Table 4. Additive effects of cigarette smoke on changes in organic acids and pH in caecal contents by the cellulose-free diet

Organic acid and pH	Cellulose-free diet Non-smoke (n = 5)	Cellulose-free diet Smoke (n = 4)	p value
Acetic acid	72.1 ± 15.2	45.5 ± 4.6#	0.014
Propionic acid	14.7 ± 3.2	14.2 ± 1.4	0.807
Butyric acid	12.8 ± 5.5	14.0 ± 3.4	0.807
Isobutyric acid	8.3 ± 5.8	5.0 ± 2.9	0.221
Valeric acid	3.1 ± 4.3	7.9 ± 6.0	0.270
Isovaleric acid	3.7 ± 3.3	7.5 ± 1.1	0.111
Succinic acid	15.6 ± 9.7	14.3 ± 10.6	0.806
pH	6.9 ± 0.3	7.6 ± 0.2#	0.028

(μ mol/g caecal contents)

Each point indicates the mean ± S.D. of 4 tor 5 animals. # $p < 0.05$: significant relative to animals without exposure by the Mann-Whitney U test.

Table 5. Effects of exposure to cigarette smoke and the cellulose-free diet on the population of micro-flora in caecal contents

Organisms	Control diet Non-smoke (n = 5) (Control)	Control diet Smoke (n = 5) (Effect of smoke)	Cellulose-free diet Non-smoke (n = 5) (Effect of cellulose-free diet)
Fusiform bacteria	10.2 ± 0.1	10.1 ± 0.3	10.6 ± 1.0
Bacteroidaceae	9.7 ± 0.4	9.6 ± 0.5	10.0 ± 0.1
Bifidobacterium	6.0 ± 0.3	5.3 ± 0.8	5.0 ± 0.4*
Lactobacillus	6.8 ± 0.5	6.9 ± 1.0	7.5 ± 0.4
Enterococcus	7.1 ± 0.4	6.9 ± 0.2	7.3 ± 0.1
Enterobacteriaceae	6.7 ± 0.8	6.5 ± 0.4	6.0 ± 0.6
Staphylococcus	6.7 ± 0.7	7.0 ± 0.1	6.5 ± 0.3

($\log_{10}$ CFU/g caecal contents)

Each point indicates the mean ± S.D. per one g of caecal contents for 5 animals. *$p < 0.05$: significant relative to control by the Mann-Whitney U test.

ra in caecal contents are shown in Table 5. Cigarette smoke tended to decrease the population of *Bifidobacterium* (6.0 ± 0.3 vs. 5.3 ± 0.8 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.175), while the cellulose-free diet significantly decreased the population of *Bifidobacterium* (6.0 ± 0.3 vs. 5.0 ± 0.4 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.016) and tended to increase *Lactobacillus* (6.8 ± 0.5 vs. 7.5 ± 0.4 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.050) (Table 5). On exposed to cigarette smoke, the cellulose-free diet tended to decrease the population of Staphylococcus (7.0 ± 0.1 vs. 6.6 ± 0.4 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.050) and inrease the population of *Bifidobacterium* (5.3 ± 0.8 vs. 6.2 ± 0.5 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.080) (Table 6), while on the cellulose-free diet, cigarette smoke induced significant increase of the population of *Bifidobacterium* (5.0 ± 0.4 vs. 6.2 ± 0.5 $\log_{10}$ CFU/g caecal contents, respectively, p = 0.020) (Table 7).

Table 6. Additive effects of the cellulose-free diet on changes in the population of micro-flora in caecal contents by exposure to cigarette smoke

Organisms	Smoke Control diet (n = 5)	Smoke Cellulose-free diet (n = 4)
Fujiform bacteria	10.1 ± 0.3	10.3 ± 0.2
Bacteroidaceae	9.6 ± 0.5	10.0 ± 0.4
Bifidobacterium	5.3 ± 0.8	6.2 ± 0.5
Lactobacillus	6.9 ± 1.0	7.5 ± 0.4
Enterococcus	6.9 ± 0. 2	7.1 ± 0.4
Enterobacteriaceae	6.5 ± 0.4	5.8 ± 0.9
Staphylococcus	7.0 ± 0.1	6.6 ± 0.4

($\log_{10}$ CFU/g caecal contents)

Each point indicates the mean ± S.D. per one g of caecal contents for 4 or 5 animals.

Table 7. Additive effects of cigarette smoke on changes in the population of micro-flora in caecal contents by the cellulose-free diet

Organisms	Cellulose-free diet Non-Smoke (n = 5)	Cellulose-free diet Smoke (n = 4)
Fujiform bacteria	10.6 ± 1.0	10.3 ± 0.2
Bacteroidaceae	10.0 ± 0.1	10.0 ± 0.4
Bifidobacterium	5.0 ± 0.4	6.2 ± 0.5*
Lactobacillus	7.5 ± 0.4	7.5 ± 0.4
Enterococcus	7.3 ± 0.1	7.1 ± 0.4
Enterobacteriaceae	6.0 ± 0.6	5.8 ± 0.9
Staphylococcus	6.5 ± 0.3	6.6 ± 0.4

($\log_{10}$ CFU/g caecal contents)

Each point indicates the mean ± S.D. per one g of caecal contents for 4 or 5 animals. *$p < 0.05$: significant relative to animals without exposure to cigarette smoke by the Mann-Whitney U test.

DISCUSSION

This study demonstrated that the cellulose-free diet suppressed the anti-oxidant capacity in mice and that the suppression was exacerbated by cigarette smoke. Both these changes in the anti-oxidant capacity were accompanied with changes in the proportion of organic acids in the gut. This study is the first experimental report that supports the results of epidemiological studies about the beneficial effects of dietary fiber among subjects exposed to cigarette smoke. The most impressive finding in this study is that the suppression in the anti-oxidant capacity by feeding with the cellulose-free diet was further decreased by cigarette smoke while in the control diet group the anti-oxidant capacity tended to be increased by cigarette smoke. In smokers the anti-oxidant levels were elevated in serum and the lung. Erythrocytes from smokers contain more glutathione and catalase and protect endothelial cells from hydrogen peroxide (Toth *et al.*, 1986). Ascorbic acid levels in alveolar macrophages from smokers are increased (McGowan *et al.*, 1984). These anti-oxidant levels are suggested to increase to compensate for increased oxidative stresses by cigarette smoke. Cavarra *et al.* (2001) demonstrated that in mice, which

are sensitive to cigarette smoke and develop to emphysema by cigarette smoke, the anti-oxidant activity during exposure to cigarette smoke was decreased to about 70% of that of non-exposed mice while in resistant mice strains the anti-oxidant activity was increased during exposure to cigarette smoke. In patients with COPD the anti-oxidant capacity was decreased to about two-thirds of that of healthy control subjects (MacNee, 2005). In the present study the anti-oxidant capacity in the smoke exposed mice with the cellulose-free diet was decreased to about a half of that in non-exposed mice with control diet. This result demonstrated that dietary fiber especially cellulose is a crucial factor in maintaining the anti-oxidant capacity to reduce oxidative stress due to cigarette smoke and preventing subjects exposed to cigarette smoke from developing COPD.

This study did not fully clarify the mechanism how the anti-oxidant capacity was suppressed by the cellulose-free diet. Dietary fiber contributes to maintain the gut environment. In patients with IBS, alterations of organic acid levels and micro-flora population, and a decrease of the anti-oxidant capacity are reported. The changes in the gut environments supposedly contribute to systemic effects in IBS (Rodriguez-Cabeza *et al.*, 2003). Additionally, dietary fiber is known to contribute to maintaining the gut environment in a normal state. Given this background, we focused on alteration of the gut environment, especially changes in the proportion of caecal organic acids besides the anti-oxidant capacity. This study demonstrated that the cellulose-free diet suppressed the anti-oxidant capacity and the suppressed anti-oxidant capacity was further declined by cigarette smoke while under feeding with control diet cigarette smoke tended to increase the anti-oxidant capacity. Therefore, at first we investigated the independent effects of the cellulose-free diet and cigarette smoke on the organic acid pattern and pH. After these investigations the additive effects of cigarette smoke on the changes by the cellulose-free diet as well as those of the cellulose-free diet on the changes by cigarette smoke were investigated.

At first, in the non-exposed mice the cellulose-free diet significantly decreased propionic acid levels and significantly increased succinic acid levels in caecal contents. Succinic acid is seldom detected under normal fermentation in the large intestine (Morita *et al.*, 1998), because it is a typically intermediate metabolite and is quickly converted to propionate or acetate by acid-utilizing bacteria. However, an abnormal fermentation in the large intestine leads to succinic acid accumulation such as in short bowel syndrome, diarrhea and acute weaning diets (Tsukahara and Ushida, 2002). On the other hand, it has been reported that succinic acid inhibited epithelial cell proliferation of colonic mucosa in rats and had cytotoxic effects on cultured cell lines (Inagaki *et al.*, 2007), and also caused lesions in ligated rabbit ileum loops resembling those of ulcerative colitis (Gaginella *et al.*, 1977). Increased succinic acid levels may contribute to alter the gut environment. Propionic acid is one of the short chain fatty acids and has some effects on the host. Dietary propionic acid decreases cholesterol levels, while enemas consisting of propionic acid, acetic acid and butyric acid improve ulcerative colitis (Patz *et al.*, 1996). However, the role of propionic acid has not been fully elucidated. In this study the suppressed the anti-oxidant capacity by the cellulose-free diet was further declined by cigarette smoke while under feeding with control diet cigarette smoke tended to increase the anti-oxidant capacity. Thinking of these results into changes in organic acids levels, acetic acid levels may be related to changes in the anti-oxidant capacity during the exposure to cigarette smoke, because cigarettes smoke under feeding with control diet increased the acetic acid levels while under feeding with the cellulose-free diet cigarette smoke decreased the acetic acid levels. Acetic acid is primarily utilized by intestinal epithelial cells as energy substrates (Clark *et al.*, 2003; Goto *et al.*, 2005; Oba *et al.*, 2004) and contributes to maintaining the gut environment.

