Daily expression of clock genes in whole blood cells in healthy subjects and a patient with circadian rhythm sleep disorder

Mieko Takimoto,1,* Akinobu Hamada,1,* Akemi Tomoda,2,* Shigehiro Ohdo,3,* Takafumi Ohmura,1 Hisao Sakato,2 Junko Kawatani,2 Takako Jodoi,2 Hiroo Nakagawa,3 Hideyuki Terazono,3 Satoru Koyanagi,3 Shun Higuchi,3 Miyuki Kimura,4 Hiroshi Tukikawa,4 Shin Irie,4 Hideyuki Saito,1 and Teruhisa Miike2

1Department of Pharmacy, Kumamoto University Hospital, and 2Department of Child Development, Graduate School of Medical Science, Kumamoto University, Kumamoto, Japan; 3Department of Clinical Pharmacokinetics, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan; and 4Kyusyu Clinical Pharmacology Research Clinic, Fukuoka, Japan

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CIRCADIAN RHYTHM EXISTS IN MOST MAMMALS, including humans, and is controlled by a circadian clock. In humans, sleep-wake, hormonal secretion (melatonin, cortisol) (6, 36), and autonomic cycles (e.g., body temperature, blood pressure, peak flow) (14, 32, 35) are regulated by the circadian clock, which is synchronized with the 24-h day by environmental time cues, especially light (16). In the rodent brain, the center of the circadian clock is located principally in the suprachiasmatic nucleus (SCN) of the hypothalamus and oscillates self-sustained (43). Recent studies reported that the circadian clock system consists of transcriptional/translational feedback loops of several clock genes, including Period (Per) 1-3, cryptochrome (Cry) 1-2, Dec 1-2, Clock, Bmal1, and casein kinase Ie (CKIe). In addition, these mammalian clock genes appear to regulate circadian expressions not only in the SCN but also in other peripheral tissues such as the extra-SCN brain region, eye, heart, kidney, lung, liver, skeletal muscle, oral mucosa, and peripheral mononuclear leukocytes (3, 26, 34, 38, 39, 48).

The sleep-wake cycle is one of the circadian rhythms that can be most readily perceived in life. In recent years, studies of circadian rhythm sleep disorders, such as delayed sleep phase syndrome and non-24-h sleep wake disorder (Non-24), have been reported (12, 45). These sleep disorders have been suggested to be caused by endogenous factors involving dysfunctions of the circadian clock and its entrainment caused by disturbances of the hypothalamic and/or brain stem (23) and by exogenous factors, including the lack of light during the day, excess light during the night, and psychological stress (44). Furthermore, any combination of factors may be responsible for circadian rhythm sleep disorders. Although patients with circadian rhythm sleep disorders are able to adapt themselves to social life, they worry about social problems, such as tardiness, absence, truancy, and withdrawal. Moreover, the patients complain frequently about indeterminate symptoms such as headache, feelings of heavy headedness, lack of appetite, nausea, stomach ache, and fatigue.

The relationship between the circadian profile of clock genes and the symptoms of circadian rhythm sleep disorders remains unclear. Usually, these patients receive various therapies, including light treatment (7, 18, 19, 33, 46), physical exercise treatment (24), and administration of vitamin B12 (25, 27, 28) and melatonin (21, 29, 41) to adjust the circadian rhythm. Presently, it is considered that the markers of circadian rhythm for these patients are the profiles of plasma melatonin, cortisol, and retinal temperature. Even if these markers show normal rhythmic patterns, certain patients suffer from circadian rhythm sleep disorders and indeterminate symptoms, suggesting that the monitoring of these markers may not be reliable information for diagnosing circadian rhythm sleep disorders.

It was recently reported that peripheral cells and tissues express the clock genes that control circadian rhythm. Bjarnason et al. (4) reported the circadian expression profiles of clock genes in whole blood cells in healthy subjects and a patient with circadian rhythm sleep disorder.
genes in human oral mucosa and skin. In addition, Boivin et al. (5) reported the fluctuation of clock genes in peripheral blood cells. We previously reported the case of a 17-year-old male subject with recurrent hypersomnia who displayed changes in the 24-h expression of the hPer2 gene in whole blood cells (42). To make an accurate diagnosis of circadian rhythm sleep disorder, the standard circadian profile of clock genes is needed. In this study, we investigated the daily expression of clock genes in human whole blood cells and compared the profile of these gene expressions in healthy subjects with a patient with circadian rhythm sleep disorder. The present paper is the first report demonstrating that the monitoring of clock gene expression in peripheral blood cells would be useful in the diagnosis of sleep disorder in patients.

