Original Article

Obesity and intermittent hypoxia increase tumor growth in a mouse model of sleep apnea

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ABSTRACT

Background: Intermittent hypoxia and obesity which are two pathological conditions commonly found in patients with obstructive sleep apnea (OSA), potentially enhance cancer progression.
Objective: To investigate whether obesity and/or intermittent hypoxia (IH) mimicking OSA affect tumor growth.
Methods: A subcutaneous melanoma was induced in 40 mice [22 obese (40–45 g) and 18 lean (20–25 g)] by injecting 10^6 B16F10 cells in the flank. Nineteen mice (10 obese/9 lean) were subjected to IH (6 h/day for 17 days). A group of 21 mice (12 obese/9 lean) were kept under normoxia. At day 17, tumors were excised, weighed and processed to quantify necrosis and endothelial expression of vascular endothelial growth factor (VEGF) and CD-31. VEGF in plasma was also assessed.
Results: In lean animals, IH enhanced tumor growth from 0.81 ± 0.17 to 1.95 ± 0.32 g. In obese animals, a similar increase in tumor growth (1.94 ± 0.18 g) was observed under normoxia, while adding IH had no further effect (1.69 ± 0.23 g). IH only promoted an increase in tumoral necrosis in lean animals. However, obesity under normoxic conditions increased necrosis, VEGF and CD-31 expression in tumoral tissue. Plasma VEGF strongly correlated with tumor weight ($\rho = 0.76$, $p < 0.001$) in the whole sample; it increased in lean IH-treated animals from 66.40 ± 3.47 to 108.37 ± 9.48 pg/mL, $p < 0.001$), while the high baseline value in obese mice (106.90 ± 4.32 pg/mL) was unaffected by IH.
Conclusions: Obesity and IH increased tumor growth, but did not appear to exert any synergistic effects. Circulating VEGF appeared as a crucial mediator of tumor growth in both situations.

1. Introduction

Obstructive sleep apnea (OSA) is characterized by recurrent disruptions of ventilation caused by an abnormal increase in upper airway collapsibility [1]. These repetitive obstructive apneas induce intermittent hypoxia, increased inspiratory efforts and sleep disruption which have been widely associated with several neurological and cardiovascular consequences [2,3]. Interestingly, it is well known that hypoxia (a hallmark challenge in OSA) enhances tumor growth [4–7]. More specifically, a recent report has indicated that intermittent hypoxia potentiates cancer progression in an animal model of OSA [8]. Similarly, data from some epidemio-logical studies also suggested a possible relationship between cancer death and severity of OSA [9,10]. Moreover, a very recent analysis of a 22-year follow-up of a population-based sample cohort has provided evidence of an association between cancer mortality and OSA [11]. Obesity, a common finding associated with OSA, is known to increase the risk of several types of neoplasia [12] and enhances tumor growth [13–15]. As for the potential mechanism(s) involved, both intermittent hypoxia and obesity can contribute to increase the vascular endothelial growth factor (VEGF) [13,16–20], which promotes angiogenesis and plays an important role in tumor growth [13,21,22].

To better understand the potential mechanisms involved in cancer growth in OSA, the aim of this work was to investigate the contribution of intermittent hypoxia and obesity to the enhancement of tumor progression in an animal model. To this end, we implanted melanoma tumor cells subcutaneously in both lean and obese mice and subjected them to a chronic pattern of intermittent

hypoxia mimicking OSA. Given that intermittent hypoxia could induce some degree of sleep loss [23], we carried out an independent set of experiments to assess whether sleep deprivation could affect tumor growth, as this could be a potential confounding factor. Besides assessing tumor growth and necrosis, we also investigated the potential mechanisms involved by determining the levels of circulating VEGF and the expression of VEGF and CD-31 in the tumor tissue as markers of angiogenesis and vascularization, respectively.