The anti-oxidant capacity in plasma significantly inversely correlated to the ratio of succinic acid levels to acetic acid levels in the caecal contents as shown in Fig. 3. Changes in the gut environment especially proportion of organic acids may contribute to changes in the anti-oxidant capacity. To clarify the effect of proportion of organic acids on the anti-oxidant capacity further investigations are needed.

Another interesting result about the additive effects of cigarette smoke on the changes by cellulose free diet was an elevation in pH level in the caecal contents. The elevation in pH may be linked to a decrease in acetic acid levels (Shimizu *et al.*, 2006). The pH in the colon of patients with colonic cancer is more alkaline, indicating a reduction in colonic carbohydrate fermentation by organic acids (Fallingborg, 1999). Cigarette smoke promoted growth of colon cancer in a mouse model (Wong *et al.*, 2009). The elevation in pH in the colon and the decreases in certain organic acids levels by cigarette smoke during feeding with the the cellulose-free diet may be related to the development and growth of colon cancer.

In this study the cellulose-free diet further reduced the decrease in body weight gain by cigarette smoke. In smoke exposed mice cellulose-free diet significantly decreased the acetice acid levels in ceacal contents.

Acetic acid is absorbed into hepato-portal flow to the liver and utilized as systemic energy sources. It has been reported that about 10% of systemic energy sources are obtained from organic acids in humans. In this study, cigarette smoke exposure deceased body weight gain. This effect of the cellulose-free diet on body weight gain during exposure to cigarette smoke may be related to a decrease in acetic acid levels in the caecum.

Although the present study did not elucidate how organic acid balance was changed, food contents, gut movement, population of micro-flora and fermentation of micro-flora *et al.* may contribute to changes in organic acid levels in the gut. In the present study we evaluated micro-flora in the caecal contents. The cellulose-free diet decreased propionic acid levels and increased succinic acid levels while the cellulose-free diet decreased the population of *Bifidobacterium* which produces mainly acetic acid, but the population of Bacteroidaceae, which mainly produces succinic acid, proved to be unchanged. Cigarette smoke increased acetic acid levels while cigarette smoke did not significantly change the population of the micro-flora. On exposed to cigarette smoke the cellulose-free diet decreased acetic acid levels and increased isovaleric acid levels while it did not significantly change the population of the micro-flora. With the cellulose-free diet cigarette smoke decreased acetic acid levels, while cigarette smoke increased the population of *Bifodobacterium*. Therefore, the changes in the organic acid levels in the present study were not able to be explained only by changes in the population of micro-flora. Other factors besides the changes in the population micro-flora in the gut, may contribute to connect with organic acid levels in caecal contents. Further investigations about the affects of the cellulose-free diet and cigarette smoke on fermentation of organic acids by micro-flora in the gut are needed to gain further insights into the changes organic acid levels in caecal contents by the cellulose-free diet and exposure to cigarette smoke.

One of the limitations of the present study is the small size of the animal groups. However, the anti-oxidant capacity remarkably decreased with statistical significance (390.8 ± 38.7 vs. 209.8 ± 31.2 μmol/ml, respectively, $p = 0.0143$). We therefore feel that the sample size of this study, although limited, is enough to provide the insight into the change in the anti-oxidant capacity by the cellulose-free diet and cigarette smoke.

In conclusion, the cellulose-free diet suppressed the anti-oxidant capacity in mice and that the suppression was further exacerbated by cigarette smoke. Both these changes in the anti-oxidant capacity were accompanied with changes in proportion of organic acids in the gut. The changes in the anti-oxidant capacity by the cellulose-free diet and cigarette smoke may be related with changes in the gut environment.

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Case Report

Autopsy case of primary myelofibrosis in which myeloid sarcoma was the initial manifestation of tumor progression

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Myeloid sarcoma (MyS) is defined as an extramedullary tumor-forming neoplasm consisting of immature myeloid cells with/without maturation. We experienced a case involving a 68-year-old Japanese male patient who had been followed-up for four years with a diagnosis of chronic idiopathic myelofibrosis/primary myelofibrosis (PMF) and noticed a painful mass in his left axilla. A wedge biopsy characterized the lesion as MyS that displayed megakaryoblastic/megakaryocytic differentiation. As his complete blood count included a few myeloid blasts (1% of WBC) and a bone marrow biopsy detected fibrosis without evidence of acute myelogenous leukemia (AML), a diagnosis of extramedullary blastic transformation of PMF was made, which was confirmed later by V617F mutation in *Janus kinase*-2 in both initial bone marrow biopsy and axillary tumor biopsy specimens. The patient died of pneumonia eight months after developing the axillary tumor. At autopsy, multiple MyS masses were detected in his soft tissue, but his bone marrow only contained fibrosis. Although MyS rarely develops before the leukemic transformation of PMF, no evidence of AML could be found in the patient's bone marrow at any point during the course of his disease. Thus, it is possible that the blasts in his peripheral blood were derived from the remaining MyS. Furthermore, the present case indicates that extramedullary blastic transformation, which is occasionally seen in CML, can also occur in PMF. Therefore, it is important to recognize that there is a wide variation in the pathogeneses of MyS and PMF.

Key words: autopsy, megakaryocytic differentiation, myeloid sarcoma, primary myelofibrosis

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Myeloid sarcoma (MyS), which was formerly referred to as chloroma, granulocytic sarcoma, or extramedullary myeloid tumor, is defined as an extramedullary tumor-forming neoplasm consisting of immature myeloid cells with/without maturation.[1] MyS can be hematopathologically subclassified into either granulocytic sarcoma, monoblastic sarcoma, megakaryoblastic sarcoma, or rarely, erythroblastic sarcoma, based on the predominant proliferative component. Myeloid sarcoma can occur as the first sign of a blast crisis in chronic myelogenous leukemia (CML) and is included in the diagnostic criteria for blast crisis in CML. However, a similar pathologic condition can also occur in myeloproliferative neoplasms (MPN)/chronic myeloproliferative disorders other than CML.

The recent discovery of a recurrent V617F mutation in *Janus kinase*-2 (*JAK*-2)[2] has brought about considerable progress in the understanding of the pathogenesis of MPN other than CML, and it is present in most of patients with polycythemia vera and in approximately a half of patients with essential thrombocythemia or primary myelofibrosis (PMF)/chronic idiopathic myelofibrosis.[2–4] Furthermore, the discovery has improved the diagnostic accuracy, because this point mutation could be detected using DNA samples from paraffin-embedded pathologic specimens.[5]

Recently, we experienced a case of MyS that developed during the course of PMF. Polymerase chain reaction (PCR) amplification could show *JAK*-2 mutation in both a bone marrow biopsy specimen obtained at the time of PMF and a tissue containing MyS. In the present report, we describe the hematopathological features of the case together with a review of the literature. The purpose of this report is to indicate that blast crises could develop not only in CML, but also in PMF.

CASE REPORT

A 64-year-old Japanese man was first admitted to our hospital because of general malaise. On physical examination,

Table 1 Peripheral blood findings

	Initial presentation	Tumor formation†	Blastic crisis	Death
Clinical course (month)	0	39	46	48
RBC (x10⁴/μL)	399	321	270	224
Hb (g/dL)	12.0	8.6	8.3	6.3
WBC (/μL)	5400	4600	6100	9800
Neutrophil (%)	73.0	72.0	75.0	6.0
Lymphocyte (%)	24.0	17.0	2.0	1.0
Blast (%)	0.0	1.0	21.0	91.0
Platelet (x10⁴/μL)	19.6	17.4	13.8	1.7
BM finding	PMF	PMF	ND	PMF

†At the time of tumor formation
ND, not done; PMF, primary myelofibrosis.

his spleen and liver were impalpable, and no peripheral lymphadenopathy was detected. His complete blood count (CBC) revealed a borderline degree of anemia (hemoglobin concentration: 12.0 g/L; red blood cell (RBC) count: 3.99 × 10^12/L), but his platelet and white blood cell (WBC) counts were within normal limits and no abnormal cells were detected on peripheral blood (PB) films. As bone marrow aspiration was unsuccessful despite several attempts, no chromosomal analysis was performed. Bone marrow biopsy revealed the proliferation of abnormal megakaryocytes with severe reticulin fibrosis (Fig. 1). The patient was diagnosed as having chronic idiopathic myelofibrosis based on the criteria given by the WHO classification-2001[6] at that time.

Four years later (at the age of 68 years), a painful mass was noticed in his left axilla. The lesion was histologically characterized as MyS with megakaryoblastic/megakaryocytic differentiation by a wedge biopsy (Fig. 2). At this time, his CBC included a hemoglobin level of 8.6 g/L, an RBC count of 3.21 × 10^12/L, and a WBC count of 4.6 × 10^9/L with 1% myeloid blasts. A bone marrow biopsy revealed fibrosis without evidence of acute myelogenous leukemia (AML). However, an increased number of myeloid blasts were noted in his PB, and they accounted for 78% of WBC at one month after the biopsy of the axillary tumor. Combination chemotherapy consisting of cytosine arabinoside (10 mg/m^2) and aclarubicin (14 mg/m^2), and local radiotherapy, resulted in reductions in the size of the tumor and the number of myeloid blasts in his PB. However, the tumor enlarged rapidly and the number of myeloid blasts in his PB had increased to 78% of WBC within a month (Table 1). The patient died of pneumonia at 8 months after developing the axillary tumor, and an autopsy was performed.

