Nocturnal hypoxic stress activates invasive ability of monocytes in patients with obstructive sleep apnea syndrome

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The first author contributed to all experiments and clinical management. Hiroshi Kimura was primary investigator of this study and the corresponding author. The other authors' roles were as follow;

-Clinical management of the patients; Motoo Yamauchi, Atsuhiko Fukuoka, Kiyoshi Makinodan, and Noriko Koyama.

-Cell biological experiment; Motoo Yamauchi, and Koichi Tomoda.

-Molecular experiment; Koichi Tomoda, and Masanori Yoshikawa

Although sleep apnea is known to be a risk factor of cardiovascular events, the precise mechanisms of how hypoxic stress influences the inflammatory process have not been elucidated. This study suggests that nocturnal hypoxic stress activates the monocyte-mediated inflammatory process.

Abstract

Backgrounds: Obstructive sleep apnea syndrome (OSAS) is known to be a risk factor of cardiovascular events. However, the precise mechanism has not been fully elucidated. OSAS-induced hypoxic stress may promote the production of inflammatory cytokines and chemokines by monocytes, which has a crucial role in the development of atherosclerosis. In addition, adhesion to the vascular endothelium and transendothelial migration of monocytes are considered to induce atherosclerosis. The aim of this study was to investigate the effects of hypoxic stress on the invasive ability of monocytes in OSAS.

Methods; Twenty-one male OSAS patients and 17 male healthy control subjects, age- and body mass index-matched, were enrolled. Venous blood samples were collected not only before and after sleep but also after CPAP titration for the purpose of monocyte isolation. The invasive ability of monocytes was evaluated by counting the number of invasive cells using a BD BioCoat Matrigel Invasion Chamber.

Results; The number of cells which represents invasive ability was significantly higher in OSAS patients as compared to control subjects in the early morning ($p<0.001$). Invasive ability in the early morning was significantly elevated as compared to that before sleep in OSAS patients ($p<0.001$), and it was positively correlated with oxygen desaturation index ($p<0.05$). CPAP titration led to alleviation of the invasive ability ($p<0.001$).

Conclusions; The results indicate that OSAS-induced hypoxic stress activates the invasive ability of monocytes, and that this phenomenon observed during sleep may contribute to the development of atherosclerosis in OSAS.

Keywords; sleep apnea, hypoxia, monocytes, atherosclerosis

Short Title; Invasive ability of monocytes in OSAS

Introduction

Obstructive sleep apnea syndrome (OSAS) is characterized by recurrent episodes of oxygen desaturation during sleep, development of daytime sleepiness, and deterioration of the quality of life. In addition, recent studies have shown that OSAS may be an important risk factor for cardiovascular morbidity and mortality.[1, 2] There is now convincing evidence that OSAS is an independent risk factor for hypertension, ischemic heat disease, and probably stroke.[3] OSAS is also frequently associated with metabolic syndrome.[4, 5] Although it is postulated that OSAS-induced hypoxic stress may contribute to the etiology of cardiovascular events, the precise mechanisms have not been fully elucidated. One of the possible mechanisms is that of OSAS-induced hypoxic stress modulating circulating inflammatory mediators.

Atherosclerosis is described as an inflammatory disease on the basis of atheromatous lesions having been established as active sites of inflammation and immune responses involving proinflammatory cytokines, chemokines and adhesion molecules.[6, 7] It is considered that adhesion to vascular endothelial cells and transendothelial migration of monocytes are crucial steps in the pathogenesis of atherosclerosis.[8]

Tumor necrosis factor (TNF)-α, the proinflammatory cytokine, is recognized as a critical factor in the development of atherosclerosis. TNF-α facilitates this process by causing vascular endothelial cells to express adhesion molecules that mediate the initial binding of monocytes to the endothelium as well as their transendothelial migration.[9] It has been demonstrated that circulating levels of TNF-α are predictive of cardiovascular events.[10] Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C family of chemokines and plays an important role in monocyte recruitment.[11] MCP-1 is upregulated in human atherosclerotic plaques, suggesting a role for MCP-1 in the development of early atherosclerotic lesions.[12, 13]

