Significant Relationship between Platelet Activation and Apnea-hypopnea Index in Patients with Obstructive Sleep Apnea Syndrome

Yusuke KONDO[*1], Ichiro KUWAHIRA[*2], Mie SHIMIZU[*3], Asuka NAGAI[*2], Tokuzen IWAMOTO[*2], Sakurako KATO[*4], Naoki HAYAMA[*4], Takuya AOKI[*4], Tetsuya URANO[*4], Fumihito YOSHII[*3], Hiroyuki KOBAYASHI[*5] and Tadashi ABE[*4]

[*1]*Department of Pulmonary Medicine, Tokai University Oiso Hospital*
[*2]*Department of Pulmonary Medicine, Tokai University Tokyo Hospital*
[*3]*Department of Neurology, Tokai University School of Medicine*
[*4]*Department of Pulmonary Medicine, Tokai University School of Medicine*
[*5]*Department of Clinical Pharmacology, Tokai University School of Medicine*

(Received May 19, 2011; Accepted July 25, 2011)

Obstructive sleep apnea syndrome (OSAS) is an independent risk factor for arterial thrombosis, which is associated with high cardiovascular morbidity and mortality. To investigate the possible involvement of activated platelets, we evaluated the relationship between severity of OSAS and appearance of platelet aggregates (a marker of activated platelets) in 35 OSAS patients. Platelet aggregates were quantitatively determined by means of flow cytometry. There was a significant correlation between platelet aggregates and apnea-hypopnea index in the severe (AHI ≥ 30 events/hour) group ($r = 0.756$, $p < 0.001$), but not in the mild-moderate ($5 \leq AHI < 30$ events/hour) group ($r = -0.032$, $p = 0.905$). The results indicate that the appearance of platelet aggregates increases with an increase in the severity of OSAS.

Key words: obstructive sleep apnea syndrome (OSAS), thrombosis, platelet aggregates, cardiovascular events, flow cytometry

INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) affects 2% of women and 4% of men in the age range of 30-60 years [1]. It is an independent risk factor for arterial thrombosis, leading to stroke and coronary heart disease, and thus is associated with increased cardiovascular morbidity and mortality [2, 3]. Enhanced platelet function has been suggested as a potential cause of the increased incidence of cardiovascular events in OSAS [4]. Accordingly, early detection of platelet activation could be important for prevention of cardiovascular events. Previously we found a significant increase in platelet activation in patients with OSAS by means of whole-blood flow cytometry using activation-dependent monoclonal antibodies [5]. Furthermore, we have developed a simple, rapid and automated method for detection of platelet hyperaggregability in patients with OSAS by using a conventional hematology analyzer (Abbott CELL-DYN 4000®, Abbott Diagnostics, Abbott Park, IL, USA) [6, 7]. We have reported that blood samples from healthy subjects did not contain platelet aggregates, whereas blood samples obtained from OSAS patients showed massive platelet aggregates in the absence of agonists [7]. Although such measurements are very useful for early detection of cardiovascular risk in patients with OSAS, they were only qualitative [6, 7]. In the present study, we examined the relationship between apnea-hypopnea index (AHI), as an indicator of severity of OSAS, and the appearance of platelet aggregates, measured by means of whole-blood flow cytometry. The aim of the present study is to evaluate whether increased platelet activation is correlated with increased severity of OSAS.

METHODS

Subjects and clinical examinations

Thirty-five patients (29 males and 6 females) with OSAS were enrolled. OSAS was diagnosed on the basis of respiratory polysomnographic study (PSG, Sleep Watcher®, ResMed Co. Ltd., Australia) according to the criteria of the American Academy of Sleep Medicine [8]. AHI, lowest oxygen saturation (lowest SpO_2), arousal index and sleep efficiency were determined. To evaluate whether patients with OSAS showed thrombotic complications, such as ischemic cerebrovascular disease, cardiovascular disease, or carotid atherosclerotic changes, we carried out head magnetic resonance imaging, cardiac and carotid sonography, and electrocardiography. Patients with asymptomatic chronic cerebral infarction and/or carotid intima-media thickness more than 1.1 mm were considered to have thrombotic complications. None of the subjects had received any anti-platelet drug. Patients with a history of acute inflammatory disease, malig-

Ichiro KUWAHIRA, Department of Pulmonary Medicine, Tokai University Tokyo Hospital, 1-2-5 Yoyogi, Shibuya, Tokyo 151-0053, Japan
Tel: +81-3-3370-2321 ext. 5626 Fax: +81-3-5354-5366 E-mail: kuwahira@tok.u-tokai.ac.jp