HEMATOPATHOLOGICAL FEATURES

The first bone marrow biopsy

The marrow space was diffusely occupied by fibrous tissue, which was positively stained by silver reticulin stain. The number of normal hematopoietic cells was decreased, but clusters of megakaryoblastoid cells were noted. The specimen was reviewed at the time of biopsy of the left axillary tumor and fibrosis was graded as either 1 or 2.[7]

Biopsy of the left axillary tumor

Dense diffuse growth of large mononuclear cells was noted among the patient's subcutaneous adipose tissue and striated muscle, and the lesion was associated with diffuse fibrosis. The proliferating cells had prominent single nuclei and relatively abundant amphophilic cytoplasm. The number of cells in mitosis was increased, and numerous apoptotic bodies were detected. In addition, giant cells resembling megakaryocytes were occasionally seen.

These cells were immunohistochemically examined using the avidin-biotin-peroxidase complex method and antibodies against cytoplasmic CD3 (cCD3; Dako Japan, Tokyo, Japan), CD10 (Leica Microsystems, Tokyo, Japan), CD20 (Dako Japan), CD34 (Dako Japan), CD42b (Nihon Millipore, Tokyo, Japan), CD43 (Dako Japan), CD68 (Leica Microsystems), CD79a (Dako Japan), α1-antichimotrypsin (AACT; Dako Japan), myeloperoxidase (MPO; Dako Japan), von Willebrand factor (vWF; Dako Japan), and glycophorin A (Dako Japan). As a result, we found that the majority of proliferating cells were positive for CD34 and CD43, and some of them were positive for AACT. The giant cells were positive for CD42b, but the proliferating cells were negative for CD3, CD10, CD20, CD68, CD79a, MPO, vWF, and glycophorin A. Based on these findings, the tumor was diagnosed as MyS with megakaryoblastic/megakaryocytic differentiation.

The second bone marrow biopsy

This was performed at the time of the biopsy of the axillary tumor. The histopathologic features of the biopsy sample were similar to those of the first biopsy, although an immunohistochemical examination revealed the occasional

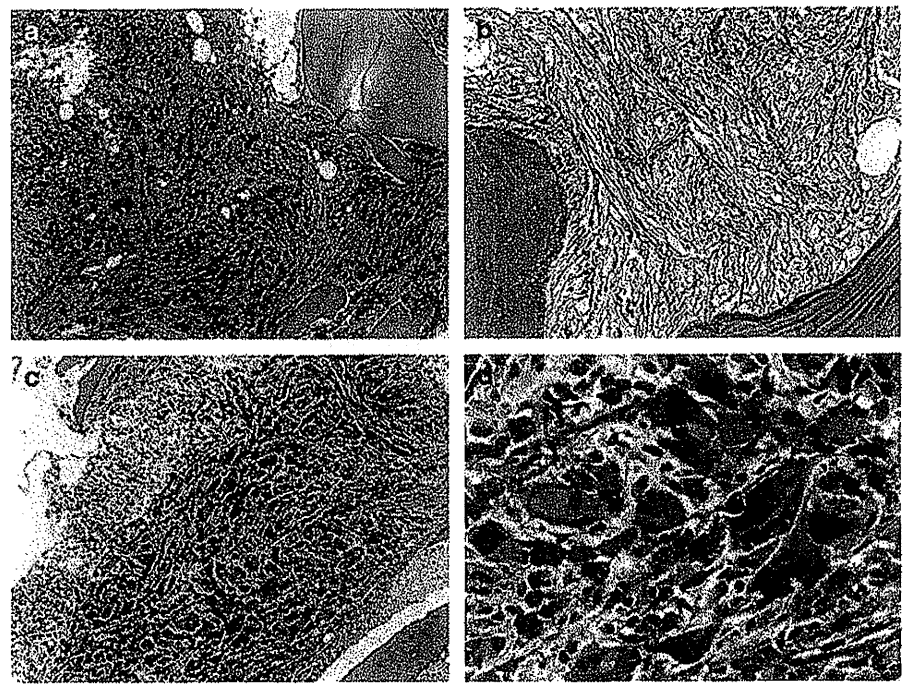

Figure 1 Bone marrow features at the first admission (**a, b**) and autopsy (**c, d**). The marrow space is subtotally occupied by cellular and fibrous growth without features of acute myeloid leukemia (AML). (a, H&E, x10). Prominent reticulin fibrosis is evident (b, silver reticulin stain, x10). At autopsy, proliferation of giant cells with abundant eosinophilic cytoplasm is noted in the fibrous background (c, H&E, x10). (d) The majority of the proliferating cells are characterized as megakaryocytes with atypical nuclei, but no features of conventional AML are present (H&E, x40).

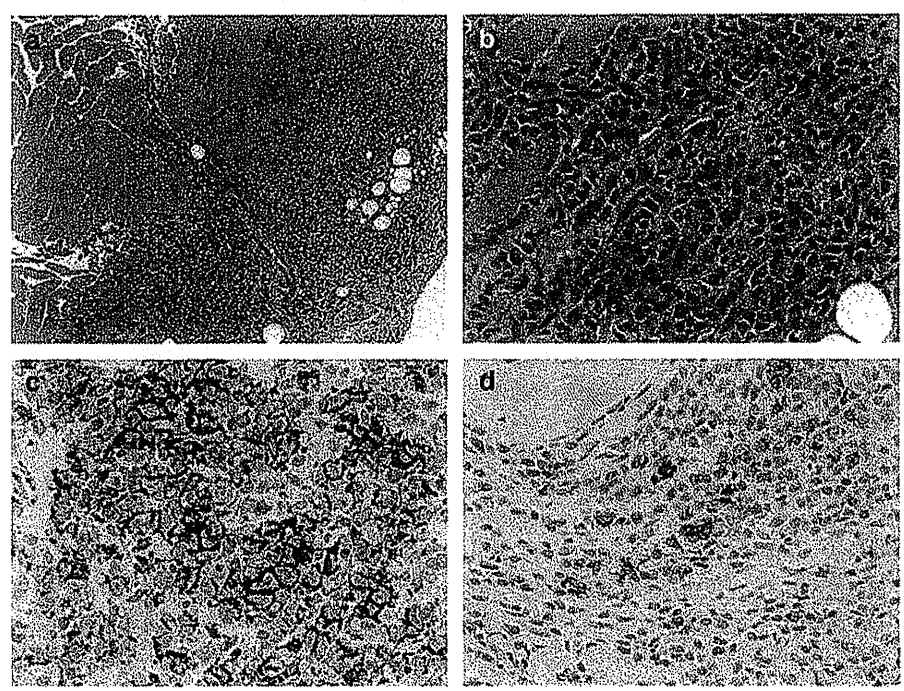

Figure 2 Histologic and immunohistochemical features of the left axillary tumor. Diffuse proliferation of mononuclear cells infiltrating into the striated muscle tissue is seen (**a**, H&E, x10). At the higher magnification, single-file pattern of growth of the cells is seen (**b**, H&E, x40). Immunohistochemically, the cells are positive for CD43 (**c**) and CD42b (**d**) (c and d, immunoperoxidase stain with hematoxylin counterstain; c ×40, d ×40).

presence of large cells that were positive for CD42b and von Willebrand factor.

Detection of the *JAK2* V617F mutation

Presence or absence of *JAK2* mutation was examined using DNA samples prepared form the paraffin embedded tissues of the first bone marrow biopsy and axillary tumor biopsy. Polymerase chain reaction (PCR) amplification was performed using the JAK2 MutaScreen™ Kit (IPSOGEN, Marseille, France) and amplicons were analyzed. Consequently, PCR amplification could show *JAK*-2 mutation in both samples (Fig. 3).

Autopsy

Multiple tissue samples from the vertebrae showed broad reticulin fibrosis with occasional collagenous fibrosis, which

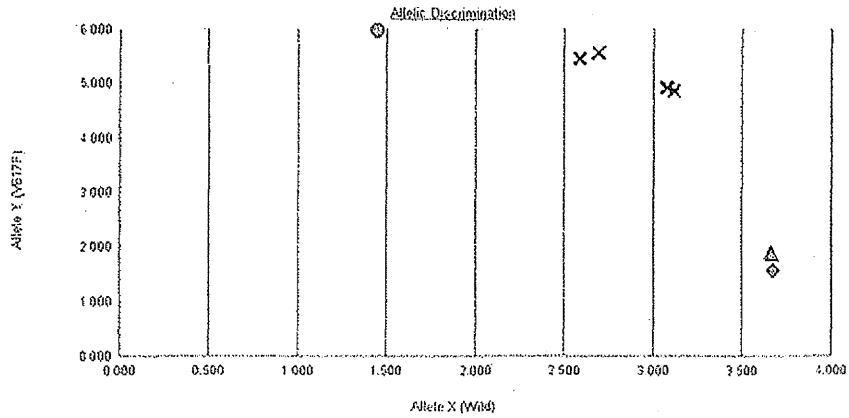

Figure 3 Scatter plot of the PCR results by JAK2 Muta*Screen*™ Kit. Fluorescent values of wild type DNA are plotted on the *x* axis, while those of DNA containing V617F on the *y* axis. Fluorescent values of both DNA samples (the initial bone marrow biopsy and axillary tumor biopsy) in duplicate are located in the upper light, indicating that one of the alleles in both DNA samples contains V617F.

