Original Article

Obesity inhibits lymphangiogenesis in prostate tumors

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Abstract: Lymphangiogenesis is the process that leads to new lymphatic vessels formation from preexisting blood vessels in the presence of appropriate inducing signals, which in pathologic conditions such as cancer, may contribute to tumor cells dissemination. The aim of the present study was to study the role of obesity, leptin and insulin in tumor lymphangiogenesis. For that, we have quantified the lymphatic vessels in prostate tumors through their immunohistochemistry staining by Lyve-1 in RM1 prostate tumors induced in different obese mice models (ob/ob, db/db and diet induced obese (DIO) and in normal weight C57BL/6J mice (control). Lymph vessels density was determined by Lyve-1 immunohistochemistry of prostate adenocarcinomas, while the percentage of the Lyve-1 stained area and lymphatic vessels number were obtained using a morphometric computerized tool. Obese ob/ob and DIO mice presented prostate tumors that were significantly larger (p<0.001) than controls, while tumors of db/db mice were significantly smaller (p=0.047). Lyve-1 expression was significantly higher in prostate tumors of DIO mice compared to tumors of db/db mice (p<0.05); furthermore Lyve-1 expression was negatively correlated with the percentage of the epididymal fat and body weight (p<0.01). No significantly correlations were found between Lyve-1 expression and tumor weight and leptin or insulin plasma levels. Our results suggest that obesity may have a protective effect against prostate cancer dissemination by inhibiting lymphangiogenesis through a still unidentified mechanism that appears not to involve leptin or insulin.

Keywords: Lymphangiogenesis, obesity, prostate cancer, leptin

Introduction

The lymphatic system constitutes a highly specialized part of the vascular system that plays an essential role in the immune system, maintenance of the blood and tissue volume, and uptake of dietary fat [1, 2].

Lymphangiogenesis is the process that leads to the formation of new lymphatic vessels from preexisting blood vessels in the presence of the appropriate inducing signals such as transcription factor Prospero homeobox protein 1 (Prox-1), vascular endothelial growth factor (VEGF-C) and lymphatic vessel endothelial hyaluronan receptor (LYVE-1) [1, 3]. There has been an increasing awareness of the importance of this process in pathological conditions, such as cancer, since lymphatic vessels may allow tumor cell metastatic dissemination [1, 2, 4]. In prostate cancer, the lymph nodes, together with bone and liver are the most common metastatic sites [5].

The lymphatic vasculature is formed by lymphatic endothelial cells (LEC), the identification of proteins expressed by these cells is important to allow the study of lymphangiogenesis in pathological conditions [1, 3, 6], among these Lyve-1 marker is one of the best-characterized marker of LEC cells, expressed in both luminal and abluminal surface during lymphangiogenesis [6, 7].

Obesity has been documented as a cancer risk factor in many epidemiological studies, even though obesity alone does not seem to increase the risk in all tissues similarly [8]. In prostate cancer, this association is still controversial; some studies reported obesity as a risk factor, while others reported a potential protective role of obesity in the severity of this type of cancer [8-11].

In obesity, the existence of a close association between lymphangiogenic processes and adipose tissue mass has been hypothesized, since
Lymphangiogenesis obesity and prostate cancer

Obese patients present increased circulating VEGF-C levels that may explain the increased risk of metastatic dissemination that has been described in some cancers, such as prostate cancer [12-15].

The aim of the present study was to evaluate the influence of different endocrine environments associated with obesity in lymphatic vessels density and tumor size of prostate tumors induced in different mouse strains.