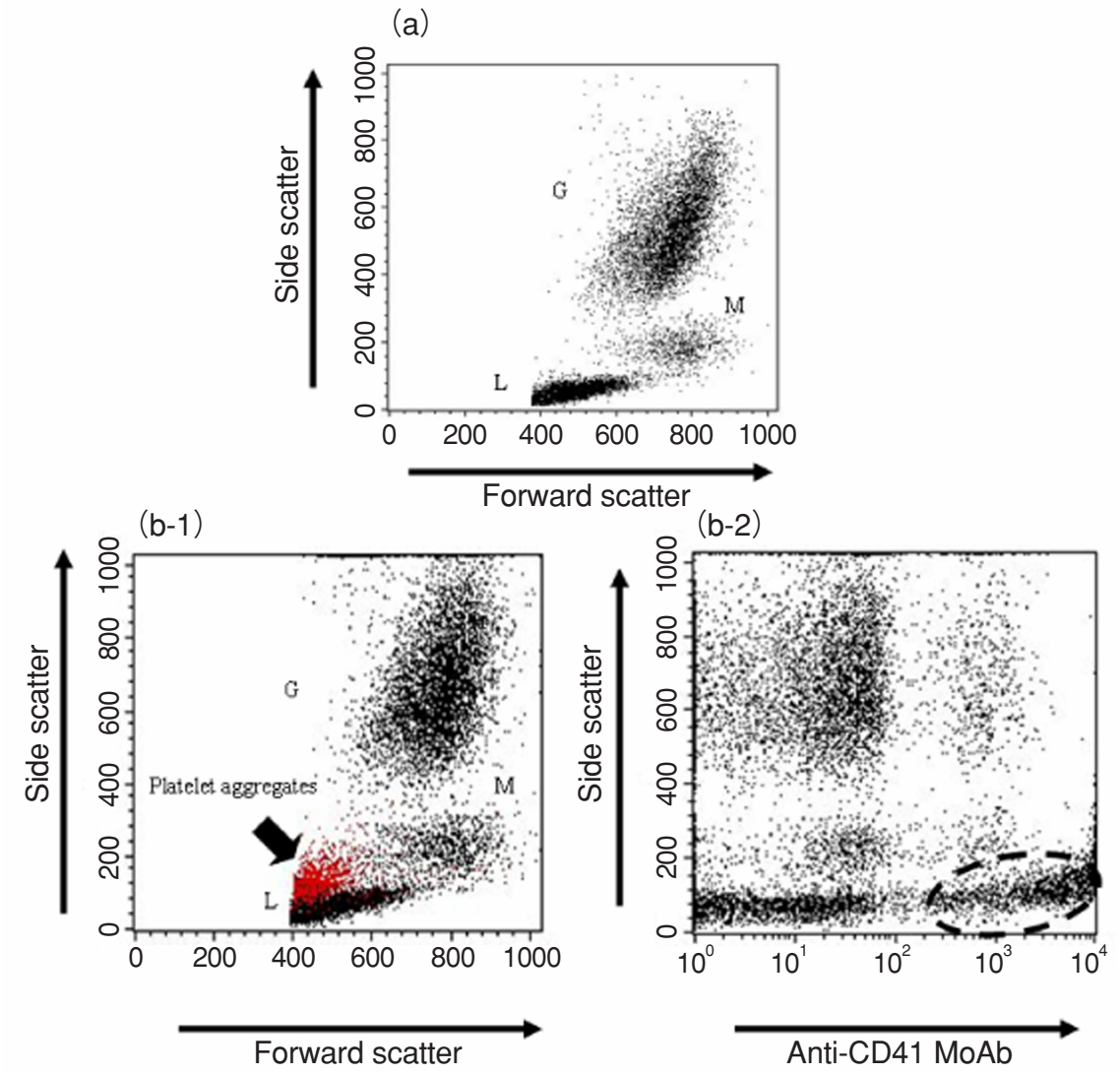

Fig. 1 Detection of platelet aggregates in the WBC region by flow cytometry.
Platelet aggregates (red dots in Fig. 1b-1), which were located within the WBC region, was seen in citrated blood of an OSAS patient (b-1), but not a healthy volunteer (Fig. 1a). Platelet aggregates (dashed circle), which are positive for MoAb-CD41a, appear in the region between lymphocytes and granulocytes on the scatter plot for the OSAS patient (b-2). G, M, and L indicate granulocytes, monocytes, and lymphocytes, respectively.

nancy or atrial fibrillation were not included in the study. The Tokai University Research Ethics Committee approved this study, and all participants gave written informed consent.

