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Original article

Morphogenetic events in the perinodal connective tissue in a metastatic cancer model

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ABSTRACT

Background: The modifications of connective tissue surrounding metastatic lymph nodes in a murine model of rectal cancer are described.
Methods: Athymic nude mice ($n = 36$) were inoculated with 10×10^5 ht-29 cancer cells into the submucosal layer of the rectum. Control mice ($n = 5$) were treated with a sterile buffer. Tumor and the involved lymph nodes were visualized in vivo by magnetic resonance imaging at 1 to 4 weeks after cell injection. After the sacrifice, the excised samples were processed for histology.
Results: After one week from cell injection all treated animals developed rectal cancer. Since the first week, neoplastic cells were visible in the nodes. In the surrounding connective tissue, the diameter of the adipocytes was reduced and a mesenchymal-like pattern with stellate cells embedded in an oedematous environment was visible. Since the second week, in the perinodal connective an enlargement of the stroma was present. The tissue was organized in cords and areas with extracellular accumulation of lipids were found. At the fourth week, we observed an enlargement of multilocular areas and lobules of elongated elements almost devoid of lipid droplets. In control animals, in absence of neoplastic masses, pelvic nodes were surrounded by a typical connective tissue characterized by unilocular adipocytes with groups of multilocular adipocytes.
Conclusions: We have developed a model of rectal cancer with nodal metastases. Using this model, the work demonstrates that around secondary lesions, the morphogenetic events follow a standard evolution characterized by an early phase with lipolysis and mesenchymalization and later phases with a brown-like phenotype acquisition.

1. Introduction

The growth of a tumor in its early stages of development necessarily involves the surrounding tissue [1]. In particular, the connective tissue, surrounding the neoplastic lesion, plays an important role because it is involved in numerous processes that are related to the development of the disease [2]. For example, we might recall the micro angiogenetic phenomena that appear very important in providing adequate metabolic support to the tumor during its development [3]. Moreover, the modifications and the metabolic pathways that lead the connective tissue to form a connective stroma, that could supply a strong support to the neoplastic cells, are very important during tumor development [1]. The connective tissue plays also an important role in modulating the inflammatory reactions that has an action in the defense against the proliferative process [4]. Therefore, the sets of reactions that occur in the connective tissue surrounding tumor are characterized not only by harmful aspects because they not only represent mere degeneration, but also have an active role that can strongly affect the characteristics of the process [1]. The role of connective tissue developed around the neoplastic lesion is obviously related to the cytological features of the connective tissue itself, considering the great morphological variability of this tissue [5]. Among the connective tissue, those with a large fatty infiltration are usually called adipose tissue. These tissues are characterized by the presence of a dominant cell type: the adipocyte. The most important characteristic of adipocyte is a conspicuous intracytoplasmic accumulation of triglycerides, which can be shaped in the form unilocular or multilocular [6]. These tissues are the most represented in the human body and constitute the environment, in which a large number of neoplastic lesions develop [7]. In relation to the development of these lesions, the type of adipose tissue present in a particular area and also the composition adipocyte lipid accumulation, may represent two important factors in the genesis and progression of cancer [8]. In this context, peri-tumoral fat tissue could play an important role in

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controlling the progression of neoplastic lesions. In literature, data concerning the morphology and/or composition of adipose tissue and the development of specific cancers in context are poor [9]. Histological studies, specifically focused on the comprehension of the variety of connective tissue around experimental secondary lesions, are absent. Probably, the difficulty in standardization of surgical techniques to obtain a tumor with spontaneous secondary lesions development leads to the paucity of animal models [10].

In this study, we developed a murine model, in which the development of secondary lesions in the peri-tumoral lymph nodes is observed since a week from cancer cell injection. The availability of this model allowed us to monitor the morphogenetic events induced in the first phase of the tumor development in the connective tissue surrounding the neoplasia. The aim of the work was the identification of morphological characteristics of the peri-tumoral fat, which may be important in understanding the biological responses of healthy tissues against a neoplastic lesion during the early stages of its development. Morphogenetic events could represent a favorable target for possible anticancer therapies implemented during the early development of a neoplasia.

2. Materials and methods

2.1. Experimental model

Male, athymic mice ($n = 41$) (Charles River Laboratories, France), 4-week-old and 30 g in weight, were housed in a temperature- and humidity- controlled environment, having free access to mouse chow and tap water. This study was carried out in strict accordance with the guide for the Care and Use of Laboratry Animals of the National Institutes of health. The protocol was approved by the Ethic Commitee of the University of Verona (Permit number 3/2010). All surgery was performed after CO_2 overdose, and all efforts were made to minimize suffering.

ht-29 cell line (ATCC, Manassas, VA) were plated in 25 cm^2 flasks with McCoy's Medium, with the addition of 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomicin (p/s). Plates were incubated at 37 °C with 5% of CO_2. When at confluence, cells were treated with 1% trypsin and incubated at 37 °C with 5% CO_2 for 1 minute and then were centrifuged at 3000 rpm for 5 minutes. The pellet was suspended in 1 ml of fresh culture medium and the cells were placed in 75 cm^2 flasks with 6 ml of McCoy's medium + 20% FBS and 1% p/s (Invitrogen, Carlsbad, CA).