2. Methods

2.1. Animals

This study, which was approved by the Ethical Committee for Animal Research of the University of Barcelona, was conducted on 60 pathogen-free, 10-week-old male mice. To assess the effects of IH and obesity, we used 40 mice from a recently established metabolic syndrome mouse model (The Pound Mouse; Charles River Laboratories, Saint Germain sur L'arbresle, France; http://www.criver.com/en-US/ProdServ/ByType/ResModOver/ResMod/Pages/PoundMouse.aspx) characterized by a deletion of the exon 2 in leptin receptor that produces some characteristic features of metabolic syndrome, such as obesity, insulin resistance, dyslipidemia, and fatty liver disease [24]. Eighteen of these mice were lean (C57BL/6NCrl) and 22 were obese (C57BL/6NCrl-Lepr^{db-lb}/Crl). At the beginning of the experiments the weights of the lean and obese animals ranged 20–25 and 40–45 g, respectively. To assess whether sleep deprivation could affect tumor growth, a second series of 20 pathogen-free, 10-week-old male mice C57BL/6J were subjected to either paradoxical sleep deprivation (PSD) ($n = 10$) or controls ($n = 10$). All animals were housed in controlled cages and were fed with standard rodent chow (Panlab, Barcelona, Spain) and tap water ad libitum.

2.2. Cancer model

A murine model of subcutaneous melanoma based on B16F10 cells (American Type Culture Collection; ATCC-CRL-6475, Manassas, VA) was used [8]. This is a cancer model widely used to investigate the effects of several experimental interventions that either increase or decrease tumor progression [6,13,25]. B16F10 cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (41966, GIBCO, USA), buffered at pH 7.2–7.4 and supplemented with 10% fetal bovine serum (GIBCO, USA), penicillin–streptomycin solution (10^4 UI and 10 mg/mL, respectively) (P4333, Sigma–Aldrich, St. Louis, MO, USA), and amphotericin B solution (250 mg/mL) (A2942, Sigma–Aldrich). Cell expansion was achieved by removing the whole medium, detaching the cells by rinsing the culture with 0.25% (w/v) Trypsin–EDTA ($1\times$) solution (25200, GIBCO, USA) and plating aliquots of the cell suspension in flasks incubated at 37 °C and 5% CO_2. To induce a melanoma in the mice, 10^6 B16F10 cells were resuspended in 150 μl of phosphate buffered saline and injected subcutaneously into the left flank region of each mouse.

2.3. Application of chronic intermittent hypoxia

The experimental setting for applying intermittent hypoxia mimicking OSA is shown in the diagram of Fig. 1, top. A continuous flow of gas circulated through a box (26 cm long, 18 cm wide, 6 cm high) by means of a distribution system based on several small orifices to achieve a uniform distribution of gas inside the mice cage. A silent pneumatic valve placed near the inlet of the box cyclically switched from the room air entrance (40s) to a gas reservoir of
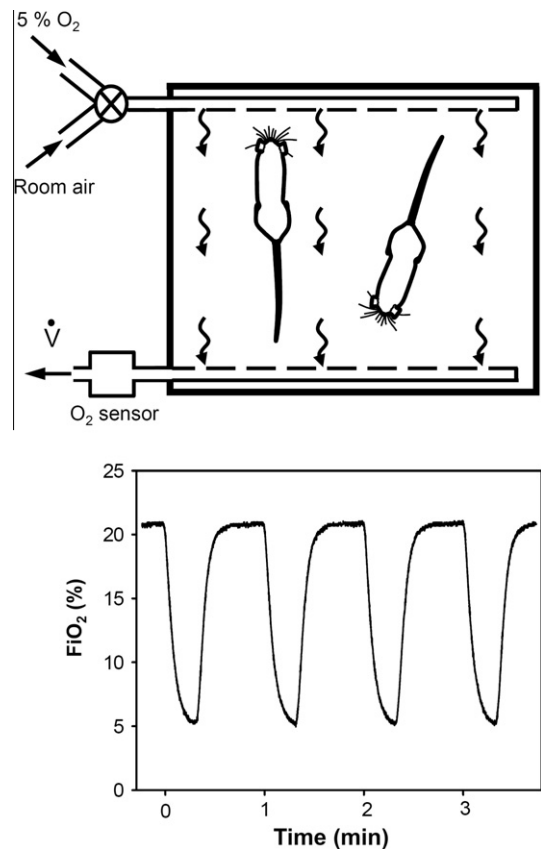

Fig. 1. (Top) Diagram of the experimental setting subjecting mice to controlled intermittent hypoxia. See text for explanation. (Bottom) Time course of oxygen fraction (FiO$_2$) of the air breathed by the mice when subjected to intermittent hypoxia.