It has already been demonstrated that circulating levels of TNF-α were elevated in patients with OSAS.[14, 15] Recent studies have shown the elevated production of TNF-α by monocytes[16] and the elevated serum levels of MCP-1 in patients with OSAS.[17, 18] We have also reported that the productions of TNF-α and MCP-1 by monocytes were elevated after sleep rather compared to those before sleep in severe OSAS patients.[19] There have been a few studies concerning the function of monocytes in OSAS.[20, 21] Monocytes of patients with OSAS readily adhered to endothelial cells in culture as compared to control subjects,[20] and repetitive hypoxia was reported to increase cholesteryl ester uptake by macrophages.[21]

The aim of the current study was to investigate the effect of hypoxic stress on the invasive ability of monocytes. To reveal the influence of hypoxic stress during sleep, we evaluated the change in invasive ability between before and after sleep. We also examined the effects of CPAP treatment on the invasive ability of monocytes.

Methods

Subjects

Twenty-one male OSAS patients and 17 male healthy control subjects were enrolled. Control subjects were matched for age and body mass index (BMI). Subjects who were taking medications for hypertension, hyperlipidemia, cardiovascular diseases, and diabetes mellitus were excluded in this study. All participants underwent overnight polysomnography (PSG). The diagnosis of OSAS was established with PSG, and the apnea-hypopnea index (AHI) in control subjects was confirmed as < 10.

Sleep study

PSG was performed using a computerized polysomnogram system (Alice 4; Respironics; Pittsburgh, PA, USA) for 2 consecutive nights. Apnea was defined as cessation

of airflow or a decrease in airflow to less than 20% of baseline for >10s, and hypopnea was defined as a discernible decrease in airflow to less than 50% of baseline associated with a fall in oxygen saturation of >=4% from baseline. Desaturation during sleep was assessed in terms of oxygen desaturation index of more than 3% desaturation (ODI), time in relation to total sleep time with SpO_2<=90％ (% time in SpO_2<=90%), and lowest SpO_2. In the night following the diagnosis of OSAS by PSG, 16 patients underwent a second PSG by which apneas were largely reduced or eliminated under nasal CPAP (REMstar Auto; Respironics; Pittsburgh, PA, USA) titration.

Isolation of peripheral blood monocytes

Whole heparinized blood was collected from all patients and control subjects both before sleep, and again after sleep with PSG in the early morning (6:30 AM). Sixteen of these patients with OSAS also had blood samples taken after CPAP treatment (6:30 AM). Peripheral blood monocytes were isolated using standard methods.[22]

Determination of invasive ability of monocytes

The invasive ability of monocytes was measured by BD BioCoat Matrigel Invasion Chamber (Discovery Labware, Bedford, MA, USA) [23] according to the manufacturer's instructions. This chamber consists of a Falcon cell culture insert in which the porous membrane is coated with a Matrigel basement membrane, a solubilized basement membrane extracted from Engelbreth-Holm-Swarm mouse sarcoma containing laminin, collagen type IV, heparan sulfate proteoglycan, entactin, and growth factors. The lower component was filled with Oxidized Low Density Lipoprotein Human (Biomedical Technology Inc., Stoughton, MA, USA) as a chemoattractant, at a concentration of 10 μg/ml. After rehydration of the basement membrane, monocyte suspensions of patients and control

subjects were seeded in the upper component of the chamber. The cell numbers of the monocyte suspensions were adjusted to 0.5×10^6 cells per well. After incubation at 37°C under 5% CO_2 for 24 hours, the cells that had invaded the chamber and migrated to the lower surface were stained with Diff-Quik (Sysmex, Kobe, Japan) and were manually counted under a microscope (200× magnification). The cells were counted in four fields of each membrane by blinded two researchers. We defined the mean of counted cells as the invasive ability.