Flow cytometric measurements and analysis of platelet aggregates

Blood sampling was carried out from 8:00 AM to 8:30 AM in all subjects after PSG was completed. The reason why blood sampling was carried out between 8:00 and 8:30 AM is that Muller *et al.* and Kamio *et al.* found a circadian rhythm of platelet activation, and platelet activation was the greatest in the morning [9, 10]. Citrate-anticoagulated whole blood was obtained from the antecubital vein with the aid of a light tourniquet. The first 2 ml of blood was discarded, and then 4.5 ml of blood was collected slowly into a plastic syringe fitted with a 21 gauge needle and containing 0.5 ml of 3.14% sodium citrate (Terumo, Tokyo, Japan). White blood cells (WBC) were simultaneously counted in EDTA-anticoagulated whole blood. A 25 μL aliquot of blood was gently added to a microcentrifuge tube containing 10 μL convenient cocktail (Oncomark; Becton Dickinson Biosciences, San Joes, CA), containing MoAb-CD45 to identify WBC, anti-glycophorin antibody to identify red blood cells and MoAb-CD41a to identify platelets. The reaction mixture was gently stirred without vortexing, followed by incubation for 15 min at room temperature in the dark. Subsequently, the cells were fixed in 500 μL of cold lysing solution and WBC were points out to pay attention to the circadian rhythm on a BD FACS Calibur (Becton Dickinson Biosciences, San Joes, CA), as shown in Fig. 1. The appearance rate of platelet aggregates (counts/μL) was calculated as (CD41a-positive population/CD45-positive population) times WBC count.

Table 1 Clinical features of 35 patients

	AHI (events/hour)	Age (year)	Sex (M/F)	BMI (kg/m²)	Lowest SpO_2 (%)	Arousal index (events/hour)	Sleep efficiency (%)	Platelet aggregates (counts/μL)
5 ≤ AHI < 30	5.0	57	M	24.7	86	11.1	84.8	208.9
(events/hour)	5.2	42	F	34.6	88	9.1	81.7	374.3
	7.3	53	M	22.2	82	25.9	97.1	29.9
	9.1	41	M	23.4	80	12.4	56.7	299.1
	10.8	41	M	22.5	83	18.7	89.1	803.0
	11.4	52	M	28.7	84	19.0	86.3	315.4
	12.3	65	M	21.0	87	35.9	66.9	787.1
	12.3	51	M	30.1	87	21.7	75.2	901.5
	13.8	59	F	26.4	83	19.0	75.7	65.5
	15.4	53	M	23.2	80	18.9	73.2	154.8
	15.7	34	M	29.5	74	20.7	97.0	104.9
	16.1	49	M	22.2	74	19.9	87.6	162.9
	17.9	54	F	28.0	79	19.0	90.6	590.9
	23.0	57	F	24.8	87	27.4	80.8	1903.1
	24.7	59	M	20.6	72	34.0	90.9	92.1
	28.2	50	F	22.7	77	33.3	94.8	258.7
AHI ≥ 30	31.1	55	M	25.3	74	19.1	69.4	23.9
(events/hour)	33.9	56	M	27.9	81	28.5	79.8	165.1
	34.5	61	M	22.2	84	26.4	73.6	89.5
	34.9	58	M	24.4	73	38.3	96.3	150.5
	37.7	69	M	24.5	87	34.7	84.9	247.8
	38.3	35	M	25.5	57	36.7	86.5	210.1
	41.3	44	M	31.6	61	25.7	95.2	60.1
	41.4	62	F	24.9	77	33.5	85.6	929.9
	43.8	30	M	30.9	76	21.4	97.9	369.9
	44.5	49	M	27.7	76	38.0	97.3	119.5
	45.6	72	M	26.6	74	33.3	88.0	472.4
	46.4	59	M	22.6	72	44.7	91.8	224.9
	47.2	48	M	27.0	79	43.7	72.2	381.4
	57.0	30	M	24.7	84	57.6	96.8	1176.4
	63.4	55	M	27.9	80	49.1	85.0	240.9
	66.8	27	M	33.6	65	31.5	88.5	1237.5
	69.8	56	M	26.9	53	59.8	90.8	839.1
	74.0	43	M	35.2	63	72.6	97.6	594.6
	88.5	39	M	34.4	75	39.5	61.3	1043.2

AHI, apnea-hypopnea index; BMI, body mass index

Statistics

All results are presented as median (interquartile range). Because the number of patients in the mild group was only 9, mild and moderate patients were combined into a single group, the mild-moderate group (n = 16, 5 ≤ AHI < 30 events/hour), for comparison with the severe group (n = 19, AHI ≥ 30 events/hour). Comparisons of age, BMI and PSG data between the two groups were carried out by using the Mann-Whitney U test. Comparison of the prevalence of thrombotic complications between the two groups was done by using the χ^2 test. The correlation between platelet aggregates and AHI was analyzed by using Spearman's correlation. A P-value < 0.05 was taken to indicate a statistically significant difference. The IBM SPSS Statistics Software version 19 (IBM Corporation, NY) was used throughout.