hypoxic air at an oxygen fraction (FiO$_2$) of 5% (20s). A zirconia solid-electrolyte cell oxygen sensor (MWL-F, 0.1–95% $O_2 \pm 1$%, Fujikura Ltd., Tokyo, Japan) was connected to the gas outlet of the box to continually measure the FiO$_2$ in the chamber. Application of intermittent hypoxia started on the same day as the injection of cancer cells. Nineteen mice (10 obese/9 lean) were placed in the intermittent hypoxia box, with food and water available for 6 h per day during the light period (10 am–4 pm) for 17 consecutive days. Fig. 1, bottom, shows the pattern of intermittent hypoxia effectively applied to these mice. A control group of 21 mice (12 obese/9 lean) was placed in a system identical to that of Fig. 1, but the reservoir of 5% O_2 air was replaced by room air (normoxia). Accordingly, both groups of melanoma-injected mice were subjected to exactly the same protocol, the only difference being the breathing of normoxic or intermittently hypoxic air.

2.4. Sleep deprivation procedure

To assess the effect of sleep deprivation on tumor growth we used the well characterized multiple platform method [26]. Briefly, the mice were placed on circular platforms (3.5 cm of diameter) located inside a cage (48 × 24 × 15 cm) into which water was introduced to a depth of ~1 cm. The animals could move freely inside the cage by jumping between platforms. Partial sleep deprivation (PSD) occurs because at the onset of each paradoxical sleep episode, the animals wake up when they fall into the water. The cage contained 12 platforms with 5 animals each. All animals were injected with melanoma cells, as described previously. After 3 days, the animals were subject to three periods of 72 h of PSD each, with

a recovery period of 24 h between them (during which the water was replaced by sawdust). At day 17, after the injection, the animals were sacrificed to measure tumor growth. Control animals followed an identical procedure, but the floor of the cage with platforms was always covered with sawdust.

2.5. Assessment of tumor tissue

At the end of the 17 days of the injection, the mice were exsanguinated and sacrificed by cervical dislocation with anesthesia and the tumors were excised, weighed, fixed in formalin and embedded in paraffin for microscopic examination. The paraffin blocks of the tumors were cut into 4-μm-thick sections and stained with hematoxylin–eosin. A section from the central part of each animal's tumor was examined under an inverted light microscope (TE-2000E, Nikon, Japan) equipped with a Charged-Coupled Device camera (CCD) (Orca C9100, Hamamatsu, Japan). Necrotic cells were identified by a loss of cell borders and presence of cellular debris. The necrotic tumor areas and the total tumor area were quantified using Metamorph image analysis software (Molecular Devices, Sunnyvale, CA). Tumor necrosis was quantified as the percentage of necrotic tumor area with respect to the total tumor area.

For the immunohistochemical detection of VEGF and CD-31, the paraffin blocks of tumors were cut at 4 μm, dewaxed, rehydrated and prepared using heat-induced epitope retrieval in citrate buffer, pH 6.0. Anti-VEGF (#ABS82 Millipore, Billerica, MA, USA) and anti-CD-31 (ab28364, Abcam, Cambridge, UK) rabbit polyclonal antibodies were used, following the manufacturer's protocol for paraffin-embedded tissues. Immunoreactivity was revealed by the labeled streptavidin biotin (LSAB) alkaline-phosphatase technique (Dako Cytomation, Denmark A/S). Sections were counterstained with hematoxylin and quantified. All the determinations from histological preparations were made by an investigator who was blind to the experimental group corresponding to each sample.

2.6. Circulating vascular endothelial growth factor

Concentration of VEGF in plasma was determined by means of colorimetric sandwich ELISA (Quantikine, R&D Systems; Minneapolis, MN), following the manufacturer's protocol.

2.7. Statistical analysis

Data are presented as mean ± SE. To discard any effect of body weight on tumor growth, tumor weight was analyzed in terms of its absolute value and by normalizing for body weight and for the body weight minus tumor weight. A two-way ANOVA was used to test the effects of obesity and intermittent hypoxia on all the variables. Multiple comparisons between groups were performed by means of Bonferroni's method.