In this study, we used oxidized low-density lipoprotein (LDL) as a chemoattractant. In OSAS patients, as invasive cells were greater with 10 μg/ml oxidized LDL than with 1 μg/ml and 100 μg/ml (data not shown), we decided to use this concentration.

The study was approved by the Human Subjects Committee of Nara Medical University, and informed consent was obtained from all participants.

Statistical analysis

Statistical analysis was performed using Stat View version 5.0 (SAS Institute Inc., Cary, NC, USA). Changes among groups were analyzed by Wilcoxon signed-ranks test, and differences between two groups were analyzed by Mann-Whitney U test. Correlations were analyzed by Pearson's correlation coefficients. Data were expressed as mean± SD. A probability value <0.05 was considered to indicate significance.

Results

Patient characteristics

The characteristics of OSAS patients and control subjects are shown in Table 1. The two groups were similar in age, anthropometric data, smoking status, lipid profile, glucose metabolism, and blood pressure. No subject was taking any medication. Sleep parameters

such as AHI, ODI, lowest SpO_2, % time in SpO_2<=90% and arousal index were significantly deteriorated in OSAS patients as compared to control subjects.

Invasive ability of monocytes

As representative cases, in a severe OSAS patient (42 yrs, male, AHI 58.0, BMI 26.3 kg/m^2; Invasive ability was 279 cells/field), large numbers of invasive cells through the chamber were observed as compared to those in a control subject (41 yrs, male, AHI 1.8, BMI 25.6 kg/m^2; Invasive ability was 90 cells/field) (Figure 1). Interestingly, the invaded monocytes morphologically appeared to have differentiated to macrophages.

On average, invasive ability of monocytes in the early morning was significantly higher in OSAS patients than in control subjects (178.2±53.9 vs. 82.2±12.1 cells/field, $p<0.001$). In both groups, there was no significant difference between current smokers (n=8 in OSAS patients, n=7 in control subjects) and non-smokers (n=13 in OSAS patients, n=10 in control subjects) (Figure 2). Invasive ability in the early morning was significantly elevated (178.2±53.9 cells/field) as compared to that before sleep (124.6±25.9 cells/field, $p<0.001$) in OSAS patients (Figure 3). On the other hand, there was no significant change before and after sleep in control subjects (88.8±5.1 to 81.2±5.2 cells/field).Invasive ability of monocytes in the early morning in OSAS patients was positively correlated with ODI ($r=0.498$, $p<0.05$) (Figure 4). It was also positively correlated with AHI ($r=0.479$, $p<0.05$), % time in SpO_2<=90% ($r=0.456$. $p<0.05$) and BMI ($r=0.459$, $p<0.05$). However, it was not significantly correlated with the lowest SpO_2 ($r=-0.228$, $p=0.324$) and arousal index ($r=0.296$, $p=0.270$).

Finally, OSAS patients showed significantly decreased invasive ability after CPAP treatment (192.5±49.3 to 127.6±48.4 cells/field, $p<0.001$) (Figure 5).

Discussion

The present study indicated that hypoxic stress induced by sleep apnea activates the invasive ability of monocytes in patients with OSAS, and that this phenomenon may contribute to the development of atherosclerosis. It was also clarified that the invasive ability was significantly decreased after CPAP titration, suggesting that CPAP treatment could play a crucial role in the prevention of atherosclerosis in OSAS patients.

With regard to the association of cardiovascular diseases and OSAS, various pro-inflammatory mediators, including TNF-α, IL-6 and adhesion molecules, induce monocyte migration into endothelial cells and subendothelial spaces. During this continuing process, monocytes change to foam cells and contribute to the progression of atherosclerosis.[6] Elevated plasma levels in the intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), L-selectin and E-selectin have been demonstrated in OSAS patients, suggesting activation of the function in vascular endothelial cells.[17, 24] Several studies have also reported that TNF-α[14-16] and MCP-1[17, 18]play crucial roles in the pathogenesis of atherosclerosis in OSAS.

