ORIGINAL ARTICLE

Prostate cancer cell proliferation and angiogenesis in different obese mice models

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Summary

Obesity has been associated with increased incidence and aggressiveness of prostate cancer. Although controversial, several studies suggest that leptin could influence tumour cell growth and proliferation. The main goal of this study was to assess cellular growth of prostate adenocarcinoma cells in obese mice with different endogenous hormonal environments in what relates to leptin circulating levels and sensitivity. Four groups of mice ($n = 6$/group) were used, namely obese mice with congenital non-functioning leptin receptor OBR (*db/db*), obese mice with congenital leptin deficiency (*ob/ob*), mice with diet induced obesity (DIO) and normal weight C57BL/6J mice (control). All groups of mice were injected subcutaneously with 3.0×10^5 RM1 cells/500 µl PBS (murine prostate carcinoma androgen insensitive cells) and tumour growth and angiogenesis were evaluated 14 days after inoculation. The tumours induced in *ob/ob* and DIO mice were significantly larger ($P < 0.001$) while those induced in *db/db* mice were significantly smaller ($P = 0.047$), when compared with controls. Morphometric analysis revealed that mitotic index and Ki-67 positive nuclear density, both cell proliferation markers, were also significantly lower in the tumours of *db/db* mice ($P < 0.001$) when compared to controls. An inverse correlation was observed between leptin plasma levels and tumour weight ($r = -0.642$, $P < 0.001$), mitotic index ($r = -0.646$, $P < 0.01$) and Ki-67 positive nuclear density ($r = -0.795$, $P < 0.001$). These results suggest that high leptin concentrations are not favourable to RM1 cell growth and proliferation. On the contrary, high plasma leptin levels were associated with less cellular proliferation and angiogenesis *in vivo*.

Keywords

leptin, morphometry, obesity, OBR, prostate cancer, RM1 cells

In the last century, obesity has taken epidemic proportions at most western populations (Hedley *et al.* 2004; Ogden *et al.* 2006; James 2008). The rising prevalence of obesity and overweight has been attributed to the increase of caloric consumption, in particular of high fat diets, together with the sedentary lifestyle of population (Crespo *et al.* 2001;

Calle & Kaaks 2004; Stubbs & Lee 2004). Obesity is a major public health problem as it is associated with increased risk for several co-morbid conditions such as hypertension, type 2 diabetes, cardiovascular disease and some types of cancer like colon, breast, and prostate among others (Calle & Kaaks 2004). Since the 1970s, epidemiological studies have disclosed the possible association between increased adiposity and increased incidence of colon (Schoen *et al.* 1999; Calle & Kaaks 2004; Pischon *et al.* 2006; Giovannucci & Michaud 2007), breast (Calle & Kaaks 2004; Majed *et al.* 2007), kidney (Asal *et al.* 1988; Calle & Kaaks 2004; Luo *et al.* 2007), oesophagus (Brown *et al.* 1995; Vaughan *et al.* 1995; Calle & Kaaks 2004; Veugelers *et al.* 2006) and endometrial (Calle & Kaaks 2004; Chia *et al.* 2007) cancers, in addition to an unfavourable prognosis and increased death rate from cancer (Calle & Kaaks 2004).

In opposite to what was thought for many years, the adipose tissue is not a passive deposit of triglycerides, it is an active tissue with many biological and endocrine functions (Trayhurn & Beattie 2001; Kershaw & Flier 2004; Ronti *et al.* 2006). It was after the discovery of leptin, the first hormone secreted by the adipocyte to be identified in 1994 that the adipose tissue was formally accepted and recognized as a new endocrine organ (Zhang *et al.* 1994). Besides leptin other hormones and factors like IL-6, TNFα and VEGF have also been found to be secreted by the adipose tissue in proportion to the tissue mass and have been related to the risk of development of some types of cancer, such as prostate cancer (Mistry *et al.* 2007). While some other factors, like adiponectin, seem to have a protective role in carcinogenesis (Goktas *et al.* 2005; Bub *et al.* 2006; Mistry *et al.* 2006).

Leptin is a protein, codified by the *ob* gene, with 16 kDa containing 167 aminoacids, it is secreted predominantly by the adipocytes of the subcutaneous adipose tissue and in most individuals circulates in plasma levels that are proportional to the adipose tissue mass as well as the nutritional state of the individual (Frederich *et al.* 1995; Boden *et al.* 1996; Haque & Garg 2004). Leptin acts at the hypothalamus where it regulates body weight and energy balance (Zhang *et al.* 1994). Leptin is also considered a mitogenic, anti-apoptotic (Hoda & Popken 2008) and angiogenic mediator (Ambrosini *et al.* 2002), because by acting on one of its transmembrane receptor (Tartaglia *et al.* 1995), OBRb, which is the long isoform, also activates many genes involved in cellular proliferation such as c-fos, c-jun, junB, egr-1 and socs3 and angiogenesis regulation, like VEGF factor (Fruhbeck 2006).

The association between prostate cancer and obesity is a controversial issue since the results obtained by different studies have been contradictory (Giovannucci *et al.* 2003).

Obesity seems to be a risk factor for the development of aggressive prostate tumours and increased mortality although low grade prostate tumours are also increased in obesity (Freedland *et al.* 2006, 2008; Ribeiro *et al.* 2006; Mistry *et al.* 2007). Increased leptin, insulin and IGF-1 plasma levels observed in obesity are potential mediators which may promote latent prostate cancer progression to an aggressive state (Amling 2004; Ribeiro *et al.* 2006; Mistry *et al.* 2007).

Since there is a controversial association of obesity and prostate cancer and on the role of leptin on cellular proliferation, angiogenesis, and in tumour development, our aim was to assess *in vivo* the degree of prostate cancer cell proliferation and angiogenesis in different strains of obese mice, namely in leptin deficient mice (*ob/ob*), leptin resistant and diabetic mice (*db/db*) and in mice with diet induced obesity.