3. Results

3.1. Tumor growth

Seventeen days after the injection of melanoma cells, the tumor weight of control non-obese animals was enhanced in a similar manner by intermittent hypoxia ($p = 0.006$, $F = 8.51$) and by obesity ($p = 0.003$, $F = 10.02$). Specifically, the tumor weight was $0.81 ± 0.17$ g in normoxic lean mice, $1.95 ± 0.32$ g ($p < 0.001$) in lean mice subjected to intermittent hypoxia and $1.94 ± 0.18$ g ($p < 0.001$) in normoxic obese animals. In obese mice, however, tumor growth was not affected by intermittent hypoxia ($1.69 ± 0.23$ g) (Fig. 2).
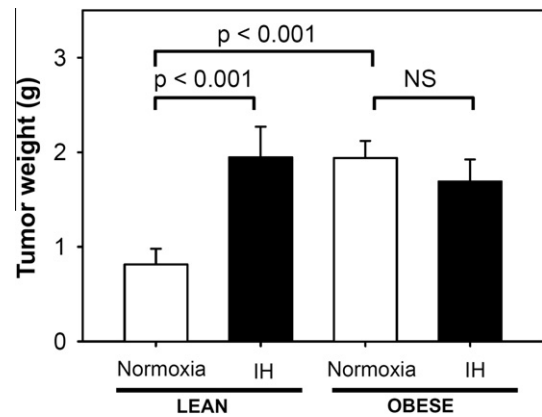

Fig. 2. The tumor weight at day 17 from lean and obese mice subjected to either normoxia or intermittent hypoxia. Results are shown as mean ± SE.
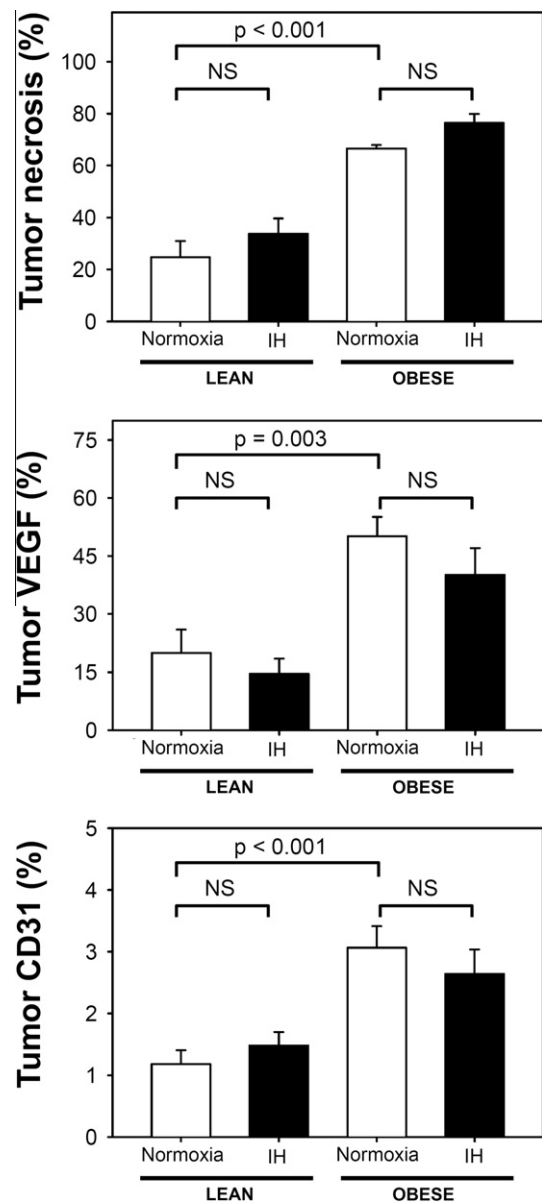

Fig. 3. Percentage of necrosis, vascular endothelial growth factor and CD-31 positive cells in tumors from lean and obese mice subjected to normoxia or intermittent hypoxia. Results are shown as mean ± SE.

The interaction between hypoxia and obesity in tumor weight was significant ($p < 0.001$, $F = 16.80$).

