It is increasingly recognized that obstructive sleep apnea (OSA) syndrome is a systematic rather than local disorder. There is also growing evidence that apart from the syndrome's major features: intermittent hypoxia and sleep fragmentation, functional activity of the immune system is altered in OSA patients, with several cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) taking active part in sleep regulation. Little is known about the effects exerted by chronic intermittent hypoxia combined with persistent pro-inflammatory activity of the immune system on the vascular micro milieu in OSA. In this study we attempted to confirm the hypothesized imbalance between pro- and anti-angiogenic factors by evaluating direct and indirect angiogenic activity of OSA patients' sera in the in vivo serum-induced angiogenesis (SIA) and leukocyte-induced (LIA) assays, respectively, in mice. Both tests revealed significantly inhibited angiogenic activity of OSA patients' sera compared with healthy controls (P<0.001). Moreover, differences related to the subject’s weight regarding in the mean number of newly-formed vessels were observed with a significantly greater inhibition in the normal-weighting apneic subjects than in the overweight or obese ones (P<0.01). The angiogenesis inhibition index was positively related to the serum IL-6 level (r=0.35; P<0.05) in the OSA group, but not to TNF-α, fasting serum leptin, or OSA syndrome severity as assessed by the AHI index. Our results demonstrate that OSA is accompanied by disturbed serum angiogenic activity, apparently resulting from an imbalance between pro- and anti-angiogenic factors, some of them being produced by the adipose tissue. The disordered angiogenic activity might be related to the pathophysiology of OSA and should be considered an important causative factor for the increased prevalence of cardiovascular diseases in OSA patients.

Key words: angiogenesis, IL-6, leptin, obstructive sleep apnea, TNF-α.
INTRODUCTION

Obstructive sleep apnea (OSA) affects approximately 2 to 4% of adults, particularly middle-aged, obese men, although its existence in women and also in lean individuals is increasingly recognized (1). The OSA syndrome is characterized by recurrent collapse of the pharynx and pauses in respiration during sleep, which leads to a fall in oxygen saturation and sleep fragmentation (2). The syndrome constitutes a substantial public health problem due to its association with cardiovascular morbidity and mortality, and its considerable impact on patients' quality of life (1, 3). To further emphasize the clinical importance of OSA, it should be mentioned that currently available treatments are associated with limited efficacy and poor compliance.

Despite the extensive literature on the role of anatomical abnormalities, the exact nature of OSA and its impact on the physiological homeostasis have not been fully elucidated. It is recognized that OSA is a systematic rather than local disorder and it is a likely manifestation of a complex metabolic syndrome (4). Accordingly, there is also growing evidence that the functional activity of the immune system in OSA patients is altered. Apart from the syndrome's major features: intermittent hypoxia and sleep fragmentation, which might undeniably affect immune homeostasis, it appears that the inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and interleukin-6 (IL-6) take active part in sleep regulation and that their production and circadian rhythm are deregulated in apneic patients (5). It has recently been established that OSA is associated with increased oxidative stress and the state of inflammatory cell activation (6, 7).

Although the bond between cardiovascular pathology and OSA is commonly accepted, there is scant evidence available on the effects exerted by chronic intermittent hypoxia combined with persistent pro-inflammatory activity of the immune system on the vascular micro milieu, including both physiological and pathological angiogenesis processes. Therefore, in this study we investigated the hypothesized imbalance between pro- and anti-angiogenic factors. We addressed the issue by evaluation of direct and indirect angiogenic activity of OSA patients' sera in the in vivo serum-induced angiogenesis (SIA) and leukocyte-induced (LIA) assays, respectively, in mice. We also measured the serum concentrations of the main angiogenesis-regulating factors, such as TNF-α, IL-6, and fasting leptin in the samples examined.

MATERIAL AND METHODS

The study was approved by the hospital's Ethics Committee and all subjects gave informed consent.

Subjects' characteristics

Subjects were recruited from those who had been referred to our hospital for suspected OSA, snoring, or excessive daytime sleepiness. There were no shift workers in the population. A total of 58 newly diagnosed patients with OSA verified by standard polysomnographic examination (Somnostar Alpha, SensorMedics Yorba Linda, CA) (apnea-hypopnea index (AHI) >10 per hour of
sleep) were selected for the study (F/M - 20/38, mean age 56.2 ±12.5 yr, AHI 30.7 ±14.1, BMI 29.7 ±6.8 kg/m²). A control group consisted of 36 healthy subjects (F/M - 12/24, mean age 51.3 ±10.6 yr, AHI 2.8 ±2.1, BMI 27.8 ±4.2 kg/m²). There were no significant differences between the control and OSA groups except the AHI values (P<0.001).