Materials and methods

RM-1 murine prostate cancer cells

RM-1 cells are a murine androgen insensitive prostate cancer cell line, which was gently offered by T Thompson (MD Anderson Cancer Center, Houston, TX, USA) for this study (Baley *et al.* 1995). The cells were grown at 25 cm^2 flasks in Dulbecco's Modified Essential Medium (DMEM – Invitrogen, New York, NY, USA) with high glucose (4500 mg/l), 50 mM EPES buffer solution (Invitrogen), sodium bicarbonate (Sigma, St Louis, MO, USA), Penicillin–Streptomycin 100 U/ml (Invitrogen) and Nystatine 100 U/ml (Sigma) in a incubator (Function Line – Heraeus Instruments, Hanau, Germany) which maintained constant humid atmosphere with 5% CO_2 at 37 °C. The cells were separated (passaged) every time they became confluent using trypsin 0.25% (Invitrogen). Mycoplasm test was performed in the cell culture medium, without any antibiotics, to exclude contamination. Cellular viability was evaluated using the trypan blue (Sigma) test and a viability superior to 90% was considered satisfactory.

RM1 Cellular characterization

Immunocytochemistry. A sample of RM1 cells in suspension, obtained at 17th passage, was analysed to check the presence or absence of leptin receptor OBRb (long form). After performing a 50 µl cytospin (Cytospin 3 – Shandon, Cheshire, UK) of the cell suspension on poly-L-lysine (Sigma) microscope slides, the cellular preparation was fixed with 10% formaldehyde. Cells were pepsin digested (0.1%) at HCl 0.01 N (pH 2.25) for 10 min and after this the endogenous peroxidase was blocked by adding H_2O_2 0.3% and

incubating for 5 min. The preparation was first incubated with the primary antibody OBR 12-A (Alpha Diagnostics International, San António, TX, USA) 1:100 BSA 1% overnight at 4 °C and then with the secondary antibody (pig antibody anti-rabbit, DaKoCytomation, Glostrup, Denmark) 1:100 BSA 1% for 1 h. At the end of this time period the preparation was incubated with avidin–biotin complex (ABC, DaKoCytomation) for 30 min and after this the immune reaction was revealed using diaminobenzidine (DAB, DaKoCytomation) during 7 min.

Internal negative control was obtained by omitting the primary antibody (OBR 12-A) and external positive and negative controls were obtained by performing the same technique in mice peritoneal exudate obtained 5 h after intra-peritoneal injection of 1.5 mL casein 10% in C57BL/6J mice.

Normal mice prostate histological study. Native prostate of normal weight C57BL/6J adult male mice were collected and processed for routine histological analysis after being fixed in a paraformaldehyde and picric acid 14% solution. Microscopy slides with tumour sections were incubated in a pepsin solution (0.1%) HCl 0.01 N pH 2.25 during 45 min at 37 °C for antigen retrieval. Endogenous peroxidase blockade was performed by peroxide hydrogen 0.3% incubation for 5 min. Unspecific bindings were eliminated by incubation during 5 min with Protein Block (Novolink Polymer Detection System, Newcastle, UK). Slides were incubated overnight with primary antibody OBR 12-A (Alpha Diagnostics International) 1:100 BSA 1% at 4 °C. After this time were washed with PBS and incubated with Post Primary Block (Novolink Polymer Detection System) during 30 min and then with Polymer (Novolink Polymer Detection System) during another 30 min and the immune reaction was revealed with DAB.

Internal negative control was done as described above except for the omission of the primary antibody.

In vivo studies

Animals. Normal weight C54BL/6J (control), *ob/ob*, *db/db* and C54BL/6J male mice with diet induced obesity (DIO) were purchased to a certified commercial breeder (Charles River Laboratories, Barcelona, Spain) and maintained in standard animal house conditions, temperature 21 ± 1 °C and constant humidity, 12 h controlled photoperiod (7—19 h) with unrestricted assess to regular rat chow (SAFE – Scientific Animal Food & Engineering) and tap water, except for DIO mice that were fed from weaning with hypercaloric diet (45% fat, Charles River). All procedures were approved by the local Ethics Board for Animal

Research and followed the European Union laws on animal protection (86/609/EC).

RM1 cellular growth dose–response curve in normal weight mice C57BL/6J. To evaluate RM1 cell growth *in vivo* seven groups of normal weight mice C57BL/6J (*n* = 6 mice/group) were inoculated subcutaneously with a suspension of RM1 cells in seven different concentrations: 1.5×10^5, 3.0×10^5, 6.0×10^5, 7.5×10^5, 9.0×10^5, 1.2×10^6 e 1.8×10^6 cells/500 µl PBS. Tumour growth and development was then followed-up and monitored for 14 days after inoculation. At the end of the experiment mice were sacrificed by 99% CO_2 atmosphere exposition, blood was collected by cardiac puncture and plasma was separated and kept at −20 °C for later hormone assays. The induced tumours were collected, weighed and processed for histological analysis.

RM1 cellular growth in obese mice. To access RM1 cell growth in obese mice, *ob/ob* mice (*n* = 6), *db/db* mice (*n* = 6), DIO mice (*n* = 6) and normal weight C57BL/6J mice (*n* = 6) (control) were subcutaneously inoculated with a suspension of RM1 at a concentration of 3.0×10^5 cells/500 µl in PBS that was chosen from the dose-response curve performed in normal weight mice. All the experimental procedures were similar to the ones described above. At the end of the experimental period besides the tumour, epididymal fat was also collected and weighed, while plasma was collected to perform the hormone assays.

Tumours histological analysis

Ki-67 Immunohistochemistry. Tumour cellular proliferation was evaluated with the aid of Ki-67 nuclear antigen immunohistochemistry which is expressed during cellular cycle at G1, S, G2 and M phases.

Tumour sections were placed on poly-L-lysine covered microscopy slides. Microwaves were used for antigen retrieval by emerging the slides in tampon citrate solution 0.01 M (pH 6.0) and exposure to microwave oven at 900 W for 20 min. Endogenous peroxidase was neutralized using hydrogen peroxide (LabVision) and unspecific bindings were blocked with ultra block serum (LabVision, Fremont, CA, USA), then slides were incubated, for 60 min, with the primary antibody Ki67 (Novocastra, NCL-L-Ki67-MM1, Newcastle, UK) (1:30) at 25 °C, followed by the biotinylated secondary antibody Goat-Antipolyvalent (Labvision) incubation for 15 min at room temperature. The immune reaction was then marked by an enzymatic polyvalent complex Streptavidin HRP (Labvision) and revelled by adding DAB substrate (Diaminobenzidine tetrahydrochloride, Sigma).