At the end of the experiment, the weight of lean (23.2 ± 0.7 g) and obese (45.5 ± 0.9 g) animals subjected to intermittent hypoxia was lower ($p = 0.008$, $F = 7.84$) with respect to the specified control groups (28.7 ± 2.0 g and 46.2 ± 1.4 g in lean and obese mice, respectively). Normalizing the tumor weight for the body weight (both in terms of total body weight and of body weight minus tumor weight) provided results concordant with the ones obtained using the absolute tumor weight (Fig. 2) and the statistical significance of the differences among groups were maintained.

3.2. Necrosis, VEGF and CD-31 in tumors

Obesity had also a considerable impact on tumor necrosis ($p < 0.001$, $F = 38.75$) and on the expression of VEGF ($p < 0.001$, $F = 24.28$) and CD-31 in the tumor ($p < 0.001$, $F = 22.45$) (Figs. 3 and 4). The obese animals presented significantly higher levels of necrotic tumoral tissue ($66.54 \pm 1.40\%$), presence of VEGF ($50.11 \pm 4.97\%$) and CD-31 ($3.06 \pm 0.35\%$) positive cells with respect to the lean animals ($24.70 \pm 6.26\%$, $20.04 \pm 6.01\%$ and $1.18 \pm 0.22\%$, respectively) ($p < 0.001$ for all these three variables). However, the intermittent hypoxia stimulus promoted a tendency to increase necrotic tissue in lean animals only (from $24.70 \pm 6.26\%$ to $33.74 \pm 5.94\%$ ($p = 0.066$, $F = 3.60$)). No added effect of intermittent hypoxia was observed in obese mice.

3.3. Relationship between circulating VEGF and tumor growth

The levels of VEGF in plasma were increased by intermittent hypoxia ($p = 0.028$, $F = 5.25$) and obesity ($p = 0.047$, $F = 4.24$) (Fig. 5, top), with statistically significant interaction between variables ($p < 0.001$, $F = 19.45$). Specifically, VEGF was increased from 66.40 ± 3.47 to 108.37 ± 9.48 pg/mL in lean animals ($p < 0.001$), although it was not further increased under obese conditions. Plasma VEGF in obese animals (106.90 ± 4.32 pg/mL) was increased, with respect to lean controls (66.40 ± 3.47 pg/mL) only under normoxia ($p < 0.001$). Interestingly, plasma VEGF presented a similar
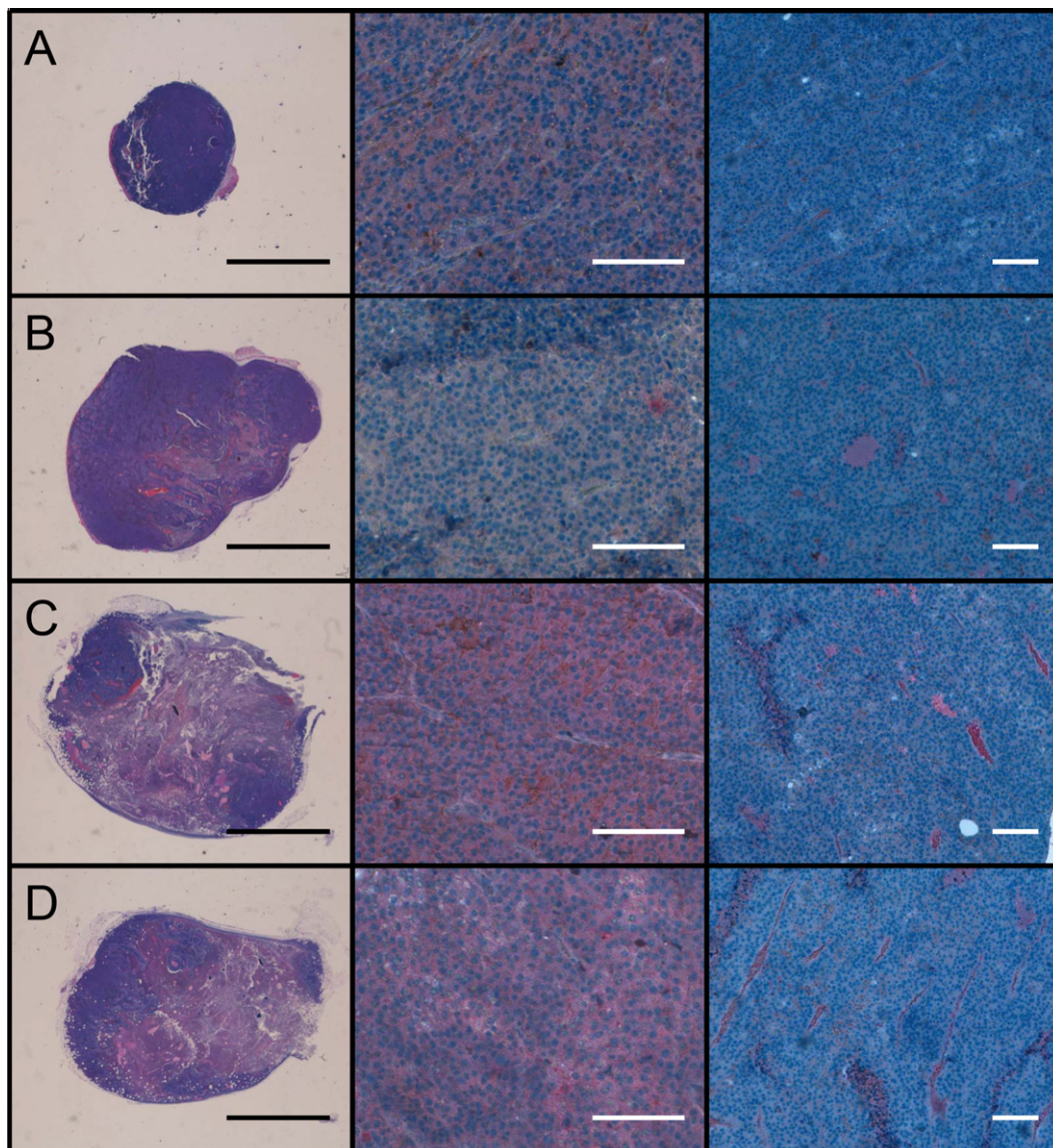

