Daily Expression of mRNAs for the Mammalian Clock Genes
Per2 and Clock in Mouse Suprachiasmatic Nuclei and Liver
and Human Peripheral Blood Mononuclear Cells

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ABSTRACT—The mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and in most peripheral tissues. Clock genes drive the biological clock. However, circadian expression variations of the human clock genes are still unclear. In this study, we analyzed the daily variations of mPer2 and mClock mRNA expression in both the mouse SCN and liver to evaluate the central and peripheral alterations in the rodent clock genes. We also examined whether there are the daily variations of the clock genes hPer2 and hClock in human peripheral blood mononuclear cells (PBMCs). The daily variation of mClock and mPer2 mRNA expression in mouse SCN and liver were determined at ZT2, ZT6, ZT10, ZT14, ZT18 or ZT22. We isolated PBMCs from 9 healthy volunteers at 9:00 and 21:00 and examined the expression of hPer2 and hClock mRNA by RT-PCR analysis. The animals exhibited a robust daily rhythm in the RNA levels of mPer2 in the SCN and liver (P≤0.01, respectively). In humans, hPer2 mRNA expression also had daily variation, and the hPer2 mRNA levels at 9:00 were significantly larger than those at 21:00 (P≤0.01). While, the Clock mRNA in both mice and humans exhibited no daily variation. These findings suggest that the variation in hPer2 mRNA expression may be useful for assessing human peripheral circadian systems.

Keywords: Clock gene, Liver, Per2, Peripheral blood mononuclear cell, Suprachiasmatic nucleus

In all living organisms, one of the most indispensable biological functions is the circadian clock (suprachiasmatic nuclei, SCN), which acts like a multifunction timer to regulate homeostatic systems such as sleep and activity, hormone levels, appetite, and other bodily functions with a 24-h cycle (1). Like any timing system, the circadian clock is made up of three components (2 – 6): an input pathway adjusting the time, a central oscillator generating the circadian signal, and an output pathway manifesting itself in circadian physiology and behavior. The daily changes in light intensities are thought to be the major environmental cue involved in circadian entrainment. Light-signals are perceived by photoreceptor cells in the retina and transmitted to neurons of the SCN via the retinohypothalamic tract (2). Recently, clock genes were identified as the genes that ultimately control a vast array of circadian rhythms in physiology and behavior (7). Three mammalian clock genes (mPer1, mPer2 and mPer3) are rhythmically expressed in the SCN. The mPer1 and mPer2 are induced in response to light (8). In particular, mPer1 induction is considered to be an initial event in light-induced resetting and entrainment of the circadian biological clock (2). The transcriptional machinery of the core clockwork regulates a clock-controlled output rhythm (3). Namely, CLOCK-BMAL1 heterodimers act through an E-box enhancer to activate the transcription of period, vasopressin and Dbp mRNA (3, 4, 9). The PER, TIM andCRY proteins (10, 11) can inhibit this activation.

The expressions of mammalian circadian clock genes are found not only in the central pacemaker of the SCN but also in other peripheral tissues (12 – 15). While the
The mechanism of the clock genes has been examined extensively in rodents that are nocturnally active (13–17). There are few reports in diurnally active humans (18, 19). Bjarnason et al. reported that clock genes such as hClock, hTim, hPer1, hCry1, and hBmal1 are expressed in human oral mucosa and skin (18). Moreover, hPer1, hCry1, and hBmal1 display a rhythmic expression profile (18). However, it is difficult to obtain samples; i.e., oral mucosa or skin, from humans, because local anesthesia is necessary for biopsies. If peripheral blood can be used, it would be convenient and easy to evaluate the changes in the expression of the peripheral clock genes. Oishi et al. reported that rPer2 mRNA expression in rats exhibited circadian oscillations in peripheral blood mononuclear cells (PBMCs) (14). On the other hand, it is unclear whether hPer2 mRNA in humans has rhythmic expression in PBMCs. In this study, we analyzed the daily variation of mPer2 and mClock mRNA expression in both the SCN and liver of mice to evaluate the central and peripheral alterations of these rodent clock genes. We also hypothesized that one of the human clock genes, hPer2, would be expressed in human PBMCs, and we investigated the daily expression of both hPer2 and hClock mRNA expression in PBMCs from healthy volunteers.

