Circadian Expression of Differentiated Embryonic Chondrocytes Expressed Genes 1 and 2 in Human Oral Squamous Cell Carcinoma HSC-3 Cells

Kazumune Arikawa,^{1, 4} Fengzhu Zhang,² Chieko Taguchi,¹ and Ujjal K. Bhawal^{3, 4}

Departments of ¹Preventive and Public Oral Health, ²Anesthesiology, ³Biochemistry and Molecular Biology, ⁴Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

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Abstract

Autonomous rhythmicity of clock genes controls the circadian rhythm and endogenous circadian rhythms regulates various biochemical, physiological, and biological processes, with a periodicity of about 24 h. Entrainment circadian clock of cells detects distinct patterns of circadian oscillation for clock genes. Although circadian disruptions have been implicated in cancer development, the altered circadian rhythm in human oral cancer remains largely unknown. Here we show that, upon entrainment by serum shock, cultured human oral epithelial cancer cell line, HSC-3, maintain an inner circadian oscillator, with key clock genes DEC1 and DEC2 oscillating in a circadian fashion. Serum-shocked HSC-3 cells exhibited an about 24 h oscillatory pattern of DEC1 and DEC2 protein and mRNA expression. Taken together, the results indicated that the transcription factors DEC1 and DEC2 modulate a rhythmic transcriptional regulation mechanism which can drive the expression of downstream genes involved in many cellular processes.

Introduction

Endogenous circadian clocks exist in most living beings that coordinate a wide variety of physiological, behavioral, and molecular circadian rhythms (1-3). Peripheral tissues contain functional endogenous clocks and circadian rhythms are driven by biological clocks (4, 5).

Circadian disruption can result in perturbed physiologic and health consequences (6-8). Epidemiologic studies revealed a correlation between cancer and circadian disruption and overnight shift work as a probable carcinogen (9, 10). Disruption of circadian rhythms has been shown to lead aging and cancer (11-13).

Rhythmic expression is driven by transcriptional and post-translational feedback loops (11) and regulated exclusively by the clock genes (14, 15). The relevance of circadian rhythms in efficacy of drugs lead to dose- and/or time-dependent effects. The dosage time as well as drug dose determine the pharmacological or toxicological effects. Adequate circadian timing of chronotherapy could improve

tolerability (16).

Expression of the clock gene Differentiated Embryonic-Chondrocytes Expressed Gene 1 (DEC1) is highly elevated in response to various environmental stimuli (17-19). The transcription factor DEC2 also plays an important role in the circadian rhythm (20). Previous studies showed a rhythmic expression of DEC1 and DEC2 mRNA in the suprachiasmatic nucleus, and peripheral tissues (21-25). DEC1 and DEC2 elicited complex rhythmic patterns in various cell types and the circadian rhythmicity of DEC1 and DEC2 in serum-shocked human oral carcinoma cells remain poorly understood.

Understanding the influence of circadian rhythms on oral carcinoma cells, we sought to take advantage of the serum shock assay to induce circadian oscillation of clock genes in HSC-3 oral carcinoma cells in this study. The pattern of circadian mRNA and protein accumulation of key clock genes DEC1 and DEC2 has been investigated. The circadian oscillation of HSC-3 cells was entrained in culture, and DEC1 and DEC2 are transcribed in a circadian fashion.

Correspondence to :

Ujjal K. Bhawal

E-mail : bhawal.ujjal.kumar@nihon-u.ac.jp

Materials and Methods

Cell culture

Human oral carcinoma cell line HSC-3 (Japanese Cancer Research Resources Bank) was cultured in Dulbecco's modified Eagles medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS; Biowest, Riverside, MO, USA). The cells were seeded in 60-mm plastic tissue culture dishes and were incubated in 5% CO₂ at 37°C. When the cells reached sub-confluence, they were harvested and sub-cultured. Serum shock experiment was carried out as described previously (25). In brief, the cells were incubated with 50% horse serum for 2 h, and the medium was then replaced with DMEM supplemented with 5% FBS. The cells were harvested at the indicated times and subjected to various analysis.