Fig. 4. Representative histological images from the central cut of the melanoma in the four groups studied: Lean animals subjected to normoxia (A) or intermittent hypoxia (B) and obese mice under normoxia (C) or intermittent hypoxia (D). For each group there are representative images of necrotic areas (left; scale bar 5 mm), expression of VEGF (central, scale bar 50 μm) and CD-31 (right; scale bar 50 μm).
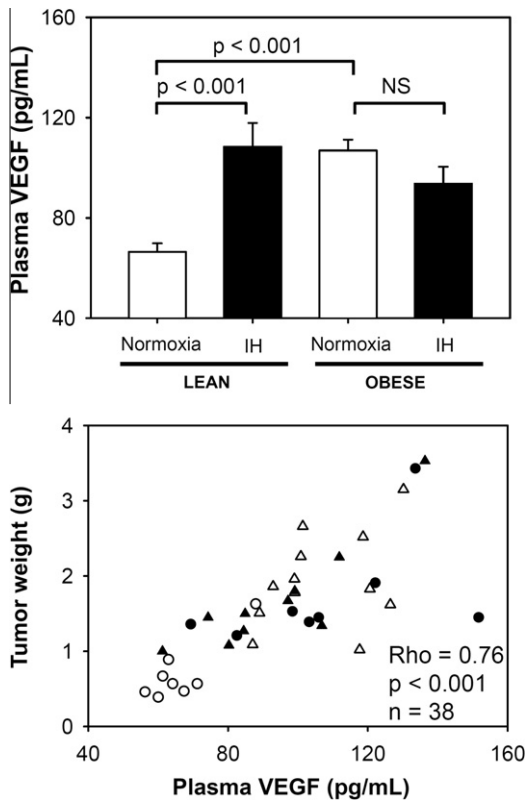
Fig. 5. (Top) Plasma VEGF from lean and obese mice subjected to normoxia or intermittent hypoxia. Results are shown as mean ± SE. (Bottom) Correlation between tumor weight and plasma VEGF from all individual cases. Open and closed symbols represent normoxia and intermittent hypoxia, respectively; circles and triangles correspond to lean and obese mice, respectively.

behavior to that observed in tumor weight. In fact, circulating VEGF strongly correlated with tumor weight ($\rho = 0.76$, $p < 0.001$) (Fig. 5, bottom).