Table 2 Reported cases of myeloid sarcoma developed before leukemic transformation

Reference number	Sex	Age(year)	Location of tumor	Period†	Diagnosis
8	F	22	Submandibular region	Two month	Granulocytic sarcoma
9	M	49	Duodenum	ND	Granulocytic sarcoma
10	M	56	Right inguinal (soft tissue)	Three month	Megakaryoblastic sarcoma
11	F	43	Inguinal lymph node	ND	Megakaryoblastic sarcoma
12	F	54	Face (soft tissue)	ND	Granulocytic sarcoma
This case	M	64	Left axilla	Seven month	Megakaryoblastic sarcoma

†A period from myeloid sarcoma to development of leukemic transformation.
ND, not described.

were confirmed by the corresponding histochemical staining examinations. Although a small number of normal hematopoietic cells including megakaryocytes remained, no foci that were indicative of blastic transformation were detected. These features were compatible with grade 2 PMF and were confirmed to be similar to those of the first and second bone marrow biopsies. No solid tumor was found in the left axillary region or at any other site. His spleen was enlarged (414 g) and displayed diffuse blastic cell proliferation in the red pulp, resulting in atrophy of the white pulp. The proliferation and/or infiltration of blastic cells without tumor formation were also seen in the esophagus, right kidney, urinary bladder, testes, and right lung (a tumor thrombus but not parenchymatous involvement).

DISCUSSION

In this case, a left axillary tumor appeared during the course of PMF, and biopsy revealed the diffuse infiltration of blastic cells with occasional cells having megakaryoblastic/megakaryocytic features. Although the lesion could be characterized as one of extramedullary tumor-forming AML or myeloid sarcoma with megakaryoblastic/megakaryocytic differentiation without other information, the lesion could be characterized as extramedullary blastic transformation of PMF, because of a history of PMF, the presence of only a few blasts in the peripheral blood and histologic features of the bone marrow (compatible with chronic phase PMF) at the time of the development of the axillary tumor, and the presence of *JAK2* mutation in both the initial bone marrow specimen and the axillary tumor. Although this interpretation seemed to be confirmed by the rapid increase in the number of blasts in his peripheral blood following the tumor biopsy, autopsy did not detect blastic transformation in the bone marrow. Thus, it is reasonable to consider that the blasts in his peripheral blood at the time of the rapid increase in their number were derived from the remaining axillary tumor, not from the bone marrow, although only vertebral bone marrow could be examined at autopsy.

Five cases of PMF in which MyS developed before leukemic transformation have been reported in the literature,[8–12]

and two of them were characterized as megakaryoblastic sarcoma[10,11] (Table 2). Leukemic transformation occurs in up to 50% of PMF,[7,13,14] but megakaryoblastic transformation is rare.[11] In our case, megakaryoblastic/megakaryocytic differentiation was only found in some parts of the tumor, while the majority of the cells remaining showed the morphological and immunohistochemical features of blasts without any phenotypically identifiable commitment to differentiation. Based on these findings, our case could be interpreted as blastic MyS with partial megakaryoblastic/megakaryocytic differentiation rather than megakaryoblastic sarcoma. Among the 92 cases of MyS reported by Pileri *et al.*,[15] 46 (50%) were characterized as belonging to the blastoid subtype, and only one case showed megakaryoblastic/megakaryocytic differentiation. Of these 92 cases, PMF had been diagnosed before the development of MyS in three cases and PMF was found at the time of MyS development in one case. On the other hand, 25 cases of MyS were not associated with any other myeloproliferative disorder, but two of these patients later developed AML. Among the 61 cases of PMF reported by Richard *et al.*,[16] three patients (5%) later developed MyS. Thus, it is indicated that both MyS with megakaryoblastic/megakaryocytic differentiation and the development of MyS during the course of PMF are rare phenomena.

The development of MyS before the leukemic transformation of PMF is a rare phenomenon, but it has been indicated that such MyS are more likely to be misdiagnosed, e.g., as malignant lymphoma or poorly differentiated sarcoma.[15] Furthermore, the axillary tumor itself could be diagnosis as MyS with megakaryoblastic/megakaryocytic differentiation, but presence of PMF history and common genetic abnormality, *i.e.*, *JAK2* V617F mutation, suggest that extramedullary blastic transformation, which is occasionally seen in CML, can also occur in PMF. Therefore, we conclude that understanding the variations in the pathogeneses of PMF and MyS is important.

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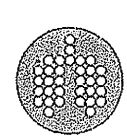

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Reduced larger von Willebrand factor multimers at dawn in OSA plasmas reflect severity of apnoeic episodes

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ABSTRACT: Plasma von Willebrand factor (VWF), produced in and released from vascular endothelial cells by various stimuli including hypoxia, induces platelet aggregation under high shear stress and plays dual pivotal roles in haemostasis and thrombosis within arterioles, which are regulated by the size of vWF multimers (VWFMs).

Patients with obstructive sleep apnoea (OSA) have increased risk of thrombotic cardiovascular events, but the pathogenesis is unclear. We examined the relationship between VWF and OSA by measuring VWF antigen (VWF:Ag), VWFMs, VWF collagen binding activity (VWF:CB) and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13. A total of 58 OSA patients were enrolled. Blood samples were collected before sleep, after sleep, and after one night of nasal continuous positive airway pressure therapy.

Based on VWFM analysis, OSA patients were classified into three groups; consistently normal VWFMs (group 1, n=29), increased high molecular weight (HMW)-VWFMs at 06:00 h (group 2, n=18), and decreased or absent HMW-VWFMs at 06:00 h (group 3, n=11). Patients in group 3 had significantly worse apnoea/hypopnoea index; VWF:CB followed a similar pattern. We observed a significant decrease in platelet count between 21:00 h and 06:00 h in OSA patients, potentially associated with reduced larger VWFMs together with decreased VWF:Ag levels. Severe OSA may contribute to an arterial pro-thrombotic state.

KEYWORDS: ADAMTS13, obstructive sleep apnoea, von Willebrand factor

Obstructive sleep apnoea (OSA) is characterised by the collapse of the upper airway and associated intermittent hypoxia during sleep [1]. OSA is associated with excessive daytime sleepiness and cardiovascular disease. Patients with OSA often suffer from obesity, hypertension, hyperlipidaemia, and impaired glucose tolerance, and OSA is an independent risk factor for cardiovascular diseases [2–4]. Consistent with this, cardiovascular risk returned to baseline in OSA patients treated with nasal continuous positive airway pressure (CPAP), whereas those with severe untreated OSA maintained a high risk [5]. Recently, some association of OSA with venous thromboembolism in regard to pulmonary embolism has been implicated [6, 7]. However, the mechanism of OSA-associated thrombosis might be multifactorial, and in fact has not been evaluated on a basis of arterial thrombosis, which is generated under high shear stress in microvasculatures, where von Willebrand factor (VWF) plays a critical role as a molecular glue that facilitates platelet aggregation or thrombi.

VWF is a macromolecular plasma protein, which is exclusively produced in and released from vascular endothelial cells, and exerts pivotal effects on both haemostasis and thrombosis. VWF assembles into unusually large VWF multimers (UL-VWFMs) consisting of identical 250 kDa subunits, before its release into the circulation. Under normal circumstances, UL-VWFMs are rapidly cleaved by a specific plasma protease, ADAMTS13 (a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13), under the high shear stress generated in the microvasculature; consequently, VWF circulates in the plasma as a heterogeneous family of multimers ranging in size from 500 to 15,000 kDa. UL-VWFMs play an essential role in primary haemostasis by binding platelets to denuded vascular endothelial tissue. However, in the absence of ADAMTS13 activity (ADAMTS13:AC) due to gene mutation or acquired autoantibodies, UL-VWFMs remain uncleaved and generate platelet hyperaggregation. Uncleaved UL-VWFMs lead to the formation of vast platelet thrombi, known as thrombotic

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TABLE 1 Characteristics of patients with obstructive sleep apnoea (OSA) and sleep controls

	OSA	Sleep controls	p-value
Sex n (M/F)	58 (55/3)	25 (22/3)	NS
Blood type			NS
A	18	12	
B	9	2	
O	26	8	
AB	6	3	
Age yrs	44.7±9.9	38.3±7.1	<0.01
BMI kg·m^{-2}	28.2±3.7	27.7±3.0	NS
AHI	50.5±22.2	4.5±2.8	<0.01
ODI3%	41.6±19.9	7.8±5.1	<0.01
Lowest Sp,O_2 %	76.0±10.0	88.8±5.0	<0.01
Systolic blood pressure mmHg	129±16	122±28	NS
Diastolic blood pressure mmHg	82±12	81±10	NS
vWF:Ag levels % at 06:00 h	103.1±61.4	143.5±63.8	<0.01
ADAMTS13:AC levels % at 06:00 h	56.8±22.6	61.7±20.6	NS

Data are presented as mean±SD, unless otherwise stated. M: males; F: females; BMI: body mass index; AHI: apnoea/hypopnoea index; ODI3%: oxygen desaturation index ≥3%; Sp,O_2: arterial oxygen saturation measured by pulse oximetry; vWF:Ag: von Willebrand factor antigen; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospondin type 1 motifs 13 activity; NS: not significant.

thrombocytopenic purpura, a life-threatening generalised disease [8–11].

It is now well established that high plasma levels of VWF antigen (VWF:Ag) are linked with an increased risk for ischaemic heart disease and ischaemic stroke [12–14]. Furthermore, the relative risks of stroke and acute myocardial infarction are higher in individuals with lower ADAMTS13:AC [14, 15]. Furthermore, hypoxia leads to increased VWF release from cultured vascular endothelial cells, both directly, by up regulating VWF expression, and indirectly *via* autocrine and paracrine signalling downstream of hypoxia-induced inflammatory cytokines including interleukin (IL)-6, IL-8, and tumour necrosis factor-α [16, 17]. Despite these important reports of hypoxia-induced VWF secretion, no subsequent studies have addressed the relationship between VWF and the severity of OSA [18, 19]. In particular, no studies have been performed on plasma samples obtained in chronological order relevant to the sleep cycle.