Internal negative control was obtained by omission of the primary antibody and by the observation of unstained muscular cells.

Caveolin immunohistochemistry. Angiogenesis was evaluated with the aid of anti-caveolin immunohistochemistry performed on the tumour sections. The technique was similar to the one described for Ki-67 immunohistochemistry except for the primary antibody used that in this case was anti-caveolin antibody (BD Biosciences, San Jose, CA, USA) 1:400.

Internal negative control was obtained by omission of the primary anti-body and absence of staining in mice pancreatic glandular cells was used as external negative control.

For each antibody, samples were processed the same day and simultaneously to decrease inter-assay variability.

Morphometric analysis

Morphometric analysis was done in 10 digital pictures per slide of 10 high magnification fields (400×, 10× ocular 40× objective) corresponding to an area of 4567.8 μm^2/picture selected by peripheral scanning of the tumour section and avoiding the central area of necrosis. Nuclei and cells in mitosis stained by Ki-67 and vascular vessels stained by caveolin immune reaction were counted in each picture with the aid of a specifically calibrated computer software acopulated to the microscope digital camera (Leica Qwin, Leica Microsystems, Wetzlar, Germany).

Hormone assays

Leptin, insulin and IGF-1 plasma levels were determined using commercial ELISA kits (EZML-82K, EZRML-13K, Linco Research and RMEE25R, BioVendor, respectively) according to the manufacturer instructions.

Statistical analysis of the results

Means, standard error of the mean (SEM), normality and homogeneity of variances were calculated for each group. Differences between means were analysed by ANOVA test with LSD *post-hoc* correction or Kruskal–Wallis test as appropriate. For correlation studies Pearson and Spearman tests were used. $P < 0.05$ was considered statistical significant. All statistically analyses were done using windows SPSS software (version 16).

Results

RM1 cellular characterization

Immunocytochemistry. RM1 cells stained positive for OBRb using the specific antibody OBR 12-A, as demonstrated by the brown coloration of the RM1 cells on the cell periphery (Figure 1a) which supports the OBRb presence at this prostate cancer cellular line. Antibody specificity was further tested by performing the same immunocytochemistry
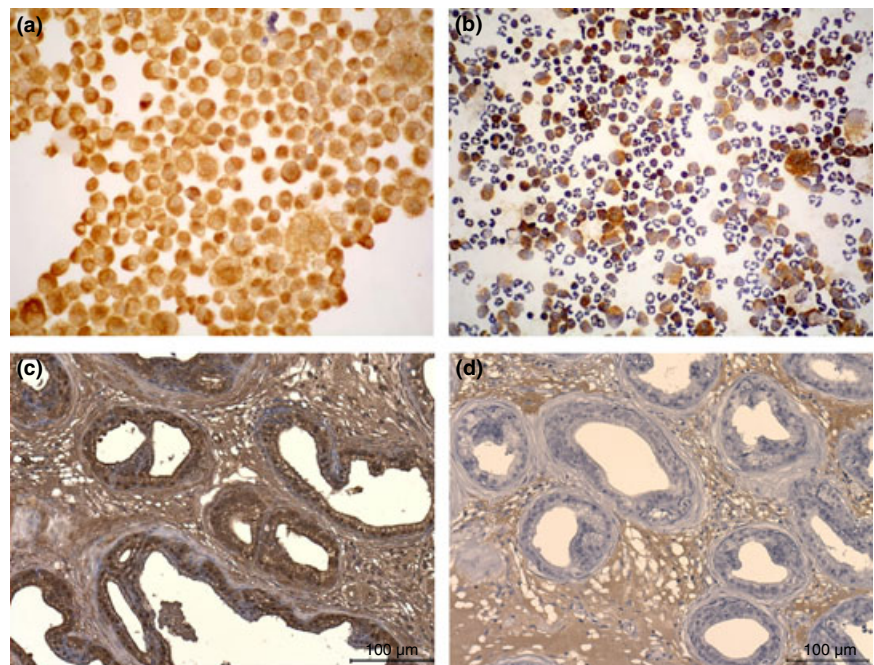

Figure 1 RM1 cells (a) show a brown coloration on the membrane of the cell which corresponds to the location of the leptin receptors after ObR immunocytochemistry. Mice peritoneal exudates (b) with macrophages marked positively for ObR (brown coloration) and neutrophils (unstained) that were used as controls (100×). Immunohistochemistry for ObR of normal C57BL/6J mice prostate (400×) showing the epithelial prostate cells positively stained (c) and negative control (d).

study on a sample of peritoneal exudates, which permitted to verify that macrophages stained positively whereas neutrophils did not stain for OBRb (Figure 1b), as anticipated since OBRb is known to be expressed by macrophages but not by neutrophils (O'Rourke *et al.* 2001; Bruno *et al.* 2005). Mice native prostate glandular epithelium also stained positive on the OBRb immunohistochemistry study (Figure 1c, d).

In vivo studies

Dose–response curve in normal weight C57BL/6J mice. Tumour growth 14 days after dorsal subcutaneous inoculation of different concentrations of RM1 cells (1.5×10^5, 3.0×10^5, 6.0×10^5, 7.5×10^5, 9.0×10^5, 1.2×10^6 and 1.8×10^6 cells in 500 µl of PBS) in C57BL/6J mice was proportional to the concentration of the inoculums'. The 3.0×10^5 cells/500 µl PBS concentration was chosen as the ideal one to be used in the obese mice study because tumours obtained after inoculation of this cellular suspension concentration since these were large enough to allow morphological analysis, presented the smaller dimension variability (SE = 0.242), a small area of central necrosis and high mitotic density (data not known).

