Fragmented Sleep Accelerates Tumor Growth and Progression through Recruitment of Tumor-Associated Macrophages and TLR4 Signaling

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Abstract

Sleep fragmentation (SF) is a highly prevalent condition and a hallmark of sleep apnea, a condition that has been associated with increased cancer incidence and mortality. In this study, we examined the hypothesis that sleep fragmentation promotes tumor growth and progression through proinflammatory TLR4 signaling. In the design, we compared mice that were exposed to sleep fragmentation one week before engraftment of syngeneic TC1 or LL3 tumor cells and tumor analysis four weeks later. We also compared host contributions through the use of mice genetically deficient in TLR4 or its effector molecules MYD88 or TRIF. We found that sleep fragmentation enhanced tumor size and weight compared with control mice. Increased invasiveness was apparent in sleep fragmentation tumors, which penetrated the tumor capsule into surrounding tissues, including adjacent muscle. Tumor-associated macrophages (TAM) were more numerous in sleep fragmentation tumors, where they were distributed in a relatively closer proximity to the tumor capsule compared with control mice. Although tumors were generally smaller in both MYD88−/− and TRIF−/− hosts, the more aggressive features produced by sleep fragmentation persisted. In contrast, these more aggressive features produced by sleep fragmentation were abolished completely in TLR4−/− mice. Our findings offer mechanistic insights into how sleep perturbations can accelerate tumor growth and invasiveness through TAM recruitment and TLR4 signaling pathways. Cancer Res; 74(5); 1–9. ©2014 AACR.

Introduction

In recent years, the possibility that sleep duration and overall sleep characteristics may affect overall cancer outcomes has been advanced (1). Indeed, in several epidemiologic studies spanning the last decade, the presence of altered sleep duration, both shortened and prolonged sleep, has been associated with higher incidence or adverse prognosis for several solid tumors (2–17). However, although the role of the circadian clock system in tumorigenesis has been extensively explored (18, 19), no animal models have thus far examined whether the association between disrupted sleep and tumorigenesis is indeed recapitulated, and if so, what potential mechanisms may underlie such associations.

In this context, some efforts to explore causal associations between a highly prevalent sleep disorder, namely obstructive sleep apnea (OSA), and cancer have also taken place (20, 21), and have operated under the assumption that the intermittent hypoxemia that characterizes patients with OSA during their sleep period is likely to mimic the biologic events that drive tumor growth (1, 22–27). The major findings from these initial studies indicate that the periodic oscillations in overall oxygenation during sleep in patients with OSA impose overall adaptive changes in the tumor metabolic cellular substrate that enhances their proliferative and invasiveness properties (28). However, these studies failed to explore another hallmark characteristic of OSA, namely sleep fragmentation (SF), that is, the presence of recurrent arousals aimed at restoring airflow that lead to sleep discontinuity.

Using a similar logical paradigm, we hypothesized that chronic sleep fragmentation, a very frequent occurrence in many human disorders, including OSA, would be associated with altered solid tumor proliferation and invasiveness in a murine model (29, 30). Furthermore, we posited that sustained sleep fragmentation would promote changes in the phenotypic distribution of tumor-associated macrophages (TAM). Indeed, TAMs have been identified as critically important constituents of cancer microenvironment, and are major contributors to cancer progression by releasing a vast array of growth factors, cytokines, inflammatory mediators, and proteolytic enzymes that underlie key components of tumor growth and invasion (31, 32).
Materials and Methods

Animals
Male C57/B6, TLR4<sup>++/−</sup>, MYD88<sup>+/−</sup>, and TRIF<sup>−/−</sup> mice, weighing approximately 25 g, were purchased from The Jackson Laboratory, housed in a 12 hours light/dark cycle (light on 7:00 am to 7:00 pm) at a constant temperature (24°C ± 1°C) and allowed access to food and water <i>ad libitum</i>. The experimental protocols were approved by the Institutional Animal Use and Care Committee and are in close agreement with the NIH Guide in the Care and Use of Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Sleep fragmentation
The custom-designed sleep fragmentation approach used to induce sleep fragmentation in rodents has been previously reported in detail (29,33), and relies on automated intermittent tactile stimulation of freely behaving mice in a standard laboratory mouse cage, using a near-silent motorized mechanical sweeper. This method obviates the need for human contact and intervention, and does not involve introduction of foreign objects or touching of the animals during sleep. To induce sleep fragmentation, we chose a 2-minute interval between each sweep, implemented during the light period (7:00 a.m. to 7:00 p.m.). Of note, 4 to 5 mice were housed in each cage to prevent the isolation stress.