All subjects had fasting blood samples taken between 7.30 and 8.30 a.m. Blood was collected on heparin and centrifuged immediately at 2000 g for 20 min. Plasma was then aliquoted and stored at -80°C until assay.

**Measurements of cytokine and leptin concentrations**

Cytokine levels were determined in the examined sera using sandwich ELISA kits (R & D Systems, Minneapolis, MN) for TNF-α, IL-6, and leptin. The optical density was measured at 450 nm using a spectrophotometric reader Elx800 (Bio-Tek Instruments, Winooski, VT). The cytokine concentration was expressed as pg/ml.

**Peripheral blood mononuclear cells (PBMC) culture**

PBMC were isolated from the healthy volunteers' peripheral, heparinized blood. The cell suspension of 10 x 10⁶/ml was preincubated with 30% of sera from OSA patients or controls for 90 min at 36°C and 5% CO₂. The cells were then washed and resuspended in the Parker medium.

**Mouse serum-induced (SIA) and lymphocyte-induced (LIA) cutaneous angiogenesis assays**

A cutaneous angiogenesis assay was performed according to the method of Sidky and Auerbach (1975) with our own modification (8, 9). The assays have been performed in 2-month old, female inbred Balb/c mice. The mice have been of a local laboratory breed, weighing ca 20 g each. The sera examined or 5 x 10⁵ of preincubated cells were injected intradermally (0.05 ml per injection, 3-6 injections per mouse, at least three mice for one tested material) into a shaved skin area in the mouse anaesthetized with chloral hydrate. To mark the injection site, the inoculated samples were dyed with 0.1% of trypan blue.

Following 72 h, the mice were killed with a lethal dose of Morbital (Biowet, Pulawy, Poland). All newly-formed blood vessels were identified and counted under a dissection microscope in the 1/3 central area of the microscopic field, at a magnification of 6 x. The identification was based on the fact that newly-formed blood vessels differ from the background vasculature by their small size, tortuosity, and divarication.

The stimulation/inhibition index was expressed as a ratio:

\[
\frac{(A_{\text{cont}}-A_{\text{ex}})}{A_{\text{cont}}},
\]

where \(A_{\text{cont}}\) is a number of new blood vessels formed upon the induction by control sera and \(A_{\text{ex}}\) is a number of new blood vessels formed upon the induction by OSA patients' sera.

**Statistical analysis**

All data analysis was performed using a commercial SPSS package. Results are expressed as means ±SD. A t-test for independent samples and one-way analysis of variance (ANOVA) were used to determine differences between groups. Pearson's correlation coefficients were used to examine the relationship between variables. A P value <0.05 was used to indicate statistical significance.

**RESULTS**

The angiogenic activity of the OSA patients sera, evaluated in the SIA test, was significantly inhibited in comparison with controls (P<0.001) (Table 1). Moreover,
differences related to the subject’s weight regarding the mean number of newly-formed vessels were observed in the examined OSA patients. The inhibition index was significantly greater in the normal-weight apneic subjects than in the overweight or obese individuals (P<0.01). No relationship between the inhibition rate and the OSA syndrome severity, as assessed by the AHI index, was noted.

To confirm the observed phenomenon, the influence of the sera from the OSA patients on human mononuclear cell proangiogenic activity in the in vivo LIA angiogenesis model was examined. PBMC were preincubated in a culture medium supplemented with 30% of the pooled sera for 2 h and then thoroughly washed and implemented intradermally into the mouse skin. As before, a significant decrease in the number of newly-formed vessels, compared with the effect of the healthy controls' sera, was observed (P<0.001) (Table 1). However, no relationship between the inhibitory index and BMI or AHI was observed.

The angiogenesis inhibition index was positively related to the serum IL-6 level (r=0.35; P<0.05) in the OSA group, but not to TNF-α and serum leptin measured by an Elisa method. No correlation between IL-6 or TNF-α and BMI has been found, while, as expected, a close relationship between leptin and BMI has been confirmed (r = 0.416; P>0.05).