RM1 cells growth in obese mice. RM1 cells (3.0×10^5 cells/500 µl PBS) where inoculated in obese *ob/ob* mice, *db/db* mice, DIO mice and normal weight C57BL/6J mice (control). Animals mean body weight was significantly higher in the obese *ob/ob* (57.5 ± 0.99 g) and in obese diabetic *db/db* mice (43.0 ± 1.69 g) when compared with DIO (30.2 ± 1.47 g) and control mice (31.7 ± 0.66 g) ($P < 0.001$) (Table 1). Epididymal fat weight for 100 g of body weight, used as a surrogate marker of fatty body content, was significantly higher at all three groups of obese mice *ob/ob* (4.59 ± 0.26 g), *db/db* (4.70 ± 0.08 g) and DIO (2.29 ± 0.03 g) when compared with control (1.19 ± 0.09 g); ($P < 0.001$, $P < 0.001$, $P < 0.01$, respectively) (Table 1).

The tumour weight 14 days after inoculation was significantly higher in *ob/ob* (2.82 ± 0.46 g) and DIO (2.78 ± 0.19 g) mice ($P < 0.001$) while *db/db* mice displayed significantly smaller tumours (0.16 ± 0.069 g) ($P < 0.05$) when compared to control mice (1.00 ± 0.24 g) tumours (Table 1).

Morphometric studies

Tumour cell proliferation as accessed by counting ki-67 positive nuclei density was higher in the *ob/ob* and DIO mice and lower in the *db/db* mice when compared with controls (Figure 2a–d), although only the difference between *db/db* mice and control has been statistically significant ($P < 0.001$) as shown on Figure 2e. The mitotic index determined by mitosis counting on the same slides stained with anti-Ki67 to allow a better visualization of mitotic nuclei was also significantly lower in the db/db mice tumours when compared to tumours of control mice group ($P < 0.001$) (Figure 3a–e).

Vascular density was significantly higher in tumours of *db/db* mice ($P < 0.05$) while vascular density of tumours of *ob/ob* and DIO mice was not statistically different from controls as it can be observed in Figure 4.

Hormonal plasma levels. Leptin plasma levels of *ob/ob* mice were undetectable while those of *db/db* mice

Table 1 Mice body weight, epididymal fat weight for percentage of body weight, tumour final weight, leptin, insulin and IGF-1 plasma levels 14 days after RM1 cell inoculation in the different experimental groups

	Control	*db/db*	*ob/ob*	DIO	*P*
Body weight (g)	31.72 ± 0.66	43.0 ± 1.69	57.5 ± 0.99	30.2 ± 1.47	$P < 0.001$*†
Epididymal fat tissue (g)	1.19 ± 0.09	4.70 ± 0.08	4.59 ± 0.26	2.29 ± 0.03	$P < 0.001$*†
					$P < 0.01$‡
Tumour weight (g)	1.00 ± 0.24	0.16 ± 0.069	2.82 ± 0.46	2.78 ± 0.19	$P < 0.001$*‡
					$P < 0.05$†
Leptin (ng/ml)	2.10 ± 0.44	26.62 ± 3.38	ND	2.92 ± 0.42	$P < 0.001$†
Insulin (ng/ml)	1.07 ± 0.24	1.90 ± 0.38	7.15 ± 1.27	0.76 ± 0.18	$P < 0.01$*
IGF-1 (ng/ml)	385.33 ± 66.51	437.16 ± 153.32	486.18 ± 216.98	360.44 ± 129.08	NS

*ob/ob vs control.
†*db/db* vs control.
‡DIO vs control.
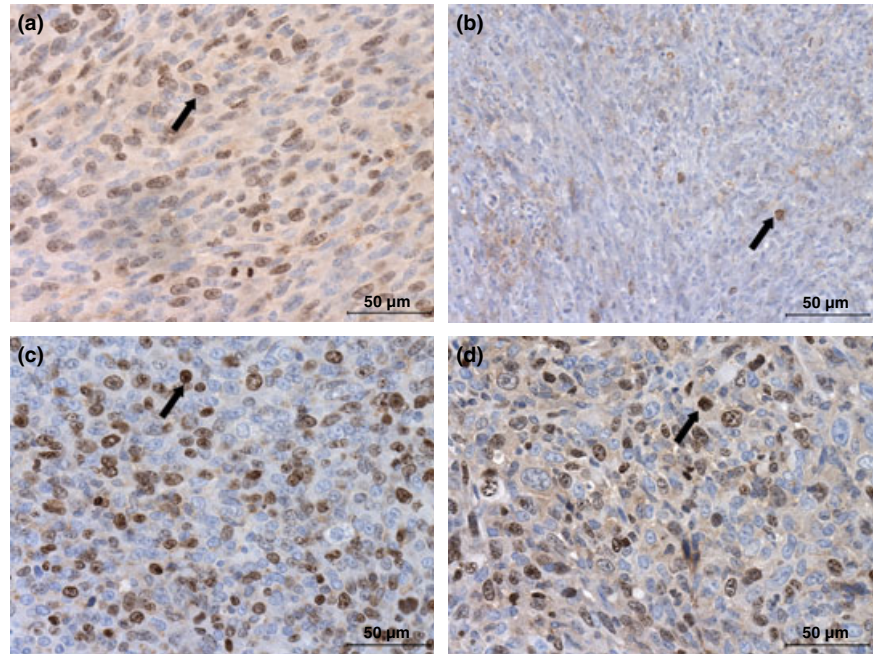ND, below detection level; NS, non-significant.