Tumor cell lines and culture medium
We used TC-1 (ATCC CRL-2785) and 3LL (ATCC CRL-1642) in all experiments. Both cell lines are derived from primary lung epithelial cells of C57/B6 mice and were cultured at 37°C, 95% air, 5% CO2 incubator in full tumor medium as recommended by the American Type Culture Collection (ATCC). TC-1 cells were cultured in RPMI-1640 medium with recommended by the American Type Culture Collection Corporation). 3LL cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, L-glutamine, aprotonin, aprotinin, and hygromycin B (Calbiochem, EMD Millipore) and selected with G418 (Gibco, Life Technologies, and selected with G418 (Gibco, Life Technologies). Cells were isolated by magnetic labeling following the manufacturer’s procedure (EasySep Mouse CD11b Positive Selection Kit; StemCell, 18770). Tumors were mechanically disrupted and incubated in collagenase IV solution 1 mg/mL for 1 hour at 37°C. Cells were filtered through a 100 μm nylon mesh cell strainer (352350; BD Falcon). Before cell identification, viable cells were selected by using the aqua-fluorescent reactive dye (L34957; Invitrogen). TAMs were defined as CD45<sup>+</sup>, CD11b<sup>+</sup>, F4/80<sup>+</sup> cells.

Isolation of TAMs

Tumors were mechanically disrupted and incubated in collagenase IV solution 1 mg/mL for 1 hour at 37°C. Cells were filtered through a 100 μm nylon mesh cell strainer and CD11b<sup>+</sup> cells were isolated by magnetic labeling following the manufacturer’s procedure (EasySep Mouse CD11b Positive Selection Kit; StemCell, 18770).

Immunofluorescence analysis

Tumors were excised, frozen in optimum cutting temperature, and stored in −80°C. Of note, 10 μm cryosections were made to minimize animal suffering and to reduce the number of animals used.

Matrix metalloproteinase <i>in vivo</i> live imaging

Twelve C57/B6 male mice (6 with sleep fragmentation and 6 with sleep conditions) were injected subcutaneously with tumor cells into the lateral aspect of the right thigh using the same protocol previously described; 28 days after tumor cell inoculation, mice were intravenously injected with 2 nmol of the matrix metalloproteinase (MMP) probe MMPSense 750 FAST (PerkinElmer; Product number: NEV10168). This MMP activatable agent is optically silent upon injection and produces fluorescent signal after cleavage by disease-related MMPs. Activation can occur by a broad range of MMPs, including MMP 2, 3, 7, 9, 12, and 13. Two days before the MMPSense injection, all the fur lining the tumor area was removed by hair removal cream, after which mice were imaged 6, 12, and 24 hours after injection using the Xenogen IVIS Spectrum (PerkinElmer) at the University of Chicago Optical Imaging Core Facility. The tumor near IR fluorescence (average radiant efficiency) of images was quantified using Living Image Software (PerkinElmer).

Local tumor invasiveness

To assess the differences in local tumor invasiveness between the sleep fragmentation and sleep conditions groups, 7 days after sleep fragmentation/sleep conditions initiation, C57/B6 mice were injected subcutaneously with TC1 cells into the lateral aspect of the right thigh using 1 × 10<sup>5</sup> cells in 0.2 mL PBS. Twenty-eight days after tumor cell inoculation, both sleep fragmentation and sleep conditions mice were euthanized and subjected to wide resection of the tumor tissue with its adjacent muscular and bone tissue of the thigh. The whole specimen was fixed in 4% paraformaldehyde after it was cut into equivalent size pieces. Those specimens were embedded in paraffin and followed by cutting of 3 to 5 μm sections and were stained with hematoxylin and eosin (H&E).