**Material and methods**

**Tumors**

Prostate adenocarcinomas were induced by subcutaneous inoculation of the murine androgen insensitive prostate cancer RM1 cell line, kindly provided by Prof. Thompson (MD Anderson Cancer Center, Houston, TX, USA) [16]. RM1 cells (3.0x10⁵ cells/500 μL of PBS) were inoculated in the dorsum of four different groups of mice models: mice with congenital leptin deficiency ob/ob (n=6), mice with congenital leptin resistance db/db (n=6) and C57BL/6J mice with diet induced obesity (DIO) (n=6) and normal weight C57BL/6J mice (n=6) used as controls, as previously described [10]. All animals were purchased to a certified commercial breeder (Charles River Laboratories, Barcelona, Spain). Fourteen days after inoculation, tumors were removed, weighed and processed for histological analysis, while plasma was collected to quantify the leptin and insulin plasma levels, as described before [10].

**Immunohistochemistry (IHC)**

IHC was performed on formalin-fixed paraffin embedded tissue sections of the tumors mounted on adhesive microscope slides Superfrost (Thermo Scientific®). Sections were successively deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method. For antigen retrieval, the sections were boiled for 3 minutes in 0.01 M-citrate buffer at pH 6.0 with 0.05% Tween 20. The endogenous peroxidase

<table>
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<tr>
<th>Table 1. Summary of mice body weight, percentage of epididymal fat, tumor size and circulating leptin and insulin [10]</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td><strong>Body weight (g)</strong></td>
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<tr>
<td><strong>Percentage of epididymal fat (%)</strong></td>
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<td><strong>Tumor weight (g)</strong></td>
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<td><strong>Leptin (ng/ml)</strong></td>
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<td><strong>Insulin (ng/ml)</strong></td>
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<sup>a</sup>db/db vs control; <sup>b</sup>ob/ob vs control; <sup>c</sup>DIO vs control; ND: below detection level.

Figure 1. RM1 cell prostate adenocarcinoma with lymphatic vessels stained by immunohistochemistry using anti-Lyve-1 antibody. In the micrograph, unstained blood vessels can be visualized as indicated by the dark arrows (200x) in contrast to the DAB stained lymphatic vessels.
Lymphangiogenesis obesity and prostate cancer

was blocked with 3% hydrogen peroxide in methanol, followed by incubation in normal serum for 30 minutes. Then the samples were incubated overnight at 4°C with the primary antibody Lyve-1 (ab33682; 1:750; Abcam). Samples were then incubated with secondary antibodies at 1:200 dilution (Polyclonal swine anti-rabbit, Dako Denmark) for 30 minutes, followed by avidin-biotin peroxidase complex (1:100, Vector Laboratories, Inc.) for 30 minutes. Diaminobenzidine was used as chromogen, and hematoxylin as nuclear counterstaining.

Computerized image analysis

Slides were scanned using the image acquisition software Olympus VS110 virtual slide scanning system. Images were analyzed using an image processing software (ImageJ, National Institutes of Health, USA) with a color deconvolution plugin which separates the stained area from the initial image allowing the quantification of the percentage of the area specifically stained with the Lyve-1 antibody. The number of the lymphatic vessels in the tumors staining for Lyve-1 was also manually counted and the number of the lymphatic vessels was expressed per squared pixel.

Statistical analysis

One way ANOVA test was used to compare the quantitative variables of the independent groups and the post-hoc Dunn test was performed to evaluate the differences between the groups, using GraphPad Prism (version 5.00). To evaluate the correlation between different parameters a Spearman test was performed, using the SPSS software (version 20.00) for Windows. p<0.05 was considered statistical significant.

Results

Obese mice of monogenetic etiology, leptin-deficient ob/ob mice and diabetic leptin-resistant db/db had a mean body weight significantly higher when compared to diet-induce obese (DIO) and control mice (p<0.001). Epididymal fat weight for 100 g of body weight, used as a surrogate marker of fatty body content, was also significantly higher in all groups of obese mice, i.e., ob/ob, db/db and DIO, when compared with control (p<0.001, p<0.001, p<0.01, respectively) (Table 1). After inoculation of mice with RM1 prostate cancer cells, obese ob/ob and DIO mice developed prostate tumors that were significantly larger (p<0.001) than those of controls, while in db/db mice tumors were significantly smaller (p<0.05). According to what was expected from the genetic mutations harbored by the genetic obese mice models used, leptin plasma levels were barely undetectable in ob/ob mice, while those in db/db and DIO mice were significantly higher when compared to controls (p<0.001). Similarly, insulin plasma levels were also significantly higher in ob/ob mice than in controls (p<0.01), although insulin levels of db/db and DIO mice were comparable to those of controls (Table 1) [10].