Figure 2 Immunohistochemistry for Ki-67 used to quantify cell proliferation, showing that the density of positively stained nuclei (arrows) was lower in the tumours of *db/db* mice (b) when compared with controls (a), while tumours developed by *ob/ob* (c) and DIO (d) mice presented a similar nuclear density and not significantly different from controls (400×).Graphic representation of Ki-67 positive nuclear density, *db/db* mice tumours presented a cell proliferation index significantly smaller when compared to the controls (ANOVA ***P < 0.001) (e).
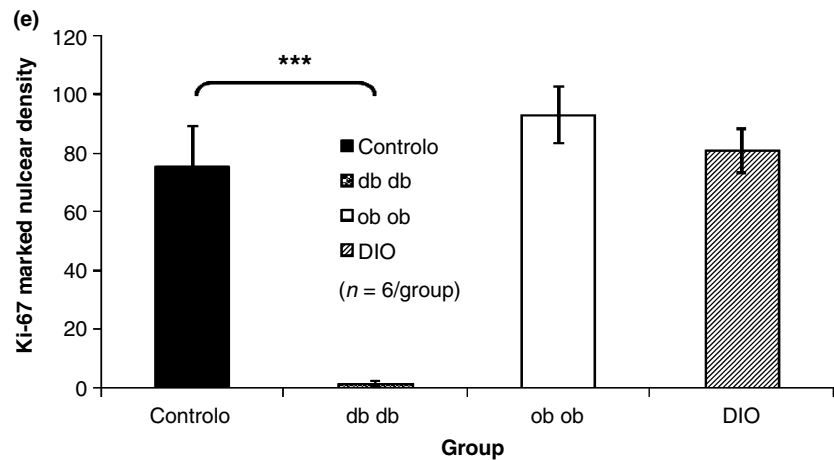
(26.62 ± 3.38 ng/ml) were significantly higher when compared with control mice (2.10 ± 0.44 ng/ml) (P < 0.001) (Table 1).

Insulin plasma levels were significantly higher in *ob/ob* mice (7.15 ± 1.27 ng/ml) when compared to the control group (P < 0.01), insulin plasma levels of *db/db* (1.90 ± 0.38 ng/ml) and DIO mice (0.76 ± 0.18 ng/ml) were similar to control group (1.07 ± 0.24 ng/ml), as shown on Table 1. IGF-1 levels were not significantly different between the four groups of animals, namely in *ob/ob* mice (486.18 ± 216.98 ng/ml), *db/db* mice (437.16 ± 153.32 ng/ml), DIO mice (360.44 ± 129.08 ng/ml) and control mice (385.33 ± 66.51 ng/ml) (Table 1).

Correlations

After obtaining these results a further statistical analysis was performed to determine whether there was any correlation between leptin levels and tumour weight and the histological parameters evaluated. A negative correlation was observed between leptin plasma levels and tumour weight that was statistically significant (r = −0.642, P < 0.001). Leptin plasma levels were also negatively correlated with Ki67 labelled nuclei density (r = −0.795, P < 0.001) and with the mitotic index (r = −0.646, P < 0.01). There was no correlation between insulin or IGF-1 levels and any of the other parameters evaluated.
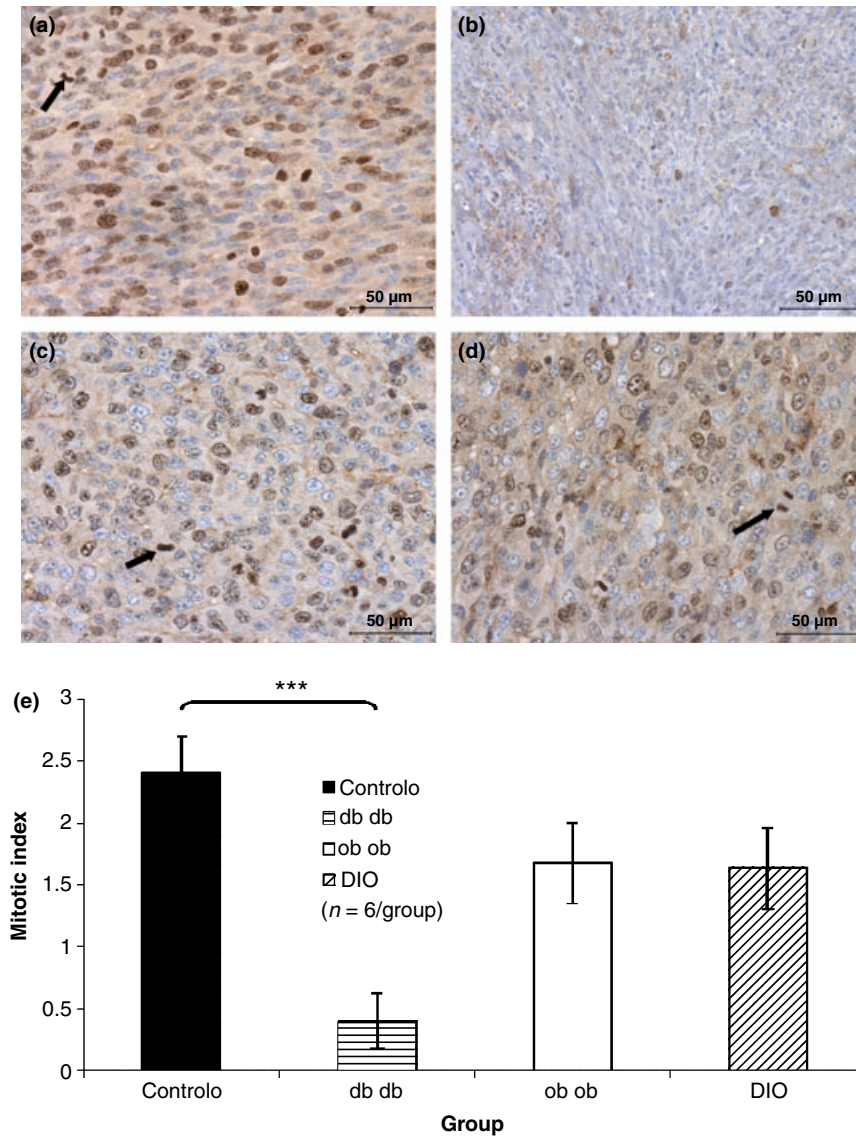
Figure 3 Immunohistochemistry for Ki-67 used to count mitosis (arrows) and to determine the mitotic index, which was significantly smaller in *db/db* mice tumours (b) when compared to controls, while the mitotic index of the tumours induced in *ob/ob* (c), DIO (d) mice were similar to the ones presented by the controls (a) (400×). Mitotic index graphic representation of the tumours presented by the different study groups, showing that *db/db* mice tumours presented a mitotic density significantly smaller than the control group (ANOVA ***$P < 0.001$) (e).