Analysis of tumor-infiltrating cells

Tumors were mechanically disrupted in small pieces and maintained overnight in TCI complete growth medium but without Geneticin (G418). After 12 hours, cells were harvested and filtered through a 100 μm nylon mesh cell strainer (352350; BD Falcon). Before cell identification, viable cells were selected by using the aqua-fluorescent reactive dye (L34957; Invitrogen). TAMs were defined as CD45<sup>+</sup>, CD11b<sup>+</sup>, F4/80<sup>+</sup> cells.

Isolation of TAMs

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Immunofluorescence analysis

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cut and stained with F4/80. Sections were washed several times in PBS, and blocked with a PBS/0.4% Triton X-100/0.5% TSA (tyramide signal amplification; PerkinElmer Life Sciences) blocking reagent/10% normal horse serum for 1 hour. Sections were then serially incubated with a rabbit anti-mouse F4/80 antibody (1:500; cat # 122604; BioLegend) at 4°C for 24 hours and then washed in PBS for six times giving 5 minutes for each wash. Sections were incubated at room temperature for 1 hour in biotinylated antibody (1:400; Vector Labs) in a PBS/0.4% TSA blocking reagent/10% horse serum solution, and then with streptavidin–horseradish peroxidase diluted at 1:100 in PBS/0.5% TSA blocking reagent. Subsequently, the sections were incubated with TSA fluorescein reagents diluted at 1:50 in amplification diluent (PerkinElmer Life Sciences) for 2 minutes. Sections were then washed and mounted onto glass slides. Sections were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

Endocan staining
Paraffin sections of sleep conditions and sleep fragmentation tumor tissue were stained immunohistochemically for endocan using anti-mouse Endocan (cat # LIA-0905; Lungin-nov) as recommended by the manufacturer.

Total RNA isolation and gene expression
Sorted TAMs were instantly frozen in liquid nitrogen after counting the amount of sorted cells. Total RNA was isolated using automated RNA extraction (Promega) and DNase-treated according to the manufacturer’s protocol. The RNA quantity and integrity were determined using a NanoDrop Spectrophotometer and Agilent 2100 Bioanalyzer Nano 6000 Lab Chip assay (Agilent Technologies). Quantitative real-time PCR (qRT-PCR) was performed using the ABI 7500 instrument (Applied Biosystems). The cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems). Of note, 500 ng of total RNA from both sleep fragmentation and sleep conditions samples were used to generate the cDNA templates for RT-PCR. TaqMan Master Mix Reagent Kit (Applied Biosystems) was used to amplify and quantify the transcripts in 20 μL reactions. Duplicate PCR reactions were performed in 96-well plates in parallel with the β-actin rRNA as a housekeeping gene. The steps involved in the reaction program included: the initial step of 2 minutes at 50°C; denaturation at 95°C for 10 minutes, followed by 45 thermal cycles of denaturation (15 seconds at 95°C) and elongation (1 minute at 60°C). Expression values were obtained from the cycle number (Ct value) using the Biosystems analysis software. These Ct values were averaged and the difference between the β-actin Ct (Avg) and the gene of interest Ct (Avg) was calculated (ΔΔCt). The relative gene expression was analyzed using the 2-ΔΔCt method. Quantitative results were expressed as the mean ± SD. The following TaqMan primer and probes were purchased from Applied Biosystems: interleukin (IL)-1β assay# Mm00434174_m1, inducible nitric oxide synthase (iNOS) assay# Mm00440502_m1, INF-γ assay# Mm01168134_m1, Arginase1 assay# Mm00475988_m1, resistin-like α (Fizz1) assay# Mm00445109_m1, Mrc1 assay# Mm00485148_m1, IL-10 assay# Mm00439614_m1, TGF-β assay# Mm01178820_m, TRL2 assay#Mm00442346_m1, TLR4 assay# Mm00445273_m1, and TLR6 assay# Mm02529782_s1.

Statistical analysis
All data are reported as mean ± SE. Comparisons for tumor growth and all other time-course experiments among sleep fragmentation and sleep conditions were performed using one-way ANOVA followed by unpaired Student t test with Bonferroni correction. Comparison of all other quantitative data between sleep fragmentation and sleep conditions was performed using unpaired Student t tests. For all comparisons, a P < 0.05 was considered as statistically significant.