The Lyve-1 molecular marker was used to stain and quantify lymphatic vessels present in the prostate tumors induced in the different groups of mice (Figure 1). Lyve-1 immunoreactive vessels were evaluated regarding the percentage of Lyve-1 stained tumors area and number of lymphatic vessels in the tumor tissue.
The tumors of the db/db and ob/ob mice were those with lesser lymphatic vessels density, while the percentage of the stained area and the number of the vessels staining with Lyve-1 antibody were significantly lower in db/db mice when compared with DIO mice (Figure 2). No significant correlation was found between tumor size and lymphatic vessel density (coefficient correlation=0.344), however the percentage of the Lyve-1 stained area was negatively correlated with the percentage of the epididymal fat (coefficient correlation=-0.615) and mice body weight (coefficient correlation=-0.618) (Table 2). No correlation has been found between the leptin and insulin plasma levels with the Lyve 1 staining (coefficient correlation=-0.153; coefficient correlation=-0.369 respectively) (Table 2).

Discussion

Previous in vivo and in vitro studies, of our research group indicated that obesity seems to have a protective role in prostate tumors cell proliferation and angiogenesis in prostate tumors [10, 17]. Lymphangiogenesis is a component of the overall angiogenic process of cancer that is known to play a critical role in tumor dissemination [1, 4]. However, there is scarce information on lymphangiogenesis with regards to its relationship with obesity. The aim of the present research was to quantify lymphangiogenesis in prostate tumors induced in normal and obese mice, with different circulating levels of leptin and insulin, in order to understand the influence of obesity and adiposity markers in the dissemination of prostate cancer.

In the present study, we evaluated the number of the vessels marked by the Lyve-1 antibody per tumor area and the percentage of the Lyve-1 stained area in prostate tumors of different mouse strains. The automatic quantification method of the Lyve-1 immunohistochemistry staining was used to remove observer associated subjectivity of manually quantified vessels.

The expression of the Lyve-1 marker was significantly lower in obese db/db mice compared to DIO mice, with similar results for both quantification methods. The groups with lower Lyve-1 expression (db/db) were also the ones that presented tumors with lowest weight, however we did not find a significant correlation between these parameters.

During the growth of solid tumors, there is a strong stimulating environment for stromal and inflammatory cells to express growth factors, which induce the formation of new lymphatic vessels in the tumor, due the tumor hypoxia [18]. However, in the present study we did not find any significant correlation between tumor weight and the expression of lymphangiogenesis markers.

Lymphangiogenesis was found to be negatively correlated with the percentage of the epididymal fat and with mice body weight, two obesity parameters that were associated with decreased lymphangiogenesis in these induced prostate tumors. In addition, lymphangiogenesis markers were similar in obese mice with high leptin levels (db/db mice) and leptin deficient (ob/ob mice). Since no correlation was found between leptin or insulin levels and Lyve-1 expression, leptin and insulin do not seem to be involved in the lymphangiogenic process.

Obesity has been associated with hypoxia due to tissue mass expansion, which could promote the progression of angiogenic and lymphangiogenic pathways [19]. Previous studies have documented an increased risk of metastatic disease in obese patients with cancer [12]. However in this study we have found a different outcome: this report shows that obesity prevents lymphangiogenesis in prostate tumors induced in mice. These results reinforce the protective role of the obesity in the prostate cancer dissemination as already suggested by our previous research [10] and some epidemiological studies [11].
In conclusion, obesity may have a protective effect against prostate cancer dissemination in mice by inhibiting lymphangiogenesis through mechanisms that are not related with circulating levels of leptin and insulin.

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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