Minoguchi et al.[16] demonstrated that spontaneous production of TNF-α by monocytes was elevated in patients with OSAS. Recently, Ryan et al.[25]showed that intermittent hypoxia activates nuclear factor kappaB (NF-kappaB), a key transcription factor in chronic inflammatory diseases,[26, 27] in cell culture (HeLa cells). They were able to clearly demonstrate a significant relationship between NF-kappaB-dependent gene, TNF-α, and sleep-related oxygen desaturation.[28] We have also shown that in severe OSAS, the productions of TNF-α and MCP-1 by monocytes were significantly elevated in the early morning after sleep as compared to before sleep, and that they were decreased after long-term CPAP treatment.[19] Taking these lines of evidence into account, we speculate that monocytes may be activated by nocturnal hypoxic stress in OSAS, and inflammatory

mediators and adhesion molecules promote their invasion ability into the subendothelial space.

Dyugovskaya et al.[20] reported that OSAS was associated with increased adherence of monocytes to human endothelial cells. They also demonstrated that expression of adhesion molecules CD15 and CD11c on monocytes were increased, and CPAP treatment downregulated the expression of adhesion molecules and decreased monocyte adherence to human endothelial cells. Lattimore et al.[21] reported that repetitive hypoxia enhances lipid uptake into human macrophages. So far, however, invasion and migration of monocytes have not been investigated in OSAS.

In the present study, we evaluated the invasive ability of monocytes using a Matrigel Invasion Chamber. This chamber is considered to be a model of the basement membrane that constitutes vascular endothelial cells.[23] This chamber has been used to study the invasion of neutrophils,[29, 30] endothelial cells,[31, 32] and smooth muscle cell.[33] We used this chamber to investigate the invasion and migration of monocytes in OSAS. As a chemoattractant, we used oxidized LDL. LDL is generally recognaized to be important in the development of atherosclerosis, and its atherogenicity may be due to oxidative modifications.[34] It was demonstrated that oxidized LDL facilitate transendothelial migration of monocytes.[35]

The invaded monocytes morphologically appeared to have differentiated to macrophages in patients with severe OSAS. We demonstrated that, in OSAS, the invasive ability of monocytes was significantly elevated after sleep, the degree of which was dependent on the oxygen desaturation index, suggesting that the augmented invasive ability is strongly related to nocturnal hypoxic stress. This invasive ability was observed to be higher in OSAS patients than in control subjects. CPAP treatment was then found to have a significantly inhibitory effect on the invasive ability. In the present study, OSAS patients and control subjects were similar in age, anthropometric data, smoking status, lipid profile, and

glucose metabolism. Neither OSAS patients nor control subjects were taking any medication. Therefore, it is strongly suggested that nocturnal hypoxic stress may activate the invasive ability of monocytes into endothelial cells, which may contribute to the development of atherosclerosis in OSAS patients. We have already reported that intima-media thickness of carotid-artery, which is an indicator of atherosclerosis, was associated with the severity of OSAS. [36] Furthermore CPAP treatment could play an important role in the prevention of atherosclerosis.

In total, the present study has major pathophysiological significance concerning the functions of monocytes/macrophages, which are simultaneously excited so as to elevate inflammatory mediators and to facilitate their hypoxic stress-induced invasive ability as well as their possible interaction with each other.

There is a potential limitation of our study. OSAS is associated with a variety of features which have been shown to cause pathophysiological responses besides hypoxia, such as hypercapnia, sleep fragmentation, frequent arousals, and sympathetic overactivity. In the present study, the influence of these factors on the invasive ability of monocytes cannot be excluded. However, we believe that nocturnal hypoxic stress can be one of the major factors to augment the invasive ability monocytes in OSAS patients. Further in vitro study is necessary to prove an association between the invasion ability of monocytes and hypoxic stress.