METHODS

Healthy human subjects. Twelve physically and mentally healthy men, age 20–41 yr (average of 27.4 ± 6.1 yr) and weight 54.9–79.9 kg (average of 64.9 ± 7.0 kg), were recruited from registrants of Kyushu Clinical Pharmacology Research Clinic. Participants gave written and informed consent, completed a questionnaire about their habitual schedule, took part in consultation, and passed a comprehensive health screening, including medical history and clinical examinations such as hematological, biochemical, serological, and urinalysis. Candidates who had irregular sleep or meal schedules or worked nights were turned off for sleeping. An in-dwelling catheter was placed in the antecubital vein for the 24-h period, and blood samples were taken at 4-h intervals beginning at 10:00 AM on the second day of hospitalization and continued until 6:00 AM of the following day.

Breakfast, lunch, and dinner at 7:00–8:00, 12:00–13:00, and 19:00–20:00, respectively, for 1 wk before the study began.

Experimental procedure. The subjects were exposed to the natural and fluorescent light of the institution during the awake time, and the lights were turned off for sleeping. An in-dwelling catheter was placed in the antecubital vein for the 24-h period, and blood samples were taken at 4-h intervals beginning at 10:00 AM on the second day of hospitalization and continued until 6:00 AM of the following day. During the sleeping time, samples were obtained under dim light (<30 Lux) without waking the subjects.

Real-time RT-PCR analysis of circadian-rhythm-related gene mRNA in whole blood cells. Blood was collected into the PAX gene Blood RNA kit manual. For quality assessment of total RNA during protocol development, DNA digestion of the samples was performed with the RNase-free DNase set (Qiagen). Synthesis of cDNA was carried out using ReverTra Ace-α-(Toyobo) for RT-PCR kit. We performed a TaqMan quantitative real-time RT-PCR using ABI Prism 7900 sequence detection system [Applied Biosystems (ABI)] to determine the expression level of hPer1, hPer2, hPer3, hBmal1, hClock, and housekeeping gene hβ-actin expression relative to hβ-actin, by means of the standard protocol described by ABI. Relative expression of clock gene was determined as the ratio of clock gene to that of β-actin for each sample. Values were normalized so that the peak value equaled 100%. TaqMan hβ-actin control reagents and the primer sets, assays-on-demand products for hPer1, hPer2, hPer3, hBmal1, and hClock, were purchased from ABI as follows: hPer1, Hs00242988_m1; hPer2, Hs00256144_m1; hPer3, Hs00213466_m1; hBmal1, Hs00154147_m1; and hClock, Hs00218571_m1.

Plasma melatonin and cortisol assay. Blood was drawn into tubes and then centrifuged to obtain plasma that was stored at −80°C until assay. Melatonin and cortisol were determined by means of commercially available radioimmunoassay and enzyme-linked immunosorbent assay, respectively (31).

Patient study and treatments. The patient, a 22-year-old man, was referred to Kumamoto University Hospital complaining of regular and recurrent difficulty sleeping and fatigue, which has continued since he was 16 yr old. This patient (with Non-24) developed a daily delay of the sleep phase, resulting in cyclic episodes of insomnia. His birth and development were unremarkable. He had no diagnosis of neurological illness, migraine headache, mental retardation, serious psychopathy, or history of drug abuse. Sleeping medication did not alleviate his sleep disorder.

A trial treatment of combined therapy was given as follows. Light therapy was initiated with illumination at 2,500–3,000 Lux for 2 h when he woke up in addition to exercise therapy. Pharmacotherapy included 0.7 mg of melatonin once per day at 21:00 and 1.5 mg of methyl B12 three times per day. Methylphenidate hydrochloride at a dose of 5–20 mg once per day was added to the regimen at the beginning of the third week. These combination therapies were continued for 1 mo in the hospital. Although sleep phase was mostly fixed within 1 wk after these treatments began, the patient continued to experience daytime somnolence; however, the somnolence also disappeared gradually throughout the second week, and the patient left the hospital.

Measurement of deep body temperature. Deep body temperature, as a surrogate of core body temperature, was assessed by a body temperature monitor (Terumo) positioned below the Lanz’s point of the anterior abdominal wall by use of a subdermal probe fixed tightly in place with adhesive medical tape. Measurements were made every 30 min for 24 h before, during, and after treatment.