3.4. Tumor growth in sleep deprivation

In animals subjected to sleep deprivation, tumor growth (2.89 ± 0.42 g) was not significantly different from that of normally sleeping control animals (2.59 ± 0.38 g; $p = 0.658$).

4. Discussion

The application of intermittent hypoxia with a frequency and relative duration that mimic OSA increased the growth of melanoma tumors in lean mice, but not in obese ones. In fact, the increased tumor growth induced by obesity was not enhanced by adding the intermittent hypoxia stimulus. The effect of intermittent hypoxia on tumoral necrosis and vascular density was smaller than the effect of obesity. Plasma levels of VEGF presented a similar behavior to that of tumor weight, resulting in a strong correlation between the two variables.

The experimental setting used allowed us to observe obese and lean mice to an intermittent hypoxia that closely mimicked the condition of patients with OSA. In contrast with most of the experimental settings used to study intermittent hypoxia in rodents, where the duration of the hypoxic and normoxic phases was relatively long [6], we applied a controlled pattern with hypoxia of lesser duration (20s) than normoxia (40s) (Fig. 1). This pattern of O_2 concentration in the air breathed by the animals was very close to the ones used in a recent study measuring arterial oxygen satu-

ration (SaO_2) in free-moving mice [27]. Accordingly, the arterial oxygen desaturation pattern of the mice in the present study was expected to range between 100% and 85% [27]. To minimize any potential effect of social stress on tumor progression [28], each group of mice was kept together in a cage, both during the application of intermittent hypoxia and throughout the rest of the day. A methodological open question is whether the application of intermittent hypoxia and cancer cells at different time points could significantly modify the results. Metabolic preconditioning resulting from chronic application of intermittent hypoxia [29] before melanoma cell injection could affect cancer progression. The application of intermittent hypoxia, once the tumor is well established, could affect the pattern of its growth. It is, however, not clear which of these possible experimental time schedules could be more realistic for studying cancer progression in patients with OSA. Though, it seems that the basic mechanisms by which intermittent hypoxia modulates tumor growth should be unaffected by the schedule of interventions. Another noteworthy issue arising from the experimental approach used in this work is the selection of the obese mouse model. The metabolic syndrome model (deletion of the exon 2 in leptin receptor) could have been replaced by mice under diet-induced obesity. In fact, a recent investigation found synergistic effects of diet-induced obesity and intermittent hypoxia on metabolic and pro-inflammatory cytokines [29]. Although studying the effects of cancer progression in obesity induced by diet in non-mutant mice would be of interest, a model of metabolic syndrome is also useful as patients with OSA usually present not only a high body mass index (BMI), but also with manifestations of metabolic syndrome [30].

It is well known that hypoxia and inflammation coincide at several points in the biology of cancer [15,21,31]. However, intermittent hypoxia triggers transcriptional responses which differ from the ones elicited by continuous hypoxia, and the duration of the hypoxic and reoxygenation periods could modulate hypoxic preconditioning. The differential effects of continuous and intermittent hypoxia, which have been described at the molecular, cellular and systemic levels [32,33], highlight the interest of investigating the effects of a high-rate intermittent hypoxia mimicking OSA on tumor growth in both lean and obese animals. A hypoxic environment triggers some adaptive cellular mechanisms that largely rely on the transcription of hypoxia-inducible factor (HIF-1α) [21,22], which can also be modulated by interaction with inflammatory molecules [14,21]. HIF-1α acts as a regulator of vascular endothelial growth factor (VEGF) in cancer, promoting angiogenesis and contributing to tumor growth [21,22]. The newly formed vascular network could, however, present structural and functional abnormalities, which lead to a reduction in both perfusion and delivery of oxygen to the tumor tissue [21,22], thereby, promoting tumor necrosis [34]. Our results indicate an upward trend in the percentage of necrotic regions in the tumors of those animals subjected to intermittent hypoxia (most significantly in the obese specimens). This increase in necrosis seems to be partly related to the reduced availability of oxygen, as a higher number of cells marked positively for VEGF in these regions [22]. The higher levels of VEGF also correlated with an enhanced vascularization of the tumors, as measured by endothelial CD-31 protein. Similar results were found in melanoma tumors of mice subjected to cyclic hypoxia [6] and in different types of obesity [13,35]. Although tumor growth was similar during obesity and intermittent hypoxia, the expression of VEGF and CD-31 and the presence of necrosis were slightly different. This could be explained by the chronic inflammation and/or interaction with other molecules related to obesity, associated with cancer growth (i.e., adipokines, insulin and others derived from metabolic syndrome) [13–15,36]. The differences observed in the expression of molecules and necrosis within the tu-

mor could modify its behavior. For instance, high levels of necrosis have been related to poorer prognosis [37].