MATERIALS AND METHODS

Animals and treatment

Male ICR mice (5-week-old) were purchased from Charles River Japan, Inc. (Kanagawa). The mice were housed 10 per cage in a light-controlled room (ZT0, light on; ZT12, light off; ZT, Zeitgeber time) at a room temperature of 24 ± 1°C and humidity of 60 ± 10% with food and water ad libitum. All mice were exposed to their light-dark cycle for 2 weeks before the experiments. During periods referred to as darkness, a dim red light was used to aid in the treatment of the mice. The 24-h rhythm of mClock and mPer2 mRNA expression in the SCN and liver were determined at ZT2 (9:00), ZT6 (13:00), ZT10 (17:00), ZT14 (21:00), ZT18 (1:00) or ZT22 (5:00).

RT-PCR analysis of mClock and mPer2 mRNA

The brain and liver were quickly removed after cervical dislocation. Coronal hypothalamic slices (500 μm) were prepared through the SCN using rodent brain matrix (RBM-2000C; ASI Instruments, Warren, MI, USA). The SCNs were punched out bilaterally from the hypothalamic slices. The mRNA levels were determined based on a previously described method (20). Total RNA from the liver of individual mice was extracted separately using the Trizol reagent (Gibco BRL, Rockville, MD, USA). In addition, total RNA from the SCN of three mice was extracted in each group to obtain an adequate amount of RNA. A SuperScript One-Step RT-PCR System (Gibco BRL) was used for the reverse transcription of approximately 100 ng of RNA, and mClock, mPer2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were amplified by PCR. PCR was performed six times on the different pooled SCN from three mice or the different livers from individual mice. PCR reactions were carried out with mClock, mPer2 and mGAPDH primers. Cycling parameters were 94°C for 3 min, 27 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. The primer pairs used for the amplification of each product were as follows: 5′-AAGATTCTGGGTCTGACAAT-3′ and 5′-TTGCAGCTTGAGACATCGCT-3′ (mClock; 1599 – 1899, 301 bp, AF000998); 5′-ACACCCACCCTTACAAGCTTC-3′ and 5′-CGCTGGATGATGTCTGGCTC-3′ (mPer2; 840 – 1619, 780 bp, AF035830); 5′-GACCTCAACTACAGCCATGGTC TACA-3′ and 5′-ACTCCACGACATCTAGCAG-3′ (mGAPDH; 155 – 332, 178 bp, M32599). The PCR products were run on 3% agarose gels (Nusieve 3:1 agarose; Bioline, Tokyo) and centrifuged for 30 min at 3000 x g at room temperature.

Healthy subjects

Nine clinically healthy volunteers (7 men and 2 women) aged 22 – 42 years (mean ± S.D.: 29.0 ± 6.1 years) were investigated. Although we did not restrict the time schedule and the daily activity in the present study, the volunteers took food between 7:00 and 8:00 at breakfast time, between 12:00 and 14:00 at lunch time, and around 20:00 at night on the average. Moreover, they usually went to sleep around 23:00 and awoke around 7:00. They did not consume alcohol or caffeine and took no medications during or on the day preceding the study. Informed consent was obtained from all participants.

Peripheral blood mononuclear cells preparation

PBMCs were isolated at both 9:00 and 21:00 from 10-ml of heparinized human venous blood, which was layered on a Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Tokyo) and centrifuged for 30 min at 300 × g at room temperature.