Statistical analyses. Daily variations of mRNA expression were analyzed statistically using ANOVA. The values of the relative expression of mRNA are presented as means ± SD. A value of $P < 0.05$ was considered significant.
RESULTS

Daily variation of melatonin and cortisol in healthy subjects. During the 24-h sampling period, all subjects showed the expected daily variation in blood melatonin and cortisol (Fig. 1). All endogenous hormones showed significant daily variation ($P < 0.05$; ANOVA). The mean plasma levels of melatonin were low between 10:00 and 22:00 ($<5 \text{ pg/ml}$). The peak levels of melatonin reached 41.7 ± 18.5 pg/ml at 2:00. The mean level of melatonin during sleep is $\sim 10$ times as high as that during wakefulness. On the other hand, the mean plasma levels of cortisol were low at $\sim 2:00$ and peaked to 18.8 ± 4.12 $\mu$g/ml in the early morning at 6:00. These data indicate that all subjects were normal for the daily variation of endocrine secretion systems as evaluated by melatonin and cortisol.

Daily variation of clock genes in whole blood cells of healthy subjects. Figure 2 shows the profiles of $hPer1$, $hPer2$, $hPer3$, $hBmal1$, and $hClock$ mRNA expressions in whole blood cells of healthy subjects. These clock genes have significant daily variations ($P < 0.05$; ANOVA). The mRNA levels of $hPer1$, $hPer2$, and $hPer3$ appeared to be significantly elevated at 6:00, and those of $hBmal1$ and $hClock$ were high at 2:00. These results indicated that the mRNA expression levels of $hPer1$, $hPer2$, and $hPer3$ in whole blood cells showed daily variations between the morning and evening hours.

Patient case study. We evaluated the case study of one patient with circadian rhythm sleep disorder (Non-24). This patient developed a daily delay of sleep phase, resulting in cyclic episodes of insomnia. Figure 3 indicates that sleep-wake rhythm is irregular before treatment; however, the daily profiles of body temperature were normal. Generally, healthy human subjects show daily variations of body temperature characterized by temperature being lowest before waking in the morning (3:00–5:00) and highest in the early evening (15:00–18:00) (35). Figure 4 shows the daily profiles of melatonin and cortisol levels in the above patient before and after the treatments. The peak level of melatonin shifted from 56 pg/ml at 6:00 to 95 pg/ml at 2:00 in response to the treatments. On the other hand, the peak level of cortisol was 21.8 $\mu$g/ml at 22:00 before the treatments and 16.6 $\mu$g/ml at 2:00 after the treatments. The daily rhythm of melatonin appeared to phase...
advance, whereas cortisol rhythm appeared to phase delay in this patient in response to the combination therapy (light, exercise, and pharmacotherapy). The normalized daily profiles of these hormones indicate that the circadian rhythm recovered as a result of the treatments. On the other hand, because the daily rhythm of deep body temperature did not differ significantly from pre- to posttreatment, we thought that the patient may have a normal rhythm of body temperature.

Figure 5 shows the daily profiles of \( h\text{Per1} \), \( h\text{Per2} \), \( h\text{Per3} \), \( h\text{Bmal1} \), and \( h\text{Clock} \) mRNA expressions in whole blood cells of this patient before and after treatment. The daily expression of these genes was almost normalized by the treatments. The peak phases of \( h\text{Per2} \) and \( h\text{Per3} \) shifted from 18:00 to 6:00 by 12 h and the peak phases of \( h\text{Per1} \), \( h\text{Bmal1} \), and \( h\text{Clock} \) also shifted to 10:00, 14:00, and 6:00, respectively. This finding indicates that the daily expression of clock gene appears to be normalized by these treatments.

**DISCUSSION**

The time-course monitoring of body temperature, cortisol, and melatonin is useful to evaluate individual circadian rhythms. These examinations are available for diagnosis of circadian rhythm sleep disorder. However, because these rhythm markers can be affected by various conditions, including external environment and endogenous stress, reliable biological markers have been required to make an accurate diagnosis in patients with circadian rhythm sleep disorder. Therefore, we examined the daily expression of human clock genes such as \( h\text{Per1} \), \( h\text{Per2} \), \( h\text{Per3} \), \( h\text{Bmal1} \), and \( h\text{Clock} \) in whole blood cells as conventional candidates for circadian rhythm diagnosis.
In healthy human subjects, the mRNA expression levels of hPer1, hPer2, and hPer3 are higher in the early morning and then decline in the late afternoon, whereas the peak levels of mRNA expression in hBmal1 and hClock are earlier than these of hPers. Bjarnason et al. (4) reported the circadian expression profiles of clock genes in human oral mucosa and skin. hPer1 and hBmal1 in these peripheral tissues showed a rhythmic expression, peaking early in the morning and at night, respectively, whereas hClock was not rhythmic. Because there is a difference in the peak level of hPer1 between mucosa and skin, the expression profile of clock genes in humans may differ among peripheral tissues. In whole blood cells, a variety of endogenous factors may influence the expression of clock genes. Boivin et al. (5) reported the circadian expression of clock genes in human peripheral blood mononuclear cells. Our data are consistent with this earlier report in that the peak levels of hPers occurred during the habitual time of activity.