Results
Sleep fragmentation induces accelerated tumor size growth
C57/B6 mice exposed to sleep fragmentation starting 1-week before flank injection with TC-1 cells showed accelerated tumor size growth with significant differences emerging at day 23 following injection and thereafter when compared with mice under control sleep conditions (Fig. 1A; n = 53/group; sleep fragmentation vs. sleep conditions: P < 0.007). Tumor weight was significantly higher at day 28 in sleep fragmentation–exposed mice (SF-57/B6: 1.955 ± 0.680 g vs. SC-C57/B6: 1.046 ± 0.479 g; P < 0.001). Similarly, mice (n = 15/group) exposed to sleep fragmentation and injected with 3LLC cells showed significantly accelerated tumor growth (P < 0.001) and tumor weight (P < 0.027) compared with sleep conditions (SF-57/B6: 1.22 ± 0.281 g vs. SC-C57/B6: 0.543 ± 0.163 g; P < 0.027; Fig. 1B).

Sleep fragmentation induces increased tumor invasiveness
When TC-1 cells were injected subcutaneously in the thigh area, increased invasiveness was apparent in SF-C57/B6 tumors compared with SC-C57/B6 tumors, with obvious penetration of surrounding tumor capsule and extension/infiltration into adjacent muscle (Fig. 2A; n = 12/group; χ²: P < 0.001). To further explore the increased invasiveness of tumors in sleep fragmentation–exposed mice, we performed live MMP imaging, which showed significantly increased MMP activity in SF-C57/B6 tumors vs. CS-C57/B6 (n = 6; P < 0.01; Fig. 2B).

TAM counts and distribution
When compared with CS-C57/B6 tumors, significant increases in TAM counts occurred in SF-C57/B6 tumors whether expressed as TAM/g tumor tissue (Fig. 3A and C; P < 0.01) or as TAM/whole tumor (Fig. 3B; P < 0.01). TAM in SF-C57/B6 tumors were preferentially distributed in close proximity to the tumor capsule, as compared with increased tumor core location in tumors from SC-C57/B6 mice (Fig. 3D and E). When endocan staining was used to identify new vessel formation in the tumor capsule, SF-C57/B6 tumors had higher expression of endocan when compared with sleep control conditions (Fig. 3F).
Sleep fragmentation induces TAM polarity shift toward M2 and higher TLR4 expression

To further understand whether changes in macrophage polarity occurred during sleep fragmentation, we performed fluorescence-activated cell sorting (FACS) of tumors for TAM (i.e., CD45+CD11b+, and F4/80+) and further assessed the proportion of those expressing CD86high, a M1 marker or CD206high, a M2 marker. A shift toward increased M2 marker expression in TAM of SC-TLR4 mice emerged, particularly in peripheral areas of the tumor (P < 0.04; Fig. 4A). TAM shift in polarity in sleep fragmentation tumors was also apparently based on increased transcriptional expression of M2 markers when compared with sleep conditions, as evidenced by higher expression of Fizz1, Arg1, and Mrc1 (P < 0.01; Fig. 4B). TAM from sleep fragmentation–exposed mice also expressed higher levels of TLR4 compared with sleep conditions–exposed mice (P < 0.02; Fig. 4C), but no changes in TLR2 or TLR6 were apparent (data not shown). On the basis of such findings, we examined TLR4 signaling as a potential pathway mediating sleep fragmentation–induced differences in tumorigenesis as described heretofore.

TLR4 signaling mediates sleep fragmentation–induced tumor progression

When compared with CS-C57/B6 mice, significant reductions in tumor size and weight occurred in SC-TLR4−/− mice (P < 0.001; Fig. 4D and E). More interestingly, the accelerated growth and increases in size induced by sleep fragmentation were completely abrogated in SF-TLR4−/− mice (Fig. 4D and E; P < 0.05 vs. SC-TLR4−/− mice). These effects were accompanied by significant reductions in TAM count in SF-TLR4−/− mice (P < 0.01; Fig. 4F). These results prompted further exploration as to whether TLR4 major downstream signaling pathways, namely MYD88 or TRIF were particularly and specifically recruited in sleep fragmentation. As shown in Fig. 4G, tumors enucleated from SF-MYD88−/− and SF-TRIF−/− mice showed reductions in tumor weight compared with SF-C57/B6 (P < 0.03, P < 0.01, respectively), but were still significantly larger than in SF-TLR4−/− mice. In addition, small, albeit statistically significant differences between sleep fragmentation and sleep conditions for MYD88 and TRIF null mice remained.