RESULTS

Table 1 summarizes the clinical features of the 35 patients and Table 2 shows the characteristics of OSAS. Arousal index was significantly higher ($p < 0.001$) and lowest SpO_2 was significantly lower ($p = 0.003$) in the severe group compared to the mild-moderate group. There was no significant difference in age, BMI, sleep efficiency or the rate of thrombotic complications between the two groups.

Fig. 1 shows the detection of platelet aggregates by flow cytometry. Platelet aggregates (indicated by red dots in Fig. 1b-1), which were located in the WBC region in citrated blood, were seen in the OSAS group (Fig. 1b-1), but not in the healthy volunteers (Fig. 1a). Platelet aggregates (dashed circle), which are positive for MoAb-CD41a, appear in the area between lymphocytes and granulocytes on the scatter plot in the OSAS group (b-2).

Fig. 2 shows the locally weighted scatterplot smooth-

Table 2 Characteristics of OSAS patients

	5 ≤ AHI < 30 events/hour (n = 16, M/F 11/5)	AHI ≥ 30 events/hours (n = 19, M/F 18/1)
Age (year)	53 (44 - 57)	55 (39 - 59)
BMI (kg/m^2)	24.1 (22.3 - 28.5)	26.9 (24.7 - 30.9)
Lowest SpO_2 (%)	83 (78 - 87)	75 (65 - 80)*
Arousal index (events/hour)	19 (19 - 27)	37 (29 - 45)*
Sleep efficiency (%)	86 (75 - 91)	88 (80 - 96)
Thrombotic complications (%)	43.8	52.6

Values are median (interquartile range), *$p < 0.05$
OSAS, obstructive sleep apnea syndrome.

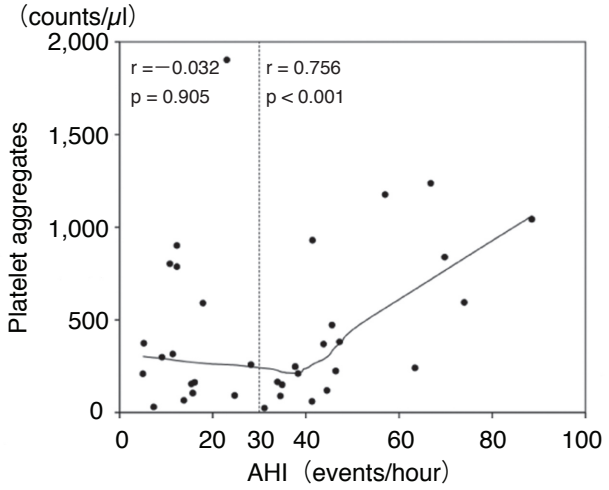

Fig. 2 Correlation and LOESS-fitted line for the relationship between platelet aggregates and AHI in patients with OSAS.
There was no correlation between platelet aggregates and AHI in the mild-moderate (5 ≤ AHI < 30 events/hour) group (Spearman's $r = -0.032$, $p = 0.905$), whereas a significant correlation was found in the severe (AHI ≥ 30 events/hour) group (Spearman's $r = 0.756$, $p < 0.001$). The locally weighted scatterplot smoothing (LOESS) curve was used to express the relationship between platelet aggregates and AHI in patients with OSAS (indicated by the line).

ing (LOESS)-fitted line for the relationship between platelet aggregates and AHI. There was a significant correlation between platelet aggregates and AHI in the severe group ($r = 0.756$, $p < 0.001$), but there was no correlation in the mild-moderate group.

DISCUSSION

The results of the present study indicate that the appearance of platelet aggregates significantly increases with increase of AHI in patients with severe OSAS. Since there is no significant difference in the rate of thrombotic complications between the two groups, platelet function might be independently enhanced because of OSAS. Previously, citrated blood samples obtained from healthy subjects and stimulated with epinephrine were tested as a model of platelet activation in OSAS [7]. Platelet aggregates were more marked at a high dose of epinephrine, which is consistent with the finding that platelet activation increases with increasing severity of OSAS. The fact that there is no correlation between platelet aggregates and AHI in the mild-moderate OSAS group suggests that there might be a threshold for platelet activation. Yaggi *et al.* have demonstrated that OSAS is associated with increased incidence of stroke or death from any cause and that the association is independent of other cardiovascular and cerebrovascular risk factors [3]. In particular, they have shown that the risk of stroke or death in patients in the most severe quartile of OSAS (AHI > 36) was three times that in the controls [3]. Marin *et al.* have demonstrated that severe OSAS increased the risk of fatal and nonfatal cardiovascular events, although mild and moderate OSAS did not [11]. The cross-sectional results of the Sleep Heart Health Study also showed that the risk of cardiovascular disease appears to increase progressively with increasing severity of OSAS [2]. Our findings in the present study are consistent with these epidemiological results.