In this study, we sequentially analysed plasma VWF:Ag levels, VWFM patterns, and ADAMTS13:AC in OSA patients not only before and after sleep, but also before and after CPAP treatment. We found that the reduced larger VWFMs together with decreased VWF:Ag levels in the plasma of OSA patients taken at dawn correlate with the clinical severity of apnoeic episodes.

PATIENTS, MATERIALS AND METHODS

Patients

Between February 2004 and April 2011, 284 patients received full standard diagnostic polysomnography (PSG) at Nara Medical University Hospital (Nara, Japan). Among them, 86 patients were diagnosed with normal or mild OSA (apnoea/

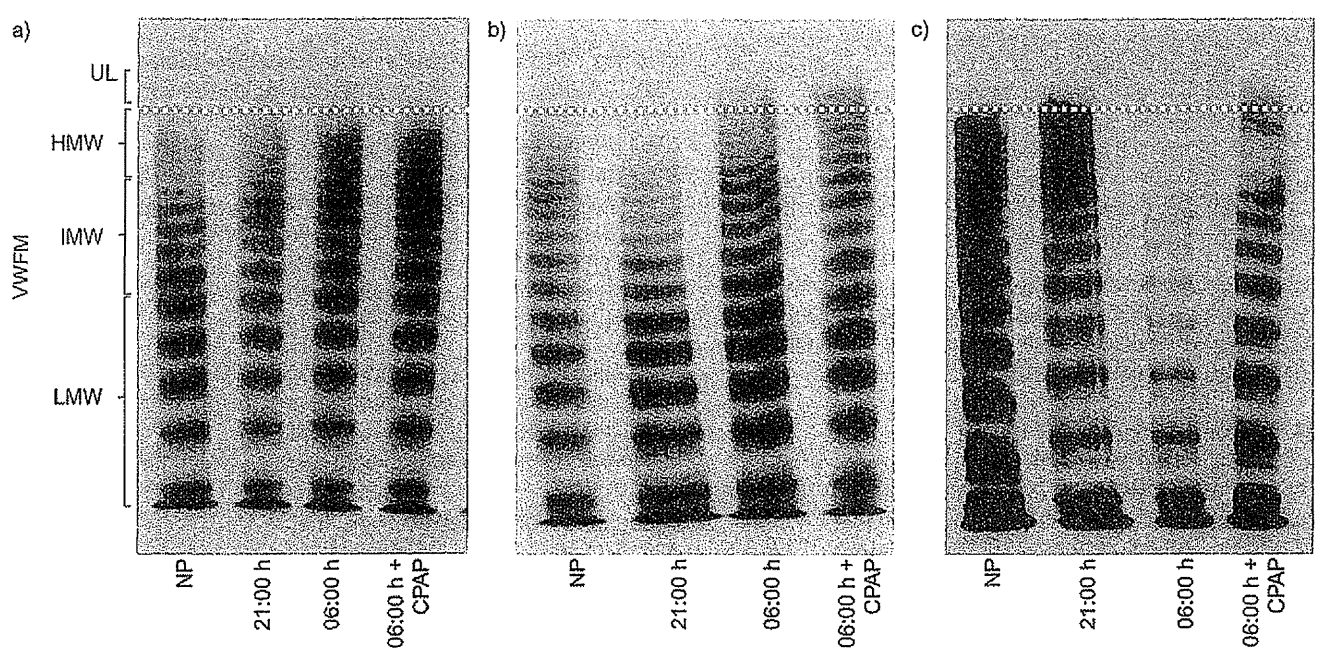

FIGURE 1. Patterns of von Willebrand factor multimers (VWFMs) corresponding to three patient groups. Obstructive sleep apnoea (OSA) patients were categorised into three groups based on the results of VWFM analysis, using sequential samples. Representative results from each group are shown. a) Group 1, patients (n=29) showed a consistently normal pattern of VWFMs. b) Group 2, patients (n=18) had increased, unusually large (UL)- and high molecular weight (HMW)-VWFMs at 06:00 h compared to 21:00 h. c) Group 3,patients (n=11) had decreased UL- and HMW-VWFM at 06:00 h compared to 21:00 h.

hypopnoea index (AHI) <15), and 198 patients were diagnosed with moderate or severe OSA (AHI ⩾15) and received nasal CPAP therapy. Within the latter group, 140 patients with the following underlying diseases were excluded: stroke, coronary artery disease, asthma, chronic obstructive pulmonary disease, arthritis, autoimmune disease, rhinitis, and malignant diseases. The 58 remaining OSA patients were enrolled in this study; detailed clinical information for these 58 patients is shown in table S1. Written informed consent was obtained from all patients, and the study was approved by the Human Subjects Ethics Committee of Nara Medical University (No. 04-012). 25 healthy volunteers (88% male), as shown in table 1, that had undergone PSG studies without OSA were also enrolled and used as the sleep controls.

Blood sampling

Plasma samples were collected from OSA patients at three time points throughout the day; 21:00 h before PSG, at 06:00 h after the PSG without CPAP, and at 06:00 h after CPAP treatment. For the sleep control subjects, plasma samples were collected at 06:00 h. Blood was collected in plastic tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing a tenth volume of 3.8% trisodium citrate an anticoagulant, and platelet-poor plasma was prepared by centrifugation at 3,000 × g for 15 min at 4°C. Aliquots were stored at -80°C prior to use. To obtain platelet counts, blood was collected into plastic whole blood tubes with spray-coated EDTA (Becton, Dickinson and Co.) tubes containing EDTA as an anticoagulant and analysed with a Coulter counter (Beckman Coulter, Tokyo, Japan).

Sleep study

PSG was performed using a computerised polysomnography system (Alice 4; Respironics, Pittsburgh, PA, USA). Data acquisition began at 21:00 h and continued until 06:00 h the following day. Apnoea was defined as a cessation of airflow for ⩾10 s, and hypopnoea was defined as a decrease in airflow at least 50% for a minimum of 10 s or a clear decrease in airflow (⩾20%) followed by either oxygen desaturation ⩾3% or signs of physiological arousal. The AHI was calculated as the number of apnoea/hypopnoea events per hour of total sleeping time. We also calculated the oxygen desaturation index ⩾3% (ODI3%), defined as the number of ⩾3% dips in oxygen saturation per hour of sleep.

During the night, following diagnostic PSG, patients were treated with nasal CPAP (REMstar Auto; Respironics), with PSG monitoring. Apnoeic episodes were substantially reduced or eliminated during treatment with nasal CPAP.

Analyses of VWF:Ag, VWF, and VWF:CB

Plasma VWF:Ag levels were measured by sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (DAKO, Glostrup, Denmark) [20]. The VWF:Ag level contained in 1 mL of pooled normal human plasma was defined as 100%; VWF:Ag levels in the 20 healthy controls were 102±33% (mean±SD) [21].

VWFMs were analysed by sodium dodecyl sulphate-1.2% agarose gel electrophoresis followed by Western blotting with luminographic detection [22, 23]. The blots were scanned and subjected to densitometric analysis using ImageJ (National Institutes of Health (NIH), Bethesda, MD, USA). Multimers were classified as low molecular weight (LMW-VWFMs; corresponding to bands 1-5 in VWFM analysis), intermediate molecular weight (IMW-VWFMs; bands 6-10), and high molecular weight (HMW-VWFMs; bands ⩾11) [24]. High molecular weight bands that were not detected in normal plasma (NP) were defined as UL-VWFMs. The levels of LMW-, IMW- and HMW-VWFMs were calculated using NIH ImageJ. For quantitative analyses, we calculated the ratios of the densities of VWFMs, LMW, IMW, and HMW relative to total VWFM density. Further, multimeric VWF:Ag levels were calculated by multiplying VWF:Ag level by the LMW, IMW, and HMW ratios.

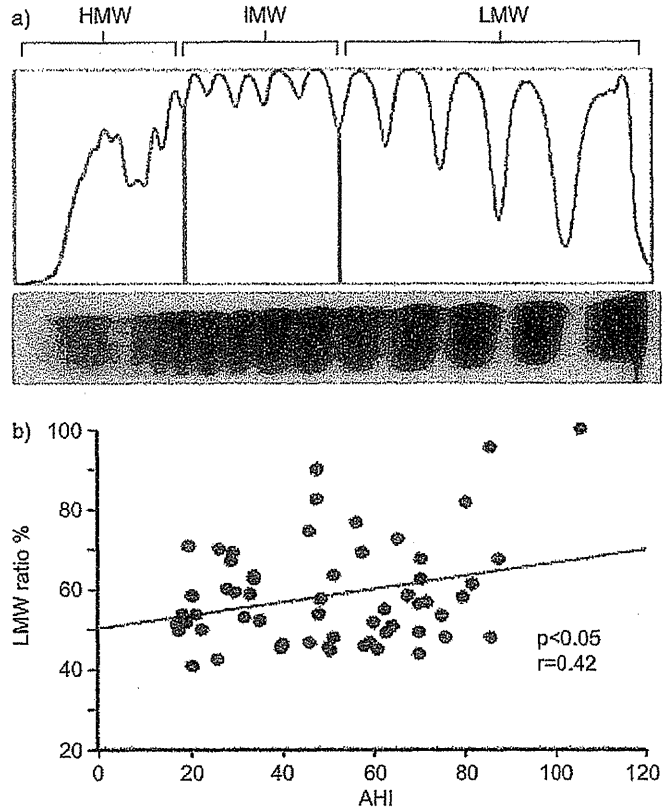

FIGURE 2. Relationship between low molecular weight (LMW) von Willebrand factor multimers (VWFMs) to total VWFMs (LMW ratio) and hypoxia. a) Quantitative analysis of VWFMs was performed by calculating the density of LMW-VWFMs relative to total M density. A representative result of VWF analysis at 06:00 h is shown. b) The LMW ratio of obstructive sleep apnoea patients was significantly correlated to apnoea/hypopnea index (AHI). IMW: intermediate molecular weight.