Discussion

This study shows that chronically fragmented sleep, a highly prevalent condition associated with a multiplicity of human disorders, leads to accelerated tumor growth and invasiveness in mice and that such adverse effects are mediated, at least in part, by changes in TAM polarity and TLR4 signaling. Indeed, increases in M2 macrophage markers were apparent in tumors...
of sleep fragmentation–exposed mice, and were accompanied by increased in vivo and in vitro MMP activity (34, 35). We further show that similar to previous reports, TLR4 signaling not only underlies significant components of tumor progression (36, 37), but also seems to mediate the differences in tumor proliferation between sleep fragmentation and sleep conditions. Taken together, these findings provide initial, yet conclusive support for sleep-mediated modulation of tumorigenesis, and suggest that host-dependent immune mechanisms constitute a major pathway of such modulatory influences.

The paramount observation of the present study is the increased tumor proliferation and marked changes in invasiveness induced by chronic sleep fragmentation exposures mimicking several sleep disorders. As such, these observations provide initial observations in a murine model on the effects of mimicking several sleep disorders. As such, these observations provide initial, yet conclusive support for sleep-mediated modulation of tumorigenesis, and suggest that host-dependent immune mechanisms constitute a major pathway of such modulatory influences.

The mechanisms mediating the increased tumor-proliferative rates induced by implementation of sleep fragmentation are unclear and likely diverse. Importantly, sleep fragmentation–induced effects occurred in both TC-1 and 3LLC tumors, indicating that increased tumor cell-proliferative rates resulting from perturbations in sleep may not be specific to a single cancerous cell type, and are potentially applicable to most solid tumors. Of note, the increased size of the tumors under sleep fragmentation conditions was also accompanied by more extensive and prominent areas of tumor core necrosis, further attesting to the changes in tumorigenesis elicited by the underlying sleep fragmentation. In parallel with the accelerated tumor expansion under sleep fragmentation conditions, increased tumor invasion to surrounding tissues was observed following both flank and thigh injections of TC-1 cells, with the latter thigh injections allowing for more accurate confirmation of the enhanced invasion process. Here again, multiple mechanisms have been described about the adaptive strategies that enable cell invasion across the physical outer boundaries of tumors. Therefore, an extensive and exhaustive survey of such mechanisms would be beyond the scope of current work. Notwithstanding, the unique clustering of TAM in the periphery of sleep fragmentation–exposed tumors...
prompted us to explore whether such changes were potentially accountable for the more aggressive tumors during sleep fragmentation conditions. Indeed, the effects of alterations in TAM polarity and in innate immunity on tumor biologic properties including growth trajectory and invasiveness to adjacent tissues have been extensively explored, and our current findings concur with such putative functions (38–41). As indicated, the number of TAMs was markedly increased in sleep fragmentation–exposed mice, and the differences were heterotopically distributed, with tumors from sleep fragmentation–exposed mice displaying preferential M2-type TAM localization in peripheral regions of the tumor. In contrast, control sleep conditions–derived tumors had increased M1-type TAM, and these were located, in their greatest proportion within the core of the tumor. Therefore, we infer that the changes in macrophage polarity and location reflect some of the changes in tumor behavior. However, it remains unclear what mechanisms are operationally activated in the context of how sleep fragmentation elicits (i) a shift in TAM polarity within the tumor, or (ii) whether sleep fragmentation fosters increased migration of TAM from the circulation into the periphery. We should point out that the overall changes in TAM polarity were unexpected, particularly when considering the marked increases in M1 macrophage markers and in total numbers of macrophages that occur in visceral adipose tissues during sleep fragmentation (30, 42). It is further possible that inflammatory changes in adipose tissues surrounding the tumors that resemble the changes in visceral white adipose tissue depots during sleep fragmentation may play a role in the shift of macrophages from the core to the periphery of the tumor, and further account for the M1/M2 phenotype shifts found here. Indeed, recent interest on the contribution of the adipose tissues surrounding tumors to tumor biologic processes such as promotion of proliferation and invasion has recently emerged (43–45). Thus, altered