RNA extraction and Quantitative Real Time-PCR (QRT-PCR)

Total RNA was extracted from HSC-3 cells using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), as described previously (26). A TURBO DNA-free™ Kit (Applied Biosystems, Foster City, CA, USA) was used to remove contaminating DNA from the RNA preparations. First-strand cDNAs were synthesized from 1 µg total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), according to the manufacturers' protocol. The relative expression levels of target mRNAs, compared to the level of β -actin RNA, were analyzed by real time PCR with the corresponding TaqMan MGB probes (Hs01041212_m1 for DEC1, Hs00229146_m1 for DEC2, and Hs01060665_g1 for β -actin) using QuantStudio 6 Real Time PCR System (Applied Biosystems). The thermal cycling conditions used were according to the TaqMan Fast Universal PCR protocol.

Western blot analysis

HSC-3 cells were lysed in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were calibrated with molecular weight markers (Bio-Rad, Hercules, CA, USA). Anti-DEC1 and Anti-DEC2 (1: 500; obtained from Professor Yukio Kato, Hiroshima University), and anti- β -actin (1: 1000; Cell Signaling Technology, Danvers, MA, USA) rabbit polyclonal antibodies were used. A

horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) was used at a dilution of 1:2000. Bound antibodies were visualized by chemiluminescence using the ECL Plus Western Blotting Detection System (GE Healthcare, Tokyo, Japan), and images were analyzed using a Luminescent ImageQuant LAS 4000 Mini (GE Healthcare).

Statistical analysis

Statistical analyses were done by one-way ANOVA and Dunnett's Test. A P-value of less than 0.05 is considered statistically significant.

Results

DEC1 and DEC2 displayed a rhythmic expression after serum shock

Serum-shocked HSC-3 cells exhibited an about 24 h oscillatory pattern of DEC1 and DEC2 protein and mRNA expression. DEC1 protein was increased at T8, gradually decreased and reached a peak at T28, then gradually decreased and increased again between T44 to T48 (Fig. 1). In contrast, DEC2 protein was gradually increased from T8, reached a peak at T24, then increased again between T44 to T48 (Fig. 1). DEC1 and DEC2 mRNA also showed the trend of circadian oscillation after serum shock (Fig. 2). There are statistically significant differences in synchronized cells compared with the T0 time point (one-way ANOVA). We observed no significant time-dependent differences in unsynchronized HSC-3 cells (data not shown).

Discussion

Molecular clock modulates normal cell function and clock genes plays a major role in the regulation of circadian rhythms. Peripheral tissues are also rhythmically expressed (27, 28). The circadian rhythm regulates a variety of biological processes (29) and circadian disruption is associated with the increased susceptibility to cancers (30). Chronotherapy has been widely used in the treatment of diseases (31). In order to understand the effects of circadian rhythms and the consequences of circadian disruption, we set out to examine whether serum-shocked cultured human oral carcinoma cell HSC-3 cells exhibit an autonomous functional inner clock. Serum shock synchronizes the circadian clock (32) and regulates rhythmic expression of genes (33). The circadian clock regulates growth factors, transcription factors, and hormones (34). HSC-3 cells

Fig. 1. DEC1 and DEC2 protein showed circadian oscillation in HSC-3 cells after serum shock treatment. HSC-3 cells were maintained in 50% FBS for 2 hours and the medium was then replaced with 5% FBS. The cells were harvested every 4 h for 48 h and the cell lysates were subjected to western blot analyses with DEC1, DEC2, and β -actin antibodies.

Fig. 2. Circadian oscillation of DEC1 and DEC2 mRNA in HSC-3 cells after serum shock treatment. Samples were collected every 4 h over a period of 48 h and analyzed by real-time PCR. Results were normalized to the endogenous control β -actin and analyzed by one-way analysis of variance. Data represent mean values from three independent experiments. The asterisks showed the level of significance compared with the 0 h.

exhibited a rhythmic pattern of