The plasma VWF collagen binding activity (VWF:CB) was measured using an enzyme immunoassay using a commercially available kit (VWF-CBA ELISA, PROGEN Biotechnik GmbH, Heidelberg, Germany) according to the manufacturer's instructions.

Assay of ADAMTS13:AC

ADAMTS13:AC was determined using a commercially available chromogenic ELISA/ACT (Kainos Co., Tokyo, Japan). The detection limit of this assay was 0.5%; the values obtained from 55 healthy controls were 99.1±21.5% (mean±SD) [25].

Statistical analysis

Laboratory data are expressed as the mean±SD. Comparisons between OSA patients and controls were analysed using the

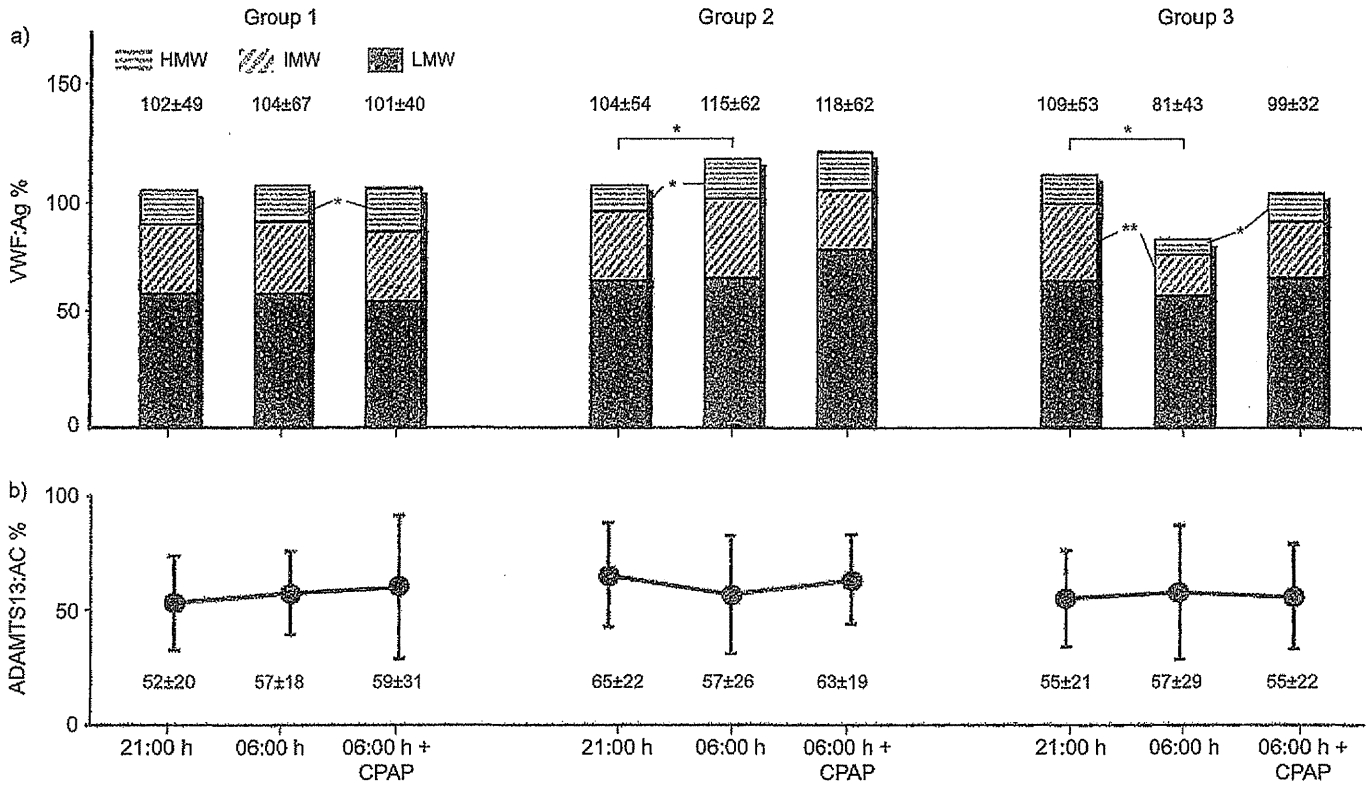

FIGURE 3. Changes in serial von Willebrand factor antigen (VWF:Ag) levels and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity (ADAMTS13:AC) in groups 1–3. VWF:Ag levels were divided into high molecular weight (HMW)-, intermediate molecular weight (IMW)-, and low molecular weight (LMW)-VWFM groups by multiplying the VWF:Ag level by the results of the multimeric analyses. Data are presented as mean±SD . Groups were first compared using the Kruskal-Wallis H-test; significantly different groups were then analysed using the Mann-Whitney U-test. *: $p<0.05$; **: $p<0.01$.

Mann-Whitney U-test or Chi-square test. All comparisons among the three groups were tested for statistical significance using the Kruskal-Wallis H-test or Chi-square test, with Yates' correction for 2×3 tables; significant differences between the three groups (overall $p<0.05$) were further analysed using the Mann-Whitney U-test or Chi-square test. All analyses were carried out using StatView (SAS Institute Inc., Cary, NC, USA). A p-value <0.05 was considered significant.

RESULTS

Characteristics of patients with OSA and controls

The demographics and sleep characteristics of patients with OSA and controls are shown in table 1. Patients with OSA were slightly older than the control population but were otherwise similar demographically. 18, seven, and four patients in the OSA group were being treated for hypertension, hyperlipidaemia, and diabetes mellitus, respectively, but no diabetic patients were receiving insulin therapy. Based on the PSG results, the two populations differed significantly with respect to AHI, ODI3%, and lowest S_{p,O_2} %.

Plasma VWF:Ag levels at 06:00 h were significantly lower in patients with OSA compared with the controls, but plasma ADAMTS13:AC at 06:00 h did not differ between these groups. Interestingly, the plasma ADAMTS13:AC at 06:00 h in both OSA patients and sleep controls were lower than those of the above mentioned healthy controls ($p<0.01$).

Chronological changes of plasma VWFM patterns categorise the patients with OSA into three groups

We analysed VWFM patterns in plasmas taken from OSA patients, obtained at 21:00 h and 06:00 h following sleep with or without CPAP. Based on these results, we categorised the patients with OSA into three groups (fig. 1). Patients in group 1 (n=29) had a consistently normal pattern of VWFM, almost indistinguishable from that of the sleep controls (n=6). Patients in group 2 (n=18) exhibited reduced HMW-VWFMs at 21:00 h and persistent UL-VWFMs at 06:00 h, with or without CPAP. Patients in group 3 (n=11) had normal VWFM patterns at 21:00 h, reduced predominantly HMW-VWFMs at 06:00 h without CPAP, and returned to a normal VWFM pattern after CPAP therapy.

The decrease in HMW-VWFMs and concomitant increase in LMW-VWFMs could reflect either enhanced proteolysis by ADAMTS13 or extensive consumption secondary to platelet aggregation. Therefore, we first calculated the ratio of LMW-VWFMs to total VWFMs (LMW ratio) at 06:00 h without CPAP (fig. 2), and subsequently determined the relationship between LMW ratio and AHI. As shown in figure 2, these two parameters are significantly correlated ($p<0.05$), suggesting that the

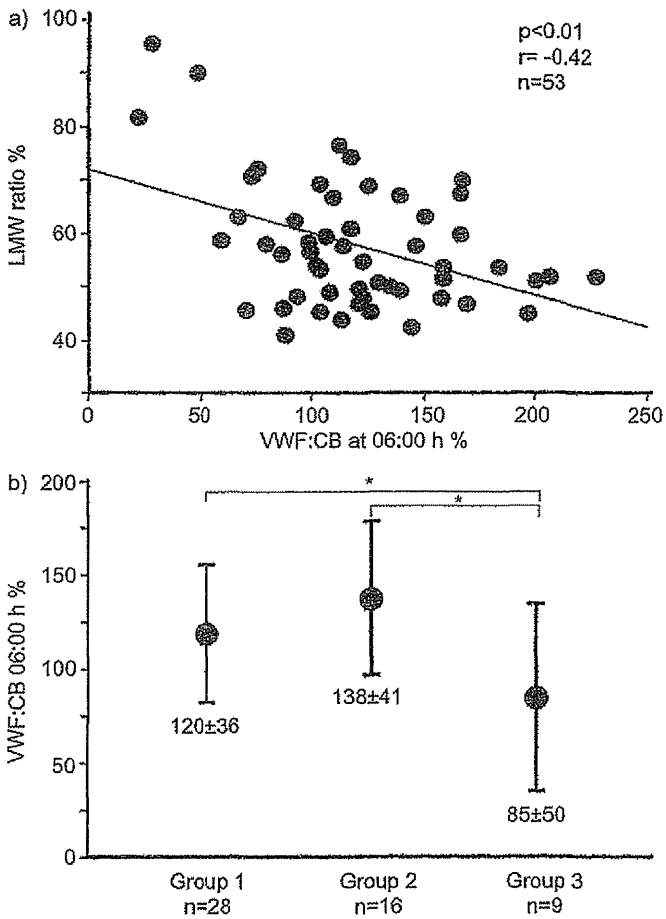

FIGURE 4. Relationship von Willebrand factor (VWF) collagen binding activity (VWF:CB) and ratio of low molecular weight (LMW)-VWF multimers (Ms) to total VWFMs (LMW ratio) and comparison of vWF:CB at 06:00 h in each group. vWF:CB was measured in 53 out of 58 obstructive sleep apnoea (OSA) patients. a. Significant inverse correlation between LMW ratio and VWF:CB at 06:00 h in OSA patients. b) VWF:CB at 06:00 h in group 3 was significantly lower than in groups 1 and 2. Data are presented as mean±SD *: $p<0.05$.

degree of hypoxia during apnoeic events is related to vFWMs processing and/or consumption.