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RT-PCR analysis of hClock and hPer2 mRNA

Total RNA was extracted from PBMCs using Isogen (Wako, Tokyo) according to the manufacturer’s recommendations. Moreover, 20 μg of total RNA was treated with deoxyribonuclease (Wako). First strand cDNA was synthesized according to the protocol of Life Technologies with 5 μg of RNA, 10 μl of 0.1 M DTT, 5 μl of 10 mM dNTP mix (Promega), 2 μl of random hexamers (Promega, Madison, WI, USA), 1 μl of RNase inhibitor (Wako Chemical, Richmond, VA, USA), 2 μl of M-MLV reverse transcriptase (Gibco BRL), and DEPC-treated H2O to yield a final volume of 50 μl. The reaction mixture was incubated for 1-h at 37°C, and the mixture was heated to 95°C for 5 min and then cooled to 4°C.

Oligonucleotide primers were selected to amplify specific regions of human Clock (18) and Per2 (Genebank accession number NM003894). Human GAPDH (21) primers were used as an internal control. The sequence of the oligonucleotide primers were as follows: hGAPDH (253 bp): 5'-GTCATCCATGACAACTTTGGTATCG-3' and 5'-GCAGGTCAAGGTCCACCACTG-3'; hClock (171 bp): 5'-AAGTTAGGGCTGAAAGACGACG-3' and 5'-GAACTCCGAGAAGAGGCAGAAG-3'; hPer2 (147 bp): 5'-GCAGGTGAAAGCCAATGAAG-3' and 5'-GACCGAAACATATCGCAT-3'.

PCR amplification was then performed with 1 μl of the cDNA supplemented with 4 μl of 10 × PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 0.05 μM of each primer, 1.5 U of Taq polymerase (Applied Biosystems), and then adjusted to 40 μl with sterile water. Cycling parameters were 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 59.7°C for 1 min, and 72°C for 7 min and 1 cycle of 72°C for 7 min. Each primer pair for hClock or hPer2 was co-amplified with hGAPDH. The PCR products were separated on 1.5% agarose gels (Wako) stained with ethidium-bromide (Wako) and photographed using a BioDoc-it System (UVP, Inc., Upland, CA, USA). Bands were quantified as a ratio of the target gene to that of the control gene by ATTO Densitograph 4.0 (ATTO Inc., Tokyo).

Statistical analyses

The differences in mClock and mPer2 mRNA expression were examined using ANOVA (Statview; Abacus Concepts, Berkeley, CA, USA). The values of the relative expression of mRNA in both hPer2 and hClock are presented as the mean ± S.D. For the assessment of differences we used the Mann-Whitney U test to compare the ratios of mRNA expression between 9:00 and 21:00. Differences were considered significant at P<0.05.

RESULTS

Daily variation of mClock and mPer2 mRNA expression in SCN and liver

To explore the daily variation of mClock and mPer2 mRNA expression in the SCN and liver, male ICR mice were housed in a light-controlled room. The mice exhibited no daily rhythm in the mClock mRNA in the SCN (Fig. 1). The relative ratio of mClock mRNA in the liver tended to be higher at ZT2 (9:00) (1.00 ± 0.38) than at ZT14 (21:00) (0.63 ± 0.20), but it was not statistically significant.

Statistical analyses

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Fig. 1. Relative mRNA levels of mClock in the SCN. Each point represents the mean ± S.E.M. in 6 mice. No significant daily variation was found in the expression of mClock mRNA in the SCN. The white bar indicates the light period, and the black bar indicates the dark period.

(A) (B) (C) (D) (E) (F) (G)

Fig. 2. Examples of RT-PCR analysis of mPer2 mRNA in the liver of mice. Lane A: 100 base-pair marker, Lane B: 9:00 (ZT2), Lane C: 13:00 (ZT6), Lane D: 17:00 (ZT10), Lane E: 21:00 (ZT14), Lane F: 1:00 (ZT18), Lane G: 5:00 (ZT22).
Figure 2 shows the results of PCR for gene expression in the liver of mice. Primers of \textit{mPer2} were co-amplified with those of \textit{mGAPDH}. As seen in Fig. 2, the expression levels of \textit{mPer2} mRNA in the liver revealed remarkable daily variation.