Discussion

Although controversial, obesity has been considered a risk factor for prostate cancer (Giovannucci *et al.* 2003; Baillargeon *et al.* 2006). Leptin, a hormone produced primarily by the adipocyte which circulates in the plasma at concentrations that are usually directly proportional to body fat content (Frederich *et al.* 1995; Maffei *et al.* 1995), has been considered as a putative link between obesity and cancer development (Chang *et al.* 2001; Mistry *et al.* 2007). This hormone acts after binding to a membrane receptor and activates intracellular pathways, such as JAK2/STAT3, MAPK/ERK and PI3K/Akt, promoting antiapoptotic, cellular proliferation, mitogenesis and angiogenesis (Baumann *et al.* 1996; Nakashima *et al.* 1997; Fruhbeck 2006). Leptin receptor isoform OBRb, localized at the cellular membrane, has been pointed as the principle responsible for activation of the pathways mentioned above. *In vitro* studies, using human prostate cancer cells in culture, namely androgen independent DU145 and PC-3 cell lines (Somasundar *et al.* 2003; Deo *et al.* 2008; Hoda & Popken 2008) and androgen dependent LNCaP cell line (Deo *et al.* 2008) performed to evaluate cellular proliferation and intracellular pathways activation in the presence of leptin have demonstrated that this hormone induces proliferation while diminishes cell apoptosis in androgen independent prostate cancer cells (DU145 and PC-3) through PI-3K and MAPK pathways activation (Somasundar *et al.* 2004; Hoda & Popken 2008).
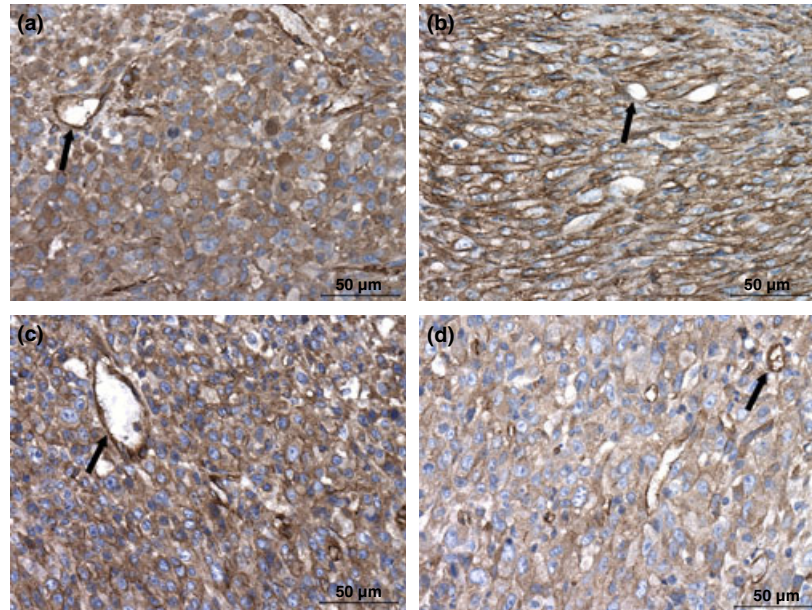
Figure 4 Tumour immunohistochemistry for caveolin used to count stained vessels (arrows) and quantify angiogenesis: these pictures show an increased capillary density in the tumours of the *db/db* group (b) when compared to tumours of the control group (a), while tumours depicted by *ob/ob* and DIO mice present a similar degree of angionegenesis (400×). Graphic representation of the vascular density determined after counting caveolin stained vessels, showing that *db/db* mice tumours had a significantly higher vascular density when compared with the ones of the control group (ANOVA *P = 0.040) (e).
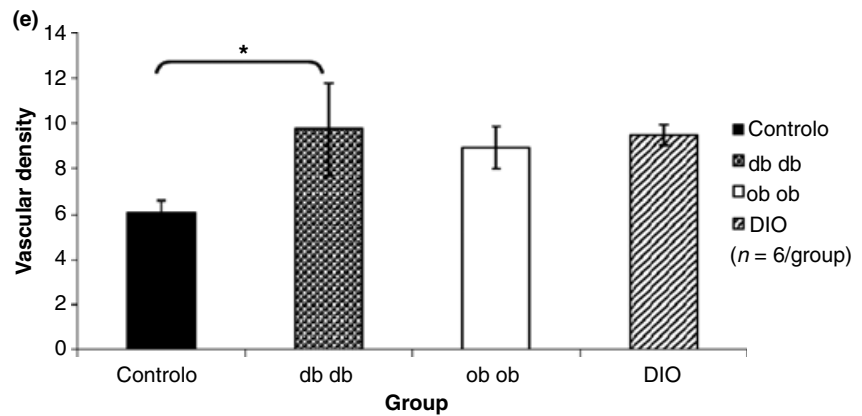
Deo *et al.* has also observed that leptin also leads to STAT-3 pathway activation in androgen dependent (DU145 and PC-3) and independent cell lines (LNCaP) and to cellular proliferation in LNCaP cells (Deo *et al.* 2008). These results diverge from the ones presented by Onuma *et al.* (2003) that have demonstrated that cell proliferation is only increased in androgen independent cells.

The aim of our study was to evaluate *in vivo*, the effect of leptin on prostate cancer cell growth, by injecting RM1 cells that are murine androgen insensitive epithelial prostate cancer cells in obese mice. We have deliberately chosen an androgen insensitive cell line to exclude androgen levels from the confounding variables, as obese individuals may present a decrease in circulating androgen levels (Cohen 2001).

Since RM1 cells had not been previously characterized in what concerns leptin receptor presentation, our first aim has been to access the OBR presence by performing an immuno-cytochemistry using a specific antibody for the long isoform (OBRb), which has confirmed the presence of the receptor by showing a positive staining of the RM1 cell membrane. This result has been confirmed by performing external controls, namely by applying the same technique to mice peritoneal exudates in which macrophages that express the long isoform of the leptin receptor were also labelled positively (O'Rourke *et al.* 2001) while neutrophils that only express the short isoform (OBRa) were not labelled (Bruno *et al.* 2005). Normal mice prostate OBRb immunohistochemistry, using the same antibody also showed that the prostate glandular epithelium labelled positively for the long isoform of the leptin receptor, as previously demonstrated by Malendowicz *et al.* (2006).