In conclusion, OSAS-induced hypoxic stress may contribute to the development of atherosclerosis via the activation of the invasive ability of monocytes/macrophages. Furthermore, our results suggest that CPAP treatment has the potential to prevent the development of atherosclerosis by attenuating the inflammatory process.

Acknowledgments

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Table 1. Characteristics and anthropometric data for evaluating the invasive ability of monocytes in OSAS and control subjects.

	Control	OSAS	p value
n	17	21	
Age (yr)	39.9±7.0	42.4±5.9	N.S.
Sex (male/female)	17/0	21/0	N.S.
BMI (kg/m^2)	26.2±2.8	28.1±3.8	N.S.
Current smoker	7/17	8/21	N.S.
Total cholesterol (mg/dl)	200.0±27.5	201.3±28.4	N.S.
HDL cholesterol (mg/dl)	51.6±8.6	46.2±7.5	N.S.
LDL cholesterol (mg/dl)	121.4±27.6	124.6±25.9	N.S.
Triglyceride (mg/dl)	135.4±71.7	153.8±55.9	N.S.
Fasting blood glucose (mg/dl)	94.9±5.7	95.8±9.8	N.S.
HbA1c (%)	5.1±0.4	5.2±0.5	N.S
Systolic blood pressure (mmHg)	128.5±13.9	132.7±14.4	N.S.
Diastolic blood pressure (mmHg)	77.0±8.3	82.4±10.6	N.S.
AHI	3.9±2.2	52.0±29.0	<0.001
ODI	7.4±3.7	48.8±26.0	<0.001
Lowest SpO_2 (%)	90.2±1.9	71.2±12.9	<0.001
% Time in SpO_2<90% (%)	0.1±0.2	25.8±27.4	<0.001
Arousal index	11.1±8.9	36.3±19.0	<0.001

BMI, body mass index (kg/m^2); AHI, apnea-hypopnea index; ODI, oxygen desaturation index more than 3% desaturation. Values are presented as mean±SD

Figure legends

Figure 1. Typical examples of invaded cells through the BD BioCoat Matrigel Invasion Chamber are shown in an OSAS patient and a control subject. Monocytes were extracted in the early morning. The invaded cells were stained with Diff-Quik (Sysmex, Kobe, Japan), and counted under a microscope (200× magnification). Large numbers of invasive cells through the chamber were observed as compared to those in the control subject. The invaded monocytes morphologically appeared to have differentiated to macrophages.

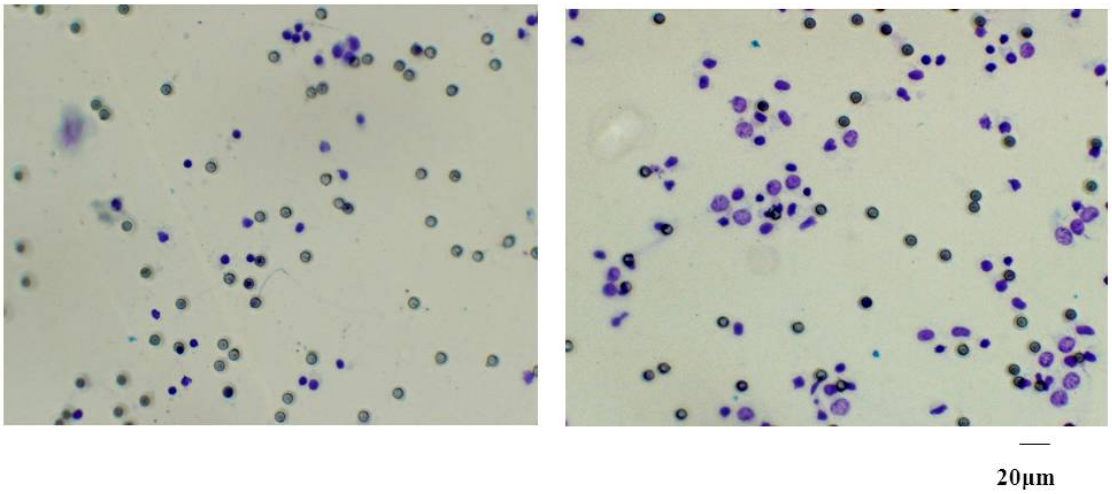
Figure 2. The invasive ability of monocytes in OSAS patients and control subjects. The invasive ability of monocytes was evaluated by counting the number of invaded cells using the BD BioCoat Matrigel Invasion Chamber. Monocytes were extracted in the early morning. The invasive ability in the early morning was significantly higher in OSAS patients than in control subjects ($p<0.001$). In both groups, there was no significant difference between current smokers and non-smokers.

