agent SonoVue® (Bracco, Italy) was reconstituted by adding 5 mL of 0.9% saline solution. The SonoVue® was injected as a bolus (0.1 mL) through a tail vein catheter followed by a 1 mL saline flush. The injection technique was carefully performed by the same researcher to avoid personal variations, and produce acceptable and reproducible results. The real-time perfusion process and the dynamic enhancement of each mammary tumor were observed in real time and continuously recorded in the ultrasound apparatus immediately after the intravenous injection of the contrast agent. Posteriorly, the qualitative and quantitative analysis of CEUS videos was performed. Qualitative analysis included the following parameters: enhancement order (centripetal, centrifugal or diffuse), margin (blurred or clear), contrast distribution (homogenous or heterogeneous) and penetrating vessels (absent or present) [23]. The quantitative analysis was performed using the time intensity curve (TIC) analysis of the ultrasound apparatus. An ovoid region of interest (ROI) was drawn in the most enhanced area of each mammary tumor and the signal was immediately plotted and fitted using the following gamma variate function:

$$I(t) = At^{c} \times exp^{(-kt)} + B,$$

where t is the time, k is a constant scale factor and A and B are parameters that define the shape of the curve. The following parameters were determined: contrast agent arrival time (AT), time to peak (TTP, defined as the time the lesions go up to the maximum contrast intensity that is related to the lesions' enhancement speed), peak intensity (PI, maximum intensity), wash-in (upslope), wash-out (downslope) and area under the curve (AUC).

2.5. Animals' sacrifice and necropsy

Immediately after ultrasonographic examination, anesthetized animals were sacrificed by exsanguination by cardiac puncture as indicated by the Federation of European Laboratory Animal Science Associations [24]. Animals were scalped and the skin was carefully evaluated under a light in order to detect mammary tumors not previously identified by palpation. All mammary tumors were collected and immersed in formalin for 24 h.