As mentioned before, we previously observed an increase in platelet activation in OSAS by means of whole blood flow cytometry using activation-dependent monoclonal antibodies [5]. The expression of activated platelet markers such as PAC-1 and CD62P was significantly higher in OSAS patients than in healthy subjects. Furthermore, we developed a simple and rapid method for detection of platelet hyperaggregability in patients with OSAS [7]. However, these methods were only qualitative, and the quantitative relationship between AHI and platelet activation was not determined. In the present study, we have shown that platelet activation increases significantly with an increase in AHI in the severe OSAS group. We have previously shown qualitatively that platelet activation in patients with OSAS is sometimes reversed by CPAP therapy, but sometimes it is not [12]. Further quantitative study is therefore needed to investigate whether platelet aggregates decrease in response to CPAP therapy.

In conclusion, we have evaluated the relationship

between severity of OSAS and the appearance of platelet aggregates in 35 OSAS patients. There was a significant correlation between platelet aggregates and AHI in the severe OSAS group, but not in the mild-moderate OSAS group. The results indicate that platelet activation increases significantly with an increase in severity of OSAS.

ACKNOWLEDGMENTS

The skillful technical assistance of Manabu Yamamoto and Tomiei Iga is gratefully acknowledged. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, No. 20659133 (I. Kuwahira).

REFERENCES

1) Young T, Palta M, Dempsey J, Skatrud J, Weber S, Badr S. The occurrence of sleep-disordered breathing among middle-aged adults. N Eng J Med 1993; 328: 1230–1235.
2) The Sleep Heart Health Study Research Group. Sleep-disordered breathing and cardiovascular disease. Cross-sectional results of the sleep heart health study. Am J Respir Crit Care Med 2001; 163: 19–25.
3) Yaggi Hk, Concato J, Kernan WN, Lichtman JH, Brass LM, Mohsenin V. Obstructive sleep apnea as a risk factor for stroke and death. N Engl J Med 2005; 353: 2034–2041.
4) Shamsuzzaman ASM, Gersh BJ, Somers VK. Obstructive sleep apnea. Implications for cardiac and vascular disease. JAMA 2003; 290: 1906–1914.
5) Shimizu M, Kamio K, Haida M, Ono Y, Miyachi H, Yamamoto M, *et al*. Platelet activation in patients with obstructive sleep apnea syndrome and effects of nasal-continuous positive airway pressure. Tokai J Exp Clin Med 2002; 27: 107–112.
6) Shimizu M, Yamamoto M, Miyachi H, Shinohara Y, Ando Y. Simple, rapid, and automated method for detection of hyper-aggregability of platelets using a hematology analyzer. Am J Hematol 2003; 72: 282–283.
7) Shimizu M, Kamio K, Iwamoto T, Ando Y, Kuwahira I. Simple, rapid and automated method for detection of hyperaggregability of platelets in sleep apnea syndrome. J Thromb Haemost 2006; 4: 920–922.
8) The Report of an American Academy of Sleep Medicine Task Force: Sleep-related breathing disorders in adults: Recommendations for syndrome definition and measurement techniques in clinical research. Sleep 1999; 22: 667–689.
9) Muller JE, Tofler GH, Stone PH. Circadian variation and triggers of onset of acute cardiovascular disease. Circulation. 1989; 79: 394–5.
10) Kamio K, Ono Y, Kamiya U, Shimizu M, Ando Y, Kuwahira I, *et al*. Platelet Activation in Obstructive Sleep Apnea Syndrome. The Journal of the Japanese Respiratory Society 2002; 40: 473–477.
11) Marin JM, Carrizo SJ, Vicente E, Agusti AG. Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. Lancet 2005; 365: 1046–1053.
12) Shimizu M, Kamio K, Haida M, Ono Y, Miyachi H, Yamamoto M, *et al*. Platelet Activation in Patients with Obstructive Sleep Apnea Syndrome and Effects of Nasal-Continuous Positive Airway Pressure. Tokai J Exp Clin Med 2002; 27: 107–112.