**DISCUSSION**

Angiogenesis, a process of new blood vessel formation by capillary sprouting from preexisting vessels, is a major physiological event crucial for the human body homeostasis, for example, throughout the reproductive cycle and pregnancy.
or during the wound healing. The angiogenic activity remains under a strict control of pro- and anti angiogenic factors that normally are balanced, being sequentially up- or down-regulated in order to stimulate or inhibit neovascularization in accordance with the local situation (10).

OSA patients are typically characterized by an increased BMI index due to the excessive fat tissue formation. Surprisingly, adipogenesis is tightly correlated with angiogenesis mostly to coordinate the fat mass development (11). Beside, hypertrophic and hyperplasic adipocytes are a proven source of cytokines and growth factors, which affects the angiogenic balance. The present study demonstrates that the overall angiogenic activity, assessed directly and indirectly in vivo in SIA and LIA tests, respectively, was diminished in the group of OSA patients. Moreover, in the more sensitive SIA model we were able to demonstrate a significant relationship between the BMI and angiogenesis inhibition index, with a more pronounced suppressive effect in the subjects with normal BMI than in overweight and obese OSA patients. Therefore, it might be concluded that while fat tissue serves as a source of pro-angiogenic stimuli, due to secreted cytokines and mediators, the OSA itself creates rather an antiangiogenic environment at least in the peripheral blood.

It is known that several cytokines of a known angiogenic regulatory potential, such as TNF-α, IL-1β, and IL-6 are involved in sleep deregulation, a major feature of the OSA syndrome (12). Moreover, disturbances of their production and circadian rhythm changes have been demonstrated in apneic patients (5). In this study, the IL-6 and TNF-α serum levels were assessed in the sera examined. A significant positive relationship between the IL-6 and the observed anti-angiogenic activity of OSA patients' sera was revealed, while there was no link with the TNF-α levels. Interestingly, circulating IL-6 was not related to BMI in either patients or controls, further proving that, accordingly to what we observed for the anti-angiogenic effect, the IL-6 effect was not dependent on the fat tissue mass. Lack of this association has also been demonstrated by others (12).

IL-6 is a known angiogenic factor that might either exert pro- or anti-inflammatory effect, depending on the local milieu and the presence of other cytokines or inflammatory mediators (13). Since the literature concerning the abnormalities in the immune system's functional activity and the angiogenesis processes in OSA patients is rather limited, we may only speculate that, due to some unknown mechanisms, IL-6 and likely some other factors diminish the physiological angiogenic balance in serum. Such candidate factors could be alterations in the oxidative balance and OSA-related metabolic deregulation (14).

The exact pathophysiological nature of OSA has not yet been fully elucidated. Excessive fat tissue, characteristic for this group of patients, serves as a potent source of cytokines, growth factors and hormones, some of them being potentially involved in the regulation of angiogenesis. Beside, continuous growth of adipocytes requires the formation of new capillaries for proper function. Leptin, a well known adipocyte-secreted cytokine, appears to play a key role in
the regulation of body weight, appetite control, sympathetic nervous system activation, hematopoiesis, and inflammatory immune response. The serum concentration of leptin has been found to be markedly increased in human obesity and positively correlated to body fat mass. In accordance, we demonstrated in this study a significant relationship between BMI and serum leptin concentration in the group of OSA patients. However, no correlation between leptin and the angiogenic activity of these patients' sera was found, although leptin has been reported to exert a proangiogenic effect in \textit{in vitro} studies. Yet, it might be speculated that a significantly smaller angiogenesis inhibition observed in obese and overweight OSA patients is due partially to a higher leptin production in these subjects. The weight-dependence observed in the antiangiogenic activity of the sera examined indirectly proves that the fat tissue is involved in the regulation of pro/antiangiogenic balance in the peripheral blood.

Our results demonstrate that OSA, as a systemic disorder, is accompanied by a disturbed serum balance of pro- and anti-angiogenic factors, apparently some of them being produced by the adipose tissue. Disturbed angiogenic activity is most likely an element of the pathophysiology of OSA and might be considered an important causative factor for the increased prevalence of cardiovascular diseases in OSA patients.

REFERENCES


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