Since these results were positive, we have proceeded to the *in vivo* study in order to evaluate leptin effect on RM1 cellular proliferation.

The RM1 cells were inoculated subcutaneously in the dorsal region of normal weight C57BL/6J mice at seven different cell concentrations and were followed up for 14 days in order to determine the most appropriate concentration to be used in the subsequent studies. Tumours obtained were analysed in what concerns mitotic index, area of necrosis and intra-group variability. Our results were similar to the results obtained by Voeks *et al.* (2002) that reported that RM1 cells, when inoculated subcutaneously, present a fast growth, conducting to high grade tumours 10 Gleason score (5+5), since cells present a high abnormality grade and are poorly differentiated (McNeal & Gleason 1991). After tumour histological analysis, we have chosen the 3.0×10^5 cells/500 μl PBS cell concentration since tumours obtained after the inoculation of this concentration presented a small intra-group variability, a high mitotic index and a necrosis area practically inexistent and thus allowing the development of tumours at ideal conditions for morphologic analysis.

To evaluate RM1 cell growth in obese animals with different endogenous environments with regards to leptin sensitivity and leptin levels, the cells were inoculated in obese mice *ob/ob* with congenital leptin deficiency due to a mutation that inactivates the leptin gene ob (Zhang *et al.* 1994); in obese *db/db* mice, diabetic and hiperleptinemic in result of congenital leptin resistance due to a spontaneous mutation on the gene of the leptin receptor db (Chua *et al.* 1996); in C57BL/6J with diet induced obesity (DIO) by feeding with hypercaloric and hyperlipidic diet (45% fat) started after weaning and thus inducing fat mass gain and increased leptin levels in animals with functional leptin receptors; and in normal weight C57BL/6J mice that were used as controls.

As expected, *db/db* and *ob/ob* mice presented body weights significantly higher when compared with control mice, while DIO mice weight was not statistically different from the control group. Nevertheless, when relative body composition was evaluated, DIO mice presented an increased epididymal fat percentage, a surrogate marker of visceral fat content (Monteiro *et al.* 2006), when compared with the control group, so in spite of DIO mice having the same body weight body as controls these mice have an increased fat mass. Likewise, as expected, leptin plasma levels were undetectable in *ob/ob* mice and were significantly higher in *db/db* mice when compared with control mice. DIO mice also presented higher leptin plasma levels than control mice but this difference was not statistically significant.

After RM1 cell inoculation, leptin deficient mice (*ob/ob*) developed significantly larger tumours than control mice, while leptin resistant mice (*db/db*) presented significantly smaller tumours when compared with control mice. This is the opposite to what was expected if leptin worked as a tumour growth factor as some authors have proposed (Chang *et al.* 2001; Somasundar *et al.* 2003, 2004; Mistry *et al.* 2008). Chang *et al.* has evaluated the association of leptin plasma levels with tumour volume at total prostatectomy in patients and has verified that after stratification for age, testosterone levels, body weight and body mass index, individuals with higher leptin levels presented a superior risk of developing larger tumours (Chang *et al.* 2001). While some other authors by conducting *in vitro* studies have verified that PC3 and DU145 cells (Somasundar 2003, 2004; Hoda & Popken 2008), both androgen independent human prostate cancer cell lines, presented increased cellular proliferation when cultivated in the presence of leptin. On the contrary, some other authors, like Stattin *et al.* (2003) in an epidemiologic study did not find any significant relationship between leptin plasma levels and risk of tumour development, while Hsing *et al.* (2001) which evaluated cellular growth of PC3 and DU145 cells in the presence of leptin also could not find any statistically significant association.

In our study, DIO mice developed tumours of similar size to those observed in *ob/ob* mice and significantly larger than the ones presented by the control group despite leptin plasma levels not being significantly higher. After morphometric analysis to evaluate the mitotic index and tumour cell proliferation, we have verified that leptin deficient *ob/ob* mice presented higher cellular proliferation indexes when compared to hyperleptinemic *db/db* mice, which presented tumours with a low cellular proliferation index, suggesting that leptin may act as a cellular proliferation repressor. A negative correlation statistically significant was also observed between leptin plasma levels and cellular proliferation and mitotic index of induced RM1 tumours.

Although vascular density of all obese mice tumours was higher than the observed in the tumours of controls, with the exception of the *db/db* mice group this failed to reach statistical significance, nevertheless we cannot exclude that the small sample size could be one of the reasons for not finding a significant difference. We were unable to find any correlation between leptin plasma levels and angiogenesis degree.

Contrary to our results, Sierra-Honigmann *et al.* have studied HUVECs cells (human umbilical vein endothelial cells) growth in the presence of leptin, after having verified that the cells presented the OBRb receptor by immunocytochemistry and that the receptor was functional by analysing OBRb tyrosine phosforilation and by determining STAT3 levels by SDS-PAGE and immunobloting. To determine

whether leptin had any effect on angiogenesis *in vitro*, cellular migration evaluation through a porous membrane in response to leptin was evaluated. The authors demonstrated that HUVECs cells formed tubules similar to capillaries in a three-dimensional collagen gel in the presence of leptin. The same effect was demonstrated in *in vivo* studies, namely in rats rendered hyperleptinemic after implantation of a polymer with leptin solution in the rats cornea and in leptin resistant OBR deficient rats (*fa/fa* Zucker rats), In these animal models a vigorous angiogenic response was observed in leptin implanted rats, while the same was absent in *fa/fa* leptin resistant rats, thus in favour of a positive effect of leptin in angiogenesis (Sierra-Honigmann *et al.* 1998). Bouloumie *et al.* have demonstrated the expression of the OBR receptor by RT-PCR in the same cell line and have also observed that leptin acted as a pro-angiogenic factor, *in vitro* and *in vivo* (Bouloumie *et al.* 1998).