Figure 3. Changes in the invasive ability of monocytes before and after sleep in OSAS patients. The invasive ability in the early morning was significantly elevated as compared to that before sleep ($p<0.001$). Open circles represent current smokers, and closed circles represent non-smokers.

Figure 4. Relationship between the invasive ability of monocytes and oxygen dsaturation indeax (ODI). The invasive ability was positively correlated with ODI ($r=0.498$, $p<0.05$).). Open circles represent current smokers, and closed circles represent non-smokers.

Figure 5. Effects of CPAP on the invasive ability of monocytes before and after short-term CPAP treatment in OSAS patients. Monocytes were extracted in the early morning. The invasive ability was significantly decreased after CPAP treatment ($p<0.001$).). Open circles represent current smokers, and closed circles represent non-smokers.

Figure 1.



Control (41y.o. male, AHI 1.8) *OSAS (42y.o. male, AHI 58.0)*

Figure 2.

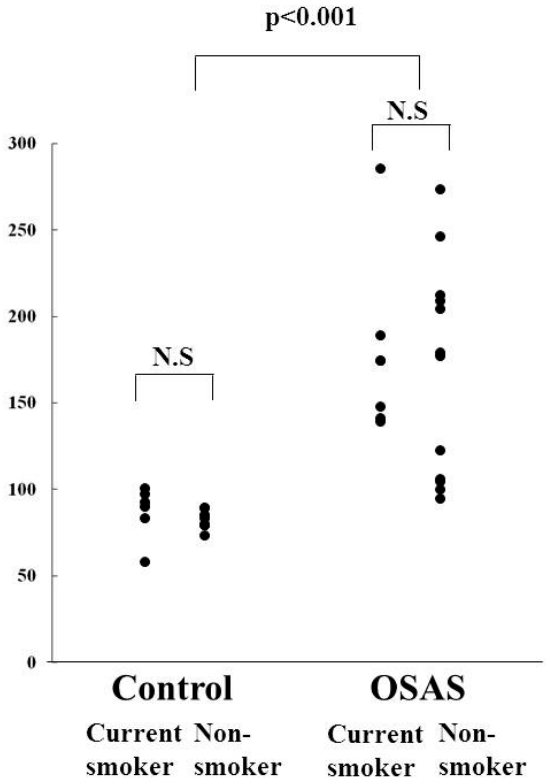

p<0.001
cells/field
N.S
N.S
300
250
200
150
100
50
0
Control
OSAS
Current smoker
Non-smoker
Current smoker
Non-smoker

Figure 3.

cells/field

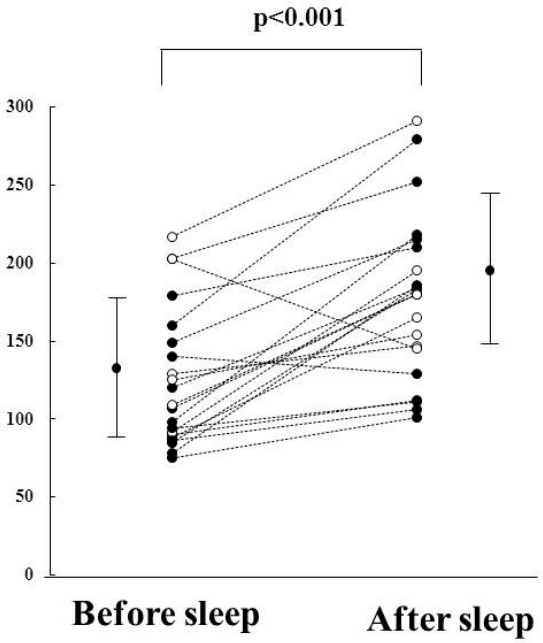


Figure 4.