Figure 3. TAM counts and distribution. A, significant increases in TAM counts in tumor tissue occurred in sleep fragmentation (SF) when compared with sleep conditions (SC) (~3-fold increase). B, almost 5-fold increase in TAM counts emerged when comparing the number of TAM/whole tumor in sleep fragmentation versus sleep conditions. C, representative FACS count of TAMs; CD45⁺, CD11b⁺, F4/80⁺ cells. D, immunofluorescence imaging of whole tumor sections from both sleep fragmentation and sleep conditions with images A and C representing both sides of the tumor capsule and B representing the tumor core, those images showing that TAMs are preferentially distributed and in close proximity to the tumor capsule in sleep fragmentation conditions, whereas in sleep conditions, increased tumor core location of TAMs was found (n = 6; blue, Hoechst-Nuclei; red, CD45⁺; Green, F4/80⁺). E, representative tumor section stained with hematoxylin and F4/80⁺ illustrating a preferential distribution of TAMs, with more TAMs in the tumor capsule in sleep fragmentation–exposed mice when compared with sleep conditions (n = 10; brown, F4/80⁺ cells). F, representative endocan stained sections showing higher immunoreactivity in the tumor capsule in sleep fragmentation conditions when compared with sleep conditions; this was apparent in low magnification (i and ii), as well as at higher magnification (iii and iv; n = 6; brown, Endocan). *P < 0.05.
sleep in the host may trigger a complex time-dependent cascade of activation and inactivation of biologically relevant pathways both systemically and regionally (46), including peritumoral fat and intratumoral cellular substrates that cumulatively orchestrate changes in tumor growth and local invasiveness.
Similarly, the increased TLR4 expression in tumors excised from sleep fragmentation–exposed mice could either reflect selective migration of M2-TLR4+ TAM cells to these tumor regions from the systemic circulation, or changes in macrophage polarity, in macrophage TLR4 expression, or in migration of M1 from the core to the tumor periphery during sleep fragmentation. Accordingly, genetic ablation of TLR4 in mice resulted in major curtailment of not only tumor growth, but also of the differences between sleep fragmentation and control sleep conditions. However, the sleep fragmentation–sleep control conditions differences in tumor size persisted in either MYD88−/+ and TRIF−/+ mice, suggesting that either both TLR4 signaling pathways are required for sleep fragmentation–induced effect on tumor growth, or that the differences between sleep fragmentation and control sleep conditions are not distinguishable once tumor growth is so markedly reduced by TLR4 genetic ablation. As with all other observations reported herein, transfer of the findings from an in vitro murine model to an in vivo model is obviously impossible, thereby hampering our ability to study mechanisms of sleep fragmentation effects in greater detail. We should also point out that the mechanisms underlying sleep fragmentation–induced activation of TLR4 signaling in macrophages, and the changes in TAM polarity are completely unknown, and this specific area will have to be explored in the future.

In summary, the present study conclusively demonstrates that perturbed sleep leads to major changes in tumorigenesis, characterized by increased tumor cell proliferation and invasion. Alterations in TAM phenotypes, particularly in the tumor periphery, and in TLR4 expression in TAM further suggest that sleep fragmentation–induced effects on tumor growth and invasion may be mediated by host-related responses, particularly those involving innate immunity, and that improved understanding of such pathways may permit improved therapeutic interventions. Considering the high prevalence of sleep disorders and cancer in middle age or older populations, there are far reaching implications to current findings about potential adverse outcomes in patients in whom the two conditions coexist.

Disclosure of Potential Conflicts of Interest

H. Shirwan is employed as a CSO in ApoVax, Inc., has ownership interest (including patents) in ApoVax, Inc., and is a consultant/advisory board member of ApoVax, Inc. No potential conflicts of interest were disclosed by the other authors.

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