Apart from the fact that tumor cells may directly sense and respond to intermittent hypoxia, systemic factors could also participate in tumor growth. Indeed, tumor cells can proliferate for two potential reasons: on the one hand, an intrinsic response to intermittent hypoxia could be sensed at the tumor level; on the other, tumor growth could be boosted by increased vascularization, enhanced by external factors such as VEGF which reach the tumor via the blood stream [31,38]. In fact, circulating VEGF is used as a cancer prognostic marker [31,39,40] and some direct inhibitors of the VEGF pathway are currently approved for use in cancer therapy [41]. This pathway could be of particular relevance in OSA since patients with this sleep breathing disorder exhibit increased VEGF in the bloodstream [16,19,20]. Our results suggest that VEGF in plasma could play an important role in tumor growth, as indicated by the strong correlation observed between these two variables. The fact that both stimuli affect similar pathways involving pro-inflammatory and pro-angiogenic factors could explain why adding both together did not further activate the previously stimulated mechanism and did not result in any synergistic effect on tumor growth in our results. It should be mentioned, however, that this result could be due to the fact that the level of obesity in our mice was extremely high. The baseline weight of the obese animals was 1.6-fold the weight of controls, which in a rough translation to humans would have represented a BMI within the very morbid range of obesity. This point emphasizes the need for future studies using animals with different levels of diet-induced obesity to study the dose-dependent interaction between obesity and intermittent hypoxia in tumor progression.

This work provides some background to recent epidemiological data that demonstrates an association between sleep-disturbed breathing (SDB) and cancer mortality [11]. A 22-year follow-up of the Wisconsin sleep study cohort of 1522 subjects indicated that, after adjusting for age, sex, body mass index and smoking, SDB was associated with cancer mortality in a dose–response fashion, particularly when considering SBD severity in terms of nocturnal desaturation. Although this dose–response association was more evident in stratified analyses, and more clearly statistically significant in non-obese compared to obese participants, the role of obesity in cancer mortality was still relevant [11]. This is not unexpected since a meta-analysis of cancer incidence has reported that, for every 5 kg/m^2 increase in BMI, the increase in the risk ratio of cancer incidence attributable to obesity ranges from 0.57 to 1.59 [12]. Moreover, a very recent pooled analysis indicated that, compared with individuals of normal weight, the relative risk of dying from cancer was 1.21 for those who were obese [42]. Even though this recent data [11] suggest an association between OSA and cancer mortality, the reports published from patient studies to date have not provided any evidence of causality. Specifically, there is still a lack of epidemiological evidence of increased cancer incidence in OSA. Another important question that remains open is whether, in addition to increasing the growth of cancer cells in a tumor, intermittent hypoxia and obesity could also enhance the process of tumorogenesis. In this regard, it could be possible that the DNA oxidative stress observed in OSA patients [43] could, per se or in combination of other tumorogenic insults, increase cell malignization.

In this context, the present animal study, carried out under well-controlled conditions to investigate the effect of intermittent hypoxia in lean and obese animals, gives support for a link between OSA and cancer progression, particularly in non-obese subjects. In conclusion, this work represents a proof of concept and more research is therefore necessary to explore cancer cell biology in OSA. It also assesses whether the results obtained in melanoma apply to other types of cancer. Clarification of these issues from pa-

tients' data would require further studies considering obesity as a specific potential factor. The relative importance of intermittent hypoxia and obesity as cancer promoters in OSA patients would depend on the severity of each of these variables, clinically measured by a pulseoximetry index and BMI, respectively.

Conflict of interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: http://dx.doi.org/10.1016/j.sleep.2012.08.012.

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