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ORIGINAL ARTICLE

Enhanced expression of melanoma progression markers in mouse model of sleep apnea

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Abstract
Introduction: Obstructive sleep apnea has been associated with higher cancer incidence and mortality. Increased melanoma aggressivity was reported in obstructive sleep apnea patients. Mice exposed to intermittent hypoxia (IH) mimicking sleep apnea show enhanced melanoma growth. Markers of melanoma progression have not been investigated in this model.
Objective: The present study examined whether IH affects markers of melanoma tumor progression.
Methods: Mice were exposed to isocapnic IH to a nadir of 8% oxygen fraction for 14 days. One million B16F10 melanoma cells were injected subcutaneously. Immunohistochemistry staining for Ki-67, PCNA, S100-beta, HMB-45, Melan-A, TGF-beta, Caspase-1, and HIF-1alpha were quantified using Photoshop.
Results: Percentage of positive area stained was higher in IH than sham IH group for Caspase-1, Ki-67, PCNA, and Melan-A. The greater expression of several markers of tumor aggressiveness, including markers of ribosomal RNA transcription (Ki-67) and of DNA synthesis (PCNA), in mice exposed to isocapnic IH than in controls provide molecular evidence for a apnea–cancer relationship.
Conclusions: These findings have potential repercussions in the understanding of differences in clinical course of tumors in obstructive sleep apnea patients. Further investigation is necessary to confirm mechanisms of these descriptive results.

Introduction

Epidemiological data connects obstructive sleep apnea (OSA) with cancer mortality and incidence[1–4] as well as with melanoma aggressivity.[5] Intermittent hypoxia (IH), a

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feature of OSA,[6] is used as a model to investigate outcomes of this disease.[7] Exposure to 6 h of one episode of hypoxia per minute to an oxygen nadir of 5% augments melanoma growth[8] and metastasis in mice.[9] In this model, both obesity and IH affect melanoma growth, with non-addictive effect.[10,11]

Several proteins are altered specifically in melanoma and have prognostic value. S100 calcium binding protein B (S100-β) is a prognostic marker for melanoma stage, disease recurrence, and low overall patient survival[12] Augmented expression of proliferation marker Ki-67 indicates increased ribosomal RNA transcription.[13] Greater expression of proliferating cell nuclear antigen (PCNA) in the cell nucleus indicates greater DNA synthesis. The presence of hypoxia-inducible factor 1-alpha (HIF-1α) suggests the exposure of the cell to hypoxia. The protein transforming growth factor beta (TGF-β) controls proliferation, differentiation, and other functions in cells. Caspase-1 induces cell apoptosis and is a marker of malignancy. Melan-A and human melanoma black 45 (HMB-45) are antigens markers of melanocytes, used to identify a tumor as a melanoma.[14]

The various stages of tumor formation and progression can be regulated by hypoxia.[15–18] Knowledge, however, focusing specifically on IH simulating OSA and its molecular effects in melanoma aggressiveness is incipient.

The aim of the present study is to test the hypothesis that an animal model simulating OSA intensifies the expression of aggressiveness markers. In this investigation, mice were exposed to 14 days of isocapnic IH, similar to severe OSA.

Methods

Animals

Twelve 2-month-old male C57BL/6 mice, weighing between 25 and 30 g at the beginning of the study, were used in the study; six exposed to isocapnic IH and six controls exposed to sham IH. Except for the gas mixture, both groups of melanoma-injected mice were subjected to the same protocol. The animals were housed under temperatures ranging between 22.5 and 24.5 °C with a 12:12-h light-dark cycle and received ad libitum standard mice chow and water. The protocol was approved by the Committee on the Ethics of Animal Experiments of the *Hospital de Clínicas de Porto Alegre* (Permit Number: 09-483) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.[19]

Isocapnic intermittent hypoxia

The isocapnic IH system was described in detail before.[20–22] In brief, for 14 days, 8 h per day, from 9 AM to 5 PM, in the lights on period, the animals were kept in the hypoxia system. During 30 s a mixture with 92% nitrogen and 8% CO_2 was released into the hypoxia chamber. A nadir of inspired oxygen fraction of $7 \pm 1\%$ and a peak of inspired carbon dioxide of $6 \pm 1\%$ were reached as confirmed by sensors inside the cage. Next, room air was insufflated during 30 s to restore normoxia. The exposure to isocapnic IH started on the same day as the injection of melanoma cells.

Melanoma cells

To induce melanoma in the mice, 1×10^6 B16F10 cells (ATCC-CRL-6475; American Type Culture Collection, Manassas, VA, USA) were used. The cells were suspended in 150 μl of phosphate buffered saline and injected subcutaneously in the left hind limb of each mouse.

On the last day of the experiment, each mouse was removed from the IH system to be immediately anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. The animals were not weighed at this moment. After deep anesthesia was confirmed, the tumors were excised and fixed. After tumor excision the mice were euthanized in a CO_2 chamber and the organs were macroscopically examined for metastasis.

Hematoxylin-eosin and immunohistochemistry

The paraffin blocks of the tumor were sectioned in 5 μm slices. Sections were mounted on glass slides, stained with hematoxylin-eosin, and the tumor diameters measured. The immunohistochemical staining was processed using primary monoclonal mouse antibodies against: (1) HIF-1α (1:200 dilution; sc-53546), (2) TGF-β (1:200 dilution; sc-398), (3) S100-β (1:400 dilution; sc-28533), (4) Melan-A (1:200 dilution; sc-20032), (5) Caspase-1 (1:200 dilution; sc-56036) (all Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), (6) HMB-45 (1:100 dilution; HMB-45; Dako, Carpinteria, CA, USA), (7) Ki-67 (1:50 dilution; SP6; Cell Marque, Rocklin CA, USA), and (8) PCNA (1:200 dilution; 307901; BioLegend, San Diego, CA, USA).

The secondary antibody was PicTure™-MAX Polymer Detection kit, 87-8983 (Invitrogen, Camarillo, CA, USA), which reacts with mouse antibodies. The color was developed with a DAB Chromogen. Sections were counterstained with hematoxylin-Harris and rinsed with ammoniacal water to obtain a light blue color.

Three pictures of each slide at 400× were quantified for immunohistochemistry staining by Adobe Photoshop™. Two different readers, blind to the exposure groups performed the staining analyses. The readings were averaged. When two measurements disagreed by more than 5%, readings from a third observer were utilized instead of the one with greater disparity among the three.[23]

Statistical analysis

Statistical analysis was performed using SPSS (SPSS, Chicago, IL). Medians and quartiles [25th–75th] were utilized to express grouped data. Mann–Whitney test was used to compare measurements of percentage of stained area. Results were considered significant when the probability of alpha error was <0.05.

Results

The median tumor diameter in the hypoxia group, was 9 mm [interquartile range (IQR): 6–11 mm], 50% larger than in the control group, 6 mm [IQR: 4–13 mm]. The difference, however, did not reach significance ($P = 0.6$). No macroscopic