Healthy human subjects who regularly maintained a sleep-wake cycle had normal circadian rhythm profiles of melatonin and cortisol, which were identical with previous reports (6, 36). Presently, the monitoring of body temperature, plasma melatonin, and cortisol as circadian rhythm markers provide information on the circadian rhythm. However, numerous exogenous and endogenous factors influence the results of these examinations. Room temperature, clothes, and bed clothes influence body temperature. Plasma concentration of melatonin is changed by oral administration of melatonin and that of cortisol varies with stress and anxiety (2, 13, 47). Thus these circadian rhythm markers occasionally did not reflect circadian rhythm per se. In addition, in humans, the phenomenon called internal desynchronization is observed (1). When a human subject is free running under temporal isolation, the cycle of sleep-wake rhythm becomes prolonged, whereas the circadian rhythms of body temperature, plasma melatonin, and cortisol keep their periodicity (15, 30). According to these facts, it has been considered that human circadian rhythm is adjusted by two different oscillators. One oscillator in SCN is regulated by the light-dark cycle and reflects circadian rhythms of body temperature and hormonal secretion. Another oscillator could be influenced by the social schedule and gives rise to sleep-wake rhythm. On the basis of this theory, the circadian rhythm markers such as body temperature, melatonin, and cortisol

![Graphs showing relative mRNA levels of clock genes in whole blood cells.](https://example.com/graphs.png)
would not be useful for the evaluation of sleep-wake rhythm and diagnosis of circadian rhythm disorders.

The patient in the present case study benefited from light therapy, exercise therapy, and pharmacotherapy. Both the daily rhythm of melatonin and cortisol and the cycle of sleep-wake rhythm were improved by the combination therapy. This patient with Non-24 showed that all peaks of clock gene expressions at 18:00 were identical before the treatment, but this combination therapy seemed to normalize daily expression of clock gene and improved his performance status. Recently, we measured daily expression of clock gene mRNA in some patients with sleep disorders (Non-24 and delayed sleep phase syndrome). Daily rhythm of clock gene varied among the patients and typical profiles of clock gene expression showed low amplitude and a delayed phase between 6 and 12 h. The relationship between daily rhythm of clock gene and pattern of sleep-wake cycles remains unknown. Further clinical investigation will be needed and analyzed to evaluate the clinical implication. The daily expression of clock genes in whole blood cells may reflect the degree of internal synchronization because the clock genes in whole blood cells should be regulated by not only the complex oscillation system in the SCN but also by peripheral cells and/or tissues. However, the mechanism of regulation of clock gene expressions in peripheral blood cells remains unclear.

In recent years, some reports have suggested that a mutation of some circadian genes such as hPer2, hPer3, hCLOCK, hCKIe, and melatonin receptors are involved with circadian rhythm sleep disorders (9–11, 20, 37, 40); however, the patient in our study does not have family history of sleep disorder. It will be of interest to investigate the mutation of circadian rhythm-related genes and the amplitude of clock gene. Therefore, structural polymorphism in the hPer3 gene may be implicated in the pathogenesis of delayed sleep phase syndrome (11). Furthermore, a missense variation in the hCKIe gene plays protective role in the development of delayed sleep phase syndrome and Non-24 (37). Further clinical study may reveal a correlation between clock gene mutations and daily rhythm of clock gene expressions. Although a patient has mutations of these genes, it may be worth monitoring clock genes in whole blood cells to understand the relationship between the sleep-wake cycle and the circadian rhythms of body temperature and/or internal secretions.

The present study suggests that the monitoring of human clock genes in whole blood cells might be useful to evaluate the internal synchronization of circadian rhythms. A diagnosis based on the monitoring of clock genes in peripheral blood cells may be useful to judge the efficacy of light therapy, exercise therapy, and pharmacotherapy in patients with circadian rhythm disorder. Further study of a large series of cases may determine the usefulness of this technique; additional studies may also elucidate this mechanism to reveal the relationship between clock gene expression in the SCN and peripheral blood cells.

REFERENCES