Chronological changes of plasma levels of VWF:Ag, VWFM ratio, and ADAMTS13:AC in three patient groups with OSA

Plasma levels of VWF:Ag at 21:00 h, 06:00 h without CPAP, and 06:00 h with CPAP were determined in all three groups of OSA patients. As shown in figure 3, plasma VWF:Ag levels were almost unchanged in group 1 patients, but significantly increased between 21:00 h and 06:00 h in group 2 patients. Notably, VWF:Ag levels remarkably decreased between 21:00 h and 06:00 h in group 3.

We then determined levels of HMW, IMW, and LMW in all three groups. In group 1, HMW-VWFM showed a slight increase at 06:00 h with CPAP, relative to 06:00 h without CPAP. In group 2, HMW-VWFMs significantly increased at 06:00 h compared to 21:00 h confirming the results of the VWFM analysis used for defining groups 1–3. Consistent with this, in group 3, the IMW-VWFMs at 06:00 h was significantly lower than that at 21:00 h; CPAP treatment reversibly increased the HMW-VWFM at 06:00 h, in accordance with the increase in plasma VWF:Ag level.

In contrast, no change in the plasma ADAMTS13:AC was seen at 21:00 h, 06:00 h, or 06:00 h with CPAP in any of the three groups. These data argue that consumption of the HMW-VWFMs occurred overnight in OSA patients.

Plasma levels of VWF:CB activity

We observed dynamic chronological changes in plasma VWF:Ag levels and VWFM patterns in our subjects, especially in group 3. VWF:CB represents a biological function of VWF, in which HMW-VWFM adheres to collagen with a higher binding affinity than IMW- or LMW-VWFM. In this study, we were able to examine plasma VWF:CB levels in 53 out of 58 OSA patients. As expected, plasma levels of VWF:CB at 06:00 h without CPAP were inversely correlated with the LMW ratio ($p<0.01$), as shown in figure 4. Furthermore, as shown in figure 4, plasma levels of VWF:CB at 06:00 h was significantly lower in group 3 (85±50%) than in either group 1 (120±36%) or group 2 (138±41%). These results argue that structurally and functionally impaired VWFMs were present at 06:00 h in group 3 patients.

Decreased platelet counts at dawn in the untreated patients with OSA

A pair of platelet counts at 21:00 h and 06:00 h without CPAP was determined in 31 of the 58 OSA patients and in six of the 25 sleep controls, all of whom were involved in the later phase of this study. To correct for a possible hydration effect during sleep, we calculated the ratio of platelet count to haematocrit. The ratios in sleep controls did not exhibit significant changes between 21:00 h and 06:00 h (fig. 5), whereas they were lower at 06:00 h in untreated OSA patients ($p<0.01$) (fig. 5). However, none of the patients who received CPAP treatment developed overt clinical signs of thrombotic complications. These results suggest that platelet consumption, to a lesser extent, might occur during sleep without distinct thrombotic symptoms in untreated OSA patients.

Patient characteristics of groups 1, 2, and 3

Table 2 summarises the demographic and measured parameters of OSA patients categorised into groups 1–3. These three groups did not differ demographically, but AHI was significantly higher in group 3 than in groups 1 and 2. ODI3% in group 3 was also significantly higher than in group 1. These results unambiguously indicate that patients in group 3, who exhibit lower levels of large VWFM at 06:00 h represent the highest severity of OSA among the three groups.

Consistent with these results, decreased plasma levels of VWF:Ag in the two different time intervals (06:00 h and 21:00 h) was remarkable in group 3, in comparison to those in groups 1 and 2. Interestingly, the differences of LMW ratio in the two times (06:00 h and 21:00 h) was significantly higher in group 3 than those of groups 1 or 2. These results indicated that decreased VWF:Ag at 06:00 h was caused primarily by the reduction in larger VWFMs. Alternatively, no significant change in ADAMTS13:AC between the two times (06:00 h and 21:00 h) was observed in group 3, whereas such a change was observed in groups 1 and 2, leaving the physiological relevance unaddressed.

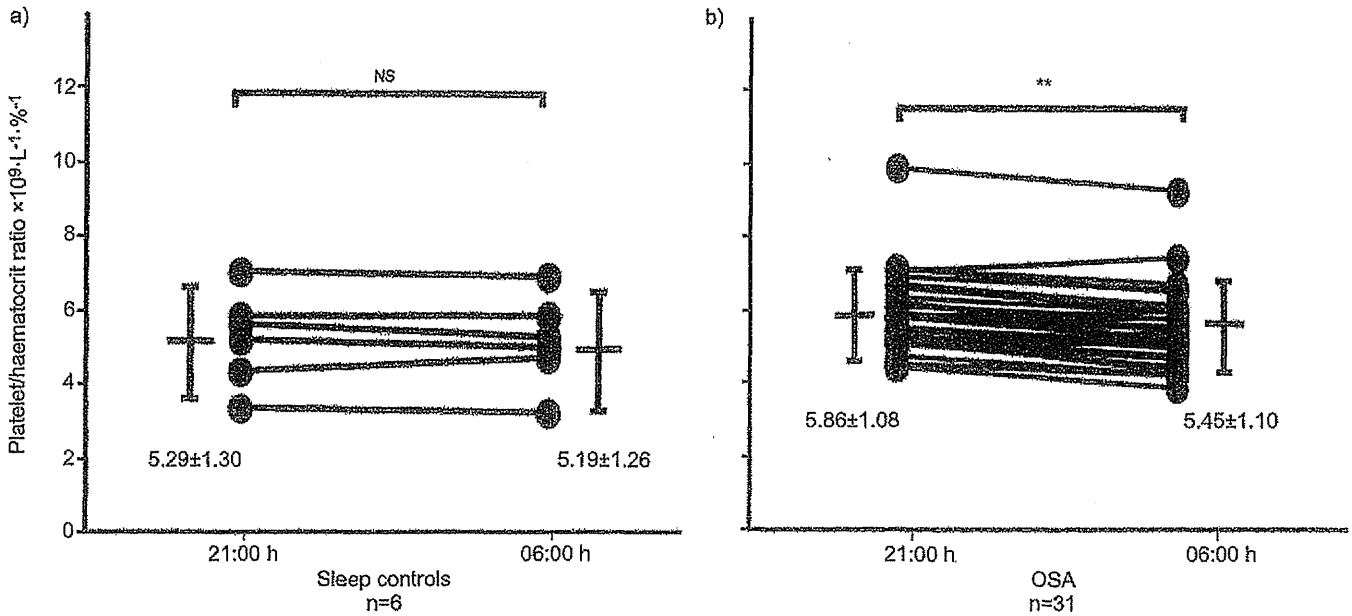

FIGURE 5. Overnight platelet counts to haematocrit ratios decreased in patients with obstructive sleep apnoea (OSA). Platelet counts were normalised to the patient's haematocrit to control for differences in hydration status. Ratios of platelet count to haematocrit were obtained at 21:00 h and 06:00 h in a) six sleep controls and in b) 31 OSA patients, both without CPAP treatment. In the sleep controls, the ratios did not change between time points. In the OSA patients, the ratio exhibited significant changes between time points. Data are presented as mean±SD. NS: nonsignificant. **: $p<0.01$.

Relationship of AHI and groups 1–3 of VWFM patterns in OSA patients

AHI is an excellent means of showing OSA severity, here we have used it to categorise three groups: moderate ($15 \leqslant AHI<30$), severe ($30 \leqslant AHI<60$), and extremely severe (AH $I \geqslant 60$). As shown in table 3, OSA patients with group 1 and 2 consisted of those with variable AHI levels. Notably, none of the OSA patients within group 3 had an AHI 15~<30, and they uniformly

TABLE 2 Characteristics and different parameter between 21:00 h and 06:00 h of patients with obstructive sleep apnoea (OSA) in groups 1–3

	Group			Overall p-value
	1	2	3	
Sex M/F	28/1	18/0	9/2	NS
Blood type				NS
A	7	4	7	
B	4	4	0	
O	14	7	4	
AB	4	3	0	
Age yrs	46.0±9.6	42.9±9.7	44.2±11.3	NS
AHI	43.1±20.0	51.4±19.6	68.7±22.6	<0.05*
ODI3%	35.7±18.2	44.1±19.3	53.2±21.5	<0.01#
Differences in time intervals 06:00 and 21:00 h				
VWF:Ag %	2.1±34.8	10.8±22.0	-28.1±40.6	<0.05¶
LMW ratio %	-0.27±5.24	-4.46±8.69	16.69±16.92	<0.01¶
ADAMTS13:AC %	4.4±13.1	-8.5±25.9	2.4±21.4	<0.05+
Plt/Ht $\times 10^9 \cdot L^{-1} \cdot \%^{-1}$	-0.045±0.036 (n=15)	-0.034±0.036 (n=10)	-0.043±0.029 (n=6)	NS

Data are presented as n or mean±SD, unless otherwise stated. M: males; F: females; AHI : apnoea/hypopnea index, ODI3%: oxygen desaturation index ⩾3%; VWF:Ag : von Willebrand factor antigen; LMW ratio: the ratio of low molecular weight-VWFMs to total VWFMs; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity; Plt/Ht: platelet count to haematocrit ratio. NS: not significant. *: $p<0.05$ between groups 1, 2 and 3; #: $p<0.01$ between groups 1 and 3; ¶: $p<0.01$ between groups 1, 2 and 3; +: $p<0.05$ between group 1 and 2.