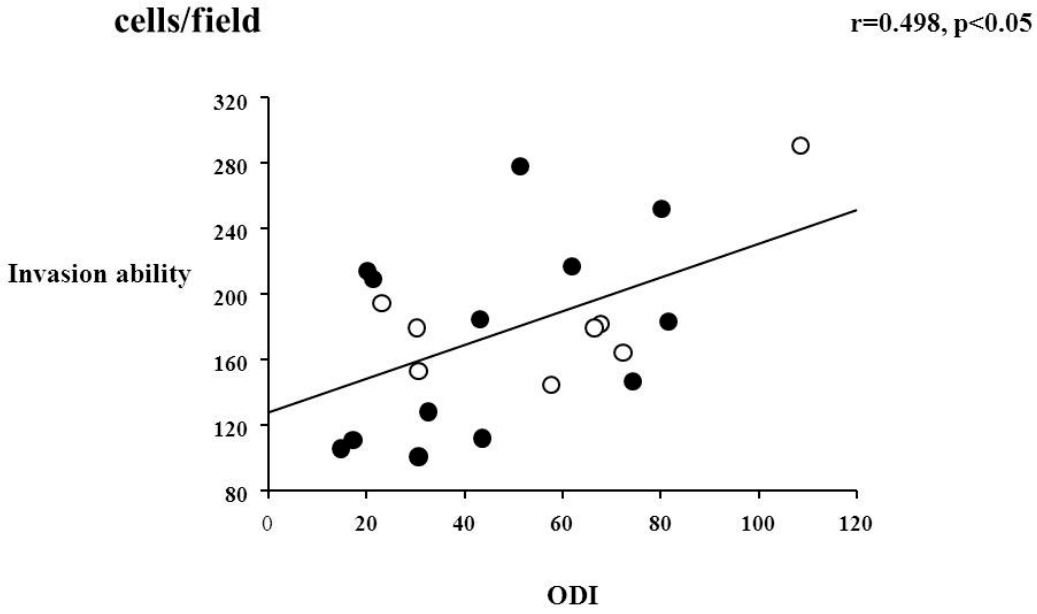
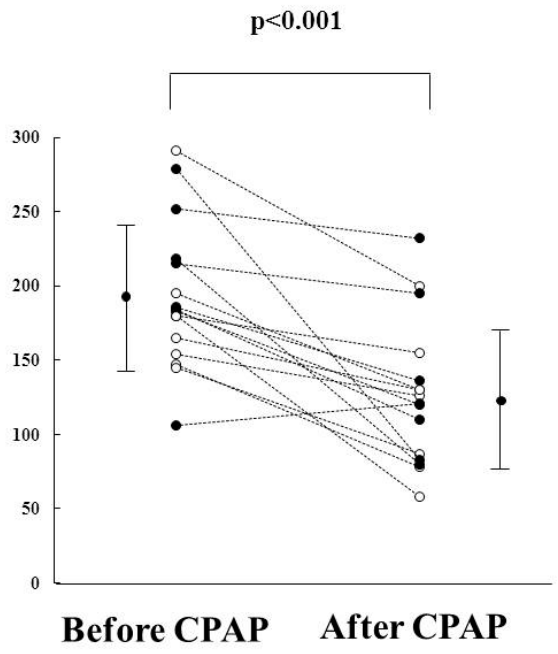

cells/field
r=0.498, p<0.05
Invasion ability
320
280
240
200
160
120
80
0
20
40
60
80
100
120
ODI

Figure 5.


p<0.001
cells/field
300
250
200
150
100
50
0
Before CPAP
After CPAP