The animals exhibited a robust daily rhythm in the \textit{mPer2} RNA levels in both the SCN and liver (\(P<0.01\), respectively, Fig. 3). The highest and lowest levels of \textit{mPer2} mRNA expression in the SCN were at ZT10 (17:00), and at ZT18 (1:00), respectively. The highest and lowest levels of \textit{mPer2} mRNA expression in the liver were at ZT14 (21:00), and at ZT2 (9:00), respectively. The \textit{mPer2} RNA oscillations in the SCN preceded those in the liver by several hours.

**Daily variation of \textit{hPer2} and \textit{hClock} in PBMCs from healthy subjects**

Figure 4 shows the results for duplex PCR for gene expression in 2 subjects. Primers of either \textit{hClock} or \textit{hPer2} were co-amplified with those of \textit{hGAPDH}. As seen in Fig. 4, \textit{hClock} mRNA remained almost stable between 9:00 and 21:00. On the other hand, as shown in Fig. 5, the expression levels of \textit{hPer2} mRNA revealed remarkable daily variation. The relative ratio of \textit{hPer2} mRNA expression was significantly higher at 9:00 (1.05 \pm 0.70) than at 21:00 (0.19 \pm 0.23) (\(P<0.01\)). However, no significant difference was found in the \textit{hClock} mRNA expression between 9:00 (1.35 \pm 0.97) and 21:00 (1.46 \pm 0.90).

**DISCUSSION**

The mice exhibited a robust daily rhythm in their RNA levels for \textit{mPer2} mRNA in both the SCN and liver. On the other hand, no significant daily variation of \textit{mClock} mRNA was detected. We have also performed semiquantitative RT-PCR analysis to examine the daily expression in the clock genes \textit{hPer2} and \textit{hClock} in PBMCs from healthy volunteers. We selected \textit{Clock} as a constitutive expression gene and \textit{Per2} as a circadian expression gene to examine in this study. Especially, the levels of \textit{rPer2} mRNA in rat PBMCs reportedly showed clear circadian change (14). We first found that \textit{hPer2} mRNA expression has a signifi-
Daily variation of mPer2 in SCN and liver

The mice exhibited no daily rhythm in the mClock mRNA in the SCN. The relative ratio of mClock mRNA in the liver tended to be higher at ZT2 (9:00) than at ZT14 (21:00), but it was not statistically significant. It is reported that the abundance of Clock is not circadianly regulated in the liver (21:00), but it was not statistically significant. It is reported that the abundance of Clock is not circadianly regulated in the liver at 21:00, but it was not statistically significant. It is reported that the abundance of Clock is not circadianly regulated in the liver at 21:00, but it was not statistically significant. It is reported that the abundance of Clock is not circadianly regulated in the liver at 21:00, but it was not statistically significant. It is reported that the abundance of Clock is not circadianly regulated in the liver at 21:00, but it was not statistically significant. 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In conclusion, we have found that hPer2 mRNA expression in PBMCs from healthy volunteers has a significant daily variation, which is different from that in mice. Several drugs can cause alterations in the circadian rhythms of physiologic phenomenon (23, 24). Thus, drug administration at certain times in a day might improve the outcome of pharmacotherapy. Although clinicians have accepted chronotherapy for the treatment of nocturnal asthma (30), most drugs are given without regard to time of day. A new concept of adverse effects due to alterations in the clock function by a drug has recently been reported (17). Ohdo et al. have shown the disruptive effect of IFN-α on the rhythm of locomotor activity, body temperature, and mPer gene mRNA expression in the periphery and the SCN. Moreover, the changes in the dosing schedule report-
edly minimized the disruptive effects of IFN-α on clock function (17). Monitoring the rhythmicity of clock genes may be useful for choosing the most appropriate time of day for the administration of drugs, which may increase their therapeutic effects and/or reduce their side effects. Although the gene expression of the peripheral clock genes is not the same as in SCN, the evidence of variation in hPer2 mRNA expression in PBMCs may be useful for detecting some drug effects on circadian systems in humans. In addition, the difference in the circadian expression of peripheral Per2 mRNA between humans and rodents should be considered in clinical pharmacology. Further studies will be needed to clarify the efficacy of peripheral clock gene expression.

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