TABLE 3 Characteristics and thrombotic parameters of patients classified with apnoea/hypopnoea index (AHI)

	15≤AHI<30	30≤AHI<60	AHI≥60	Overall p-value
Patients n	15	22	21	
Sex M/F	15/0	21/1	19/2	NS
Age yr	43.7±12.0	42.9±9.7	44.2±11.3	NS
ODI3%	19.2±4.9	36.2±10.9	63.3±9.4	<0.01**
VWFM group				
1	12 (80)	8 (36)	9 (43)	<0.05*
2	3 (20)	10 (45)	5 (24)	NS
3	0	4 (18)	7 (33)	<0.05#
VWF:Ag at 06:00 h %	98.5±49.1	98.5±55.7	111.3±75.5	NS
ADAMTS13:AC at 06:00 h %	58.1±20.2	55.2±21.9	57.6±25.6	NS
VWF:CB at 06:00 h U·mL^{-1}	1.29±0.39 (n=13)	1.23±0.50 (n=19)	1.09±0.38 (n=19)	NS
Plt/Ht at 06:00 h ×10^{9}·L^{-1}·%$^{-1}$	0.526±0.093 (n=10)	0.549±0.138 (n=13)	0.561±0.087 (n=8)	NS

Data are presented as mean±SD or n (%), unless otherwise stated. M: males; F: females; ODI3%: oxygen desaturation index ≥3%; VWFM: von Willebrand factor multimer; VWF:Ag : von Willebrand factor antigen; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity; Plt/Ht: platelet count to haematocrit ratio; NS: not significant. *: p<0.05 between 15≤AHI<30 and 30≤AHI<60, AHI≥60, **: p<0.01 between all AHI groups; #: p<0.05 between 15≤AHI<30 and AHI≥60.

had AHI ≥30 and more predominantly with AHI ≥60. The incident for group 1 patients was lower in AHI groups of 30≤AHI<60 and AHI≥60 than those of 15≤AHI<30 (p<0.05). In contrast, the incident for group 3 was higher in AHI≥60 than those of 15≤AHI<30 (p <0.05). No significant relationship between AHI score and each parameter such as VWF, ADAMTS13, or platelet count was found.

DISCUSSION

Plasma VWF:Ag levels increase after the age of 40 yrs in normal individuals; by the age of 60 yrs they can have reached ~120–140% of the healthy normal baseline [26]. The mean age of OSA patients enrolled in this study was 44.7 yrs, whereas that of control subjects was 38.3 yrs. However, the plasma VWF:Ag levels collected at 06:00 h were significantly lower for OSA patients than for control subjects (table 1). In contrast, plasma ADAMTS13 activity decreases after the age of 40 yrs in normal individuals [27]. Among our study patients and controls, plasma ADAMTS13:AC was lower than in healthy controls aged between 20-40 yrs (p<0.01), indicating that these two groups did not significantly differ (table 1).

Given the observed differences in VWF:Ag levels between OSA patients and control subjects, we analysed VWFM patterns chronologically at three time points: at 21:00 h and at 06:00 h either with or without overnight CPAP treatment. As expected, a majority of OSA patients (29 (50%) out of 58) had consistently normal VWFM patterns, categorised as group 1. Two smaller groups of patients had increased UL- and HMW-VWFM (18 (31%) out of 58) or decreased UL- and HMW-VWFM (11 (19%) out of 58) at 06:00 h; these were categorised as group 2 or group 3, respectively. The ratio of LMW-VWFM to total VWFM, termed the LMW ratio, is a determination of the relative amount of degraded VWFM; in our study population, the LMW ratio correlated significantly with the AHI.

The increased LMW ratio seen in OSA patients could arise from reduced production of VWF by vascular endothelial cells, increased clearance of HMW-VWFM from the circulation, or consumption during thrombosis. However, *in vitro* studies have clearly shown that VWF expression by cultured vascular endothelial cells is increased under conditions of hypoxia; it is unlikely that patients with OSA, a condition of intermittent hypoxia, would exhibit decreased expression of VWF overnight [17]. Additionally, no differences were seen in the plasma ADAMTS13:AC in any group at any time-point, suggesting that enhanced proteolysis of HMW-VWFM was not occurring. Therefore, we hypothesised that the elevated LMW ratio seen in our OSA patients was likely to be due to an enhanced degradation or consumption of HMW-VWFM.

The cause of thrombotic complications in OSA patients might be multifactorial, but in this study we have clearly indicated that VWF appears to play an essential role in the thrombogenesis in a certain population categorised as group 3. Although the mechanism is not yet fully elucidated, the high VWFMs released upon hypoxia from vascular endothelial cells is a most plausible factor. Thus, severe OSA could be a risk factor for both arterial and venous thrombosis as described in the introduction.

To better understand whether some degree of thrombosis was occurring overnight in untreated OSA patients, we determined platelet counts in 31 out of 58 patients; we observed a significant decrease in platelet count between 21:00 h and 06:00 h. This decrease was associated with reductions in both the plasma VWF:Ag levels and HMW-VWFM in group 3. Quantitative analyses of VWFMs in group 3 showed that levels of HMW-VWFMs increased significantly after CPAP treatment, compared with measurements taken at 06:00 h without CPAP. This is consistent with low-level consumption of UL- and HMW-VWFMs by microvascular thrombus formation and/or platelet aggregation during sleep in OSA patients; CPAP therapy might reduce such consumption. However, no patients have developed overt clinical signs of thromboembolic complications; therefore, we prefer to use the term "pre-clinical platelet consumption"

to describe this phenomenon. This may represent a baseline pro-thrombotic state in OSA patients that can be corrected by CPAP therapy.

In this study, the chronological analyses have unanimously indicated that reduced large VWFMs in plasmas at dawn reflect the clinical severity of apnoea in OSA patients. The results obtained by VWFM analysis were solid, but the procedure was time consuming and requires a high technical skill to perform. A reliable high-throughput method would be necessary for routine clinical use. In this regard, the assay for VWF:CB is a promising candidate for such a method, because HMW-VWFM adheres to collagen with a higher binding affinity than IMW- or LMW-VWFM. Our results indicated that VWF:CB at 06:00 h correlated well with VWFM patterns, and was consistent with earlier assignment of subjects to groups 1–3. Thus, through this study we have provided the first convincing evidence that VWF at dawn in group 3 was impaired not only structurally but also functionally, presumably due to hypoxia-induced release and consumption of VWF. This process might also involve platelet aggregation and consumption, even though the patients were asymptomatic. Thus, large scale studies, together with chronological measurements of platelet counts and VWF:CB, would be the focus in the following studies.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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PLoS one

Ghrelin Treatment of Cachectic Patients with Chronic Obstructive Pulmonary Disease: A Multicenter, Randomized, Double-Blind, Placebo-Controlled Trial

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Abstract

Background: Pulmonary cachexia is common in advanced chronic obstructive pulmonary disease (COPD), culminating in exercise intolerance and a poor prognosis. Ghrelin is a novel growth hormone (GH)-releasing peptide with GH-independent effects. The efficacy and safety of adding ghrelin to pulmonary rehabilitation (PR) in cachectic COPD patients were investigated.

Methodology/Principal Findings: In a multicenter, randomized, double-blind, placebo-controlled trial, 33 cachectic COPD patients were randomly assigned PR with intravenous ghrelin (2 μg/kg) or placebo twice daily for 3 weeks in hospital. The primary outcomes were changes in 6-min walk distance (6-MWD) and the St. George Respiratory Questionnaire (SGRQ) score. Secondary outcomes included changes in the Medical Research Council (MRC) scale, and respiratory muscle strength. At pre-treatment, serum GH levels were increased from baseline levels by a single dose of ghrelin (mean change, +46.5 ng/ml; between-group $p<0.0001$), the effect of which continued during the 3-week treatment. In the ghrelin group, the mean change from pre-treatment in 6-MWD was improved at Week 3 (+40 m, within-group $p=0.033$) and was maintained at Week 7 (+47 m, within-group $p=0.017$), although the difference between ghrelin and placebo was not significant. At Week 7, the mean changes in SGRQ symptoms (between-group $p=0.026$), in MRC (between-group $p=0.030$), and in maximal expiratory pressure (MEP; between-group $p=0.015$) were better in the ghrelin group than in the placebo group. Additionally, repeated-measures analysis of variance (ANOVA) indicated significant time course effects of ghrelin versus placebo in SGRQ symptoms ($p=0.049$) and MEP ($p=0.021$). Ghrelin treatment was well tolerated.

Conclusions/Significance: In cachectic COPD patients, with the safety profile, ghrelin administration provided improvements in symptoms and respiratory strength, despite the lack of a significant between-group difference in 6-MWD.

Trial Registration: UMIN Clinical Trial Registry C000000061

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Introduction

Pulmonary cachexia is common in the advanced stage of chronic obstructive pulmonary disease (COPD), and it is an independent risk factor for death in such patients [1,2]. Based on the notion that advanced COPD affects the whole body and causes wasting syndromes, many different therapeutic approaches have been attempted to improve this syndrome [1,3].

Pulmonary rehabilitation (PR) including exercise training is well accepted to improve exercise performance and quality of life in COPD patients [4], and it has been regarded as a nutritional adjunct therapy [5].

During the 1970s and 1980s, many gut peptides were identified [6]. Ghrelin, first discovered in 1999 as a novel growth hormone (GH)-releasing peptide isolated from the stomach, has been identified as an endogenous ligand for GH secretagogue receptor