When at confluence, cells were treated with 1% trypsin and incubated at 37 °C with 5% CO_2 for 1 minute. The cell solution was centrifuged at 3000 rpm for 5 minutes. After centrifugation, cells were suspended in 1 ml of McCoy's medium and counted. Then the solution was diluted with culture medium to obtain final concentrations of $10 \times 10^5$of cell/100 μl.

Thirty-six animals were anesthetized by 1% isofluorane inhalation in a mixture of oxygen and nitrogen. After anesthetization, the animals were positioned prone with the anal orifice in front of the operator. With a plastic microtube of 25 μm in diameter and using a binocular lens to increase precision, the anal orifice was gently enlarged and then 10×10^5 ht-29 cells/100 μl were injected into the rectal submucosa using a 1 ml syringe with a hand angle of inclination of about 45 degrees. This surgical transanal cell injection made it possible to deposit ht-29 cancer cells directly into the rectal submucosa. Moreover, five mice belonging to the control group were treated with sterile phosphate buffer with the same protocol described before.

At different time points after injection of ht-29 cell, all the animals belonging to be treated groups were observed by magnetic resonance imaging (MRI) using a 4.7 T horizontal magnet (Bruker, Karlsruhe, Germany). At each time points mice were sacrificed at the end of MRI acquisitions for tumor and lymph node excision. The explanted samples were processed for histological evaluations. The animals belonging to the treated group were observed 1, 2, 3, 4 weeks after cancer cell injection, whereas the animals in control group were observed at 4 weeks and then they were sacrificed for histological evaluation.

After MRI, the animals treated with ht-29 cells were sacrificed at different time points, reported before, to excise primary tumors and lymph nodes. All lymph nodes were classified on the basis of a recent nomenclature according to Van den Broeck et al. [11]. After excision, tumors and lymph nodes were fixed in 10% formalin for 2 h. Following fixation and embedding in paraffin, 5 μm sections were cut and stained with hematoxylin and eosin. The sections were observed using an optical microscope equipped with ProPlus 7.0 software. Sections were observed at different magnifications (× 4, × 40) to analyze both the primary tumor and lymph node tissue.

3. Results

3.1. Primary tumours

All animals treated with ht-29 cells developed rectal tumors. In the course of the study, no mortality or other complications were observed and no relevant changes of body weight and intestinal function were registered. At MRI, primary tumors were localized around the rectal canal and protruded towards the anal orifice. In T2 weighted images, the primary tumor mass appeared hyper-intense with respect to muscle tissue but hypo-intense with respect to fat. The main tumor mass developed in the anal-rectal zone of the animal, and it was characterized by dissemination along the first colon tract (Fig. 1). At MRI, the longitudinal diameter of the tumor was about 1.2 mm, after one week and 22.4 mm after

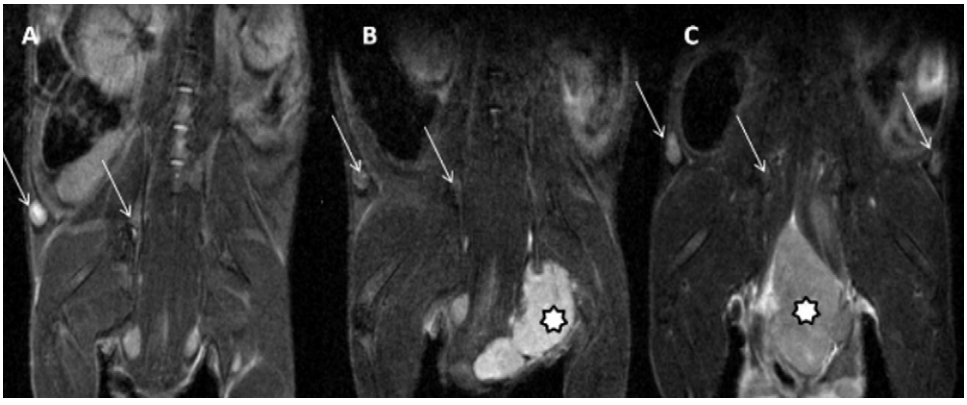

Fig. 1. MRI of mice. A. Control. B. ht-29 treated, 2 weeks. C. ht-29 treated, 3 weeks. Lymph nodes are marked by arrows and tumors by stars.