In our study, since the results obtained after immunocytochemistry for the long isoform of the leptin receptors supported the presence of OBRb in RM1 cells, and leptin levels were negatively correlated with tumour size, these results suggest that leptin is not a tumour growth factor for RM1 cells *in vivo*, and could even function as RM1 proliferation inhibitor. To further clarify the role of the OBR receptor in RM1 cell proliferation activated by leptin it would be pertinent to evaluate cellular growth in the presence of leptin *in vitro* and to study the intracellular pathways of signal activation like MAPK, STAT3 and PI3-K similarly to what has been done with other cell lines (Deo *et al.* 2008; Hoda & Popken 2008).

Our results are in agreement with the results obtained by Hsing *et al.* (2001) and Stattin *et al.* (2003) that have not found any statistically significant association between leptin plasma levels and risk of prostate cancer development in clinical studies. The same authors in turn attribute the risk association to other circulating factors, namely insulin and IGF-1, which are frequently increased in the obese state (Hsing *et al.* 2001), nevertheless we also could not find any association between IGF-1 levels and tumour growth in our study.

Hoda and Popken (2008) in *in vitro* studies using two androgen independent human prostate cancer cell lines, DU145 and PC-3, grown in the presence of leptin have demonstrated that leptin promotes cellular proliferation and inhibits apoptosis, while Deo *et al.* (2008) that studied the same two cellular lines mentioned above and also the LNCaP androgen independent cell line, has verified that leptin only enhanced the cellular growth in the last one.

Nunez *et al.* to dissociate the effect of obesity in carcinogenesis from other hormones that are frequently increased in obesity, studied breast and skin cancer development in 'Fatless A-ZIP/F-1' lipodistrophic transgenic mice, which is an animal model with practically inexistent white adipose tissue and low leptin levels, but presenting insulin resistance and increased insulin levels. Fatless mice develop more breast and skin tumours than wild mice suggesting that insulin, IGF-1 and IL-6 which levels are elevated in these mice as result of insulin resistance and pro-inflammatory state might be the principal link factors between obesity and cancer (Nunez *et al.* 2006). Ablamunits *et al.* (2006) to study leptin and adipose tissue effects on tumour development have also induced skin cancer in *ob/ob* and A-ZIP/F-1 mice and verified that leptin deficient mice when compared to control mice did not present augmented carcinogenesis while lipodistrophic and leptin deficient mice presented higher carcinogenesis suggesting adipose tissue could function has protector factor for tumour development.

In our study, and as expected, *ob/ob* mice, which are hyperphagic, obese and insulin resistant, presented plasma insulin levels significantly higher than control mice while *db/db* mice, which have a similar phenotype to *ob/ob* mice, but besides presenting insulin resistance are diabetic due to pancreatic beta cell insufficiency, present insulin plasma levels not significantly different from control mice. In spite of these differences in insulin levels we also could not find any association between insulin and tumour growth in our current study.

Cox *et al.* (2009) has demonstrated the presence of insulin receptors in human prostate primary tumours suggesting that hyperinsulinemia associated to obesity could have an adverse effect in this carcinoma development. Insulin binds to its receptor and activates several intracellular pathways RAS/RAF/MEK/ERK which stimulate mitogenesis and Akt by PI-3K phosphorilation that inhibit apoptosis and promote carcinogenesis (Frasca *et al.* 2008). Insulin also acts at the autonomous nervous system, in hyperinsulinemic conditions, insulin promotes sympathetic nervous system activation which in turn stimulates catecolamines production which could have a trophic effect in tumour cells by decreasing apoptosis (Nandeesha 2008). Leitzmann *et al.* in a prospective epidemiologic study followed up a cohort of 33 088 men, aged between 55 and 74 years during 8.9 years and has verified an inverse relation between diabetes and prostate cancer development risk, total risk (non-aggressive and aggressive forms) and non-aggressive risk, which suggests that diabetes could act as a protector for initial prostate cancer states (Leitzmann *et al.* 2008).

To our knowledge the presence of insulin receptors in RM1 cells has not been assessed it would be pertinent in future studies, to explore this signalling pathway, namely by

verifying whether the cells express the insulin receptor and by determining the role of insulin in *in vitro* and *in vivo* cellular proliferation. Also, we could not find any association between insulin levels and any of the parameters evaluated in this study.

Obesity is a pathological state characterized by altered circulating levels of several hormones, growth factors and cytokines with potential influence on cellular proliferation, thus from the results obtained in our study we cannot exclude that other factors such as adiponectin (Goktas *et al.* 2005; Lihn *et al.* 2005; Mistry *et al.* 2006; Michalakis *et al.* 2007) or IL-6 (Fried *et al.* 1998; Stienstra *et al.* 2007) could be involved on tumour cell proliferation. These factors and pathways were not evaluated in this study.

Our results could also be due to the fact that the chosen animal models diverge not only in leptin levels but also in other circulating factors, such as insulin among others previously mentioned, with a potential to interfere on tumour growth. In future studies it would be important to repeat this study using normal weight mice rendered hyperleptinemic by exogenous leptin administration to allow the evaluation of the leptin role on tumour cell proliferation independently of other factors which are also altered in obesity. Testosterone levels could also potentially interact with leptin levels and lead to different patterns of tumour growth in this animal model, as we know that obese animals have lower androgen levels than lean controls it would be important to further characterize whereas testosterone levels are able to modulate the response to leptin regarding tumour growth and angiogenesis. However, the RM1 cells used in this study are androgen insensitive and not suitable to study such interaction which would require the use of an androgen dependent murine prostate cell line, namely TRAMP cells.

In conclusion, in our research work performed to evaluate the influence of obesity and leptin on prostate cancer cell growth and angiogenesis *in vivo*, we verified that androgen insensitive murine prostate cancer RM1 cells when inoculated in obese hyperleptinemic *db/db* mice induced the development of small tumours with a low proliferation index and angiogenesis while in obese leptin deficient *ob/ob* mice induced larger tumours with high cell proliferation index when compared to control mice, suggesting that high leptin levels are not favourable to this androgen insensitive prostate cancer cell line and that the hormone will rather limit the growth of this type of prostate tumours.

Acknowledgement

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