Immunoexpression of VEGF-A in chemically-induced mammary tumors

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Abstract

Vascular endothelial growth factor (VEGF)-A is a member of VEGF family that plays an important role on angiogenesis of several human cancers, namely in breast cancer. Its expression has been correlated with poor outcome. Twenty-five female Sprague-Dawley rats were divided into two groups: MNU (n=15) and control (n=10). At seven weeks of age, animals from group MNU received an intraperitoneal injection of the carcinogen agent N-methyl-N-nitrosourea (MNU) at a concentration of 50 mg/kg. Thirty-five weeks later, all survived animals were humanely sacrificed and the mammary tumors were excised, fixed in formalin for 12 hours and histologically evaluated. Sections were incubated with VEGF-A primary antibody; the immunoexpression, the staining intensity and the microvessels density were evaluated. Survived animals from group MNU developed 28 mammary lesions that were histologically classified as: epidermal cysts, tubular adenoma, papillary carcinoma (non-invasive and invasive), cribriform carcinoma (non-invasive and invasive) and comedo. All mammary lesions showed cytoplasmic expression of VEGF-A. The majority of the mammary tumors exhibited 50-75% of neoplastic cells stained (grade 3) and a moderate intensity of staining (level 2). In these tumors was it was counted a mean number of 11.82 ± 1.09 blood vessels. Rat mammary tumors exhibited a high expression of VEGF-A, being a good model to study human mammary tumors. Once the immunoexpression was high, it is possible to conclude that these tumors have a high angiogenic potential.

INTRODUCTION

The process of angiogenesis is regulated by a balance between proangiogenic and antiangiogenic factors. The vascular endothelial growth factor (VEGF)-A is one of the most important angiogenic factor. It acts stimulating the proliferation and migration of endothelial cells, preventing the regression of newly formed vessels and increasing microvascular permeability. Physiologically, VEGF-A is responsible for the wound healing, the development of blood vessels during the menstrual cycle and promotion of monocyte chemotactic action. Previous studies found that VEGF-A expression promotes the neoplastic progression, increases the risk of developing metastasis and it is an indicator of poor prognosis in the lung, esophagus, colon and breast cancer. This work aimed to assess the immunoexpression of VEGF-A in chemically-induced mammary tumors in a rat model.

MATERIALS AND METHODS

Experimental protocol

Twenty-five female Sprague-Dawley rats, with 4-5 weeks of age were obtained from Harlan Interfauna Inc. (Barcelona, Spain). Animals were housed under controlled conditions of temperature (23±2°C), humidity (50±10%), air system filtration (10-20 ventilations/hour) and on a 12:12-h light:dark cycle. Tap water and a basic standard laboratory diet (4RF211, Mucedola, Italy) were supplied ad libitum during the study. Cages were cleaned and water was changed once per week. All procedures were done in accordance with European and National Legislation (European Directive 2010/63/EU and National Decree-law 113/2013). All procedures were approved by the Direção Geral de Alimentação Veterinária (Approval no. 008961).

Necropsy

Thirty-five weeks later, all survived animals were humanely sacrificed by an intraperitoneal injection of ketamine (75 mg/kg; Imalgene® 1000, Merial S.A.S., Lyon, France) and xylazin (10 mg/kg; Rompum® 2%, Bayer Healthcare S.A., Kiel, Germany), followed by exsanguination by cardiac puncture as indicated by the Federation for Laboratory Animal Science Associations. All animals...
were scalped and the skin was observed under a light for the detection of small mammary tumors. All mammary tumors and organs were immersed in buffered formalin at 10% during 12 hours.

**Histology and immunohistochemistry**

After fixation, mammary tumors were routinely processed for histological analysis. The sections were stained with hematoxylin and eosin and histologically classified considering the predominant lesion. It was followed the classification previously proposed by Russo and Russo. Additionally, the immunohistochemical detection of VEGF-A was performed using the protocol of Novoclin™ Max Polymer Detection System (Leica Biosystems, Newcastle, UK). Sections were incubated with the antibody for VEGF-A (clone JH121, Merck Millipore, Darmstadt, Germany) at a dilution of 1:100, overnight at 4°C.

**Immunoreactivity evaluation**

The immunoreactivity of VEGF-A was evaluated in a semiquantitative way in terms of extension (percentage of positive/negative cells) and intensity. The VEGF-A immunoreactivity was assessed to five levels: grade 0 (no staining detected), grade 1 (1-24% of neoplastic cells showed positive staining), grade 2 (25-49% stained), grade 3 (50-75% stained) and grade 4 (>75% stained). The staining intensity was also evaluated as: level 0 (unstained), level 1 (weak staining), level 2 (moderate staining) and level 3 (intense staining). The microvessels were counted in the three most vascularized areas of the lesion, known as hot spots, in 400× magnification fields from which the mean was obtained in order to determine the microvessels density (MVD). Areas of fibrosis, necrosis and inflammation and vessels with muscular walls were not counted. The rat kidney was used as positive control. The immunoreactivity of VEGF-A and the number of vessels were evaluated independently by two researchers.

**Statistical analysis**

Data were statistically analyzed with SPSS® version 17 (Chicago, IL, USA) using χ2 test. Data were expressed as mean ± standard error (S.E.); p-values lower than 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

During the experiment died five animals: four animals from MNU group and one animal from control group. The animals from MNU group died due to the process of carcinogenesis. As expected, animals from control group did not develop any mammary tumor. Animals from MNU group developed a total of 28 mammary lesions that were histologically classified as: epidermal cysts (n=1), tubular adenoma (n=1), papillary noninvasive carcinoma (n=13), papillary invasive carcinoma (n=4), papillary invasive carcinoma (n=2) and comedo invasive carcinoma (n=2). As previously reported, the papillary carcinomas were the most frequently identified mammary lesion.

All mammary lesions exhibited a cytoplasmic and homogeneous immunolabelling. The majority of the mammary tumors exhibited 50-75% of cells stained (grade 3) and a moderate intensity of staining (level 2) (p<0.05) (Table 1). The tumor from this study exhibited higher immunostaining than that observed by Saminathan and coworkers (16.2±0.86). These different results may be related with the duration of the experimental protocol, the present study ended 35 weeks after MNU administration while the study performed by them finished 28 weeks after the carcinogen administration. The MVD is frequently used to quantify mammary cancer angiogenesis. In this work was counted a mean number of 11.82 ± 1.09 blood vessels, lower than that observed in the study conducted by Saminathan and coworkers described above. These differences may be related with the number of mammary lesions, they used higher number of animals and consequently identified 44 mammary tumors, of which 38 were classified as malignant.

**CONCLUSION**

Rat mammary tumors exhibited a high expression of VEGF-A, being a good model to study human mammary tumors. Once the immunoreactivity of VEGF-A was high, it is possible to conclude that these tumors have a high angiogenic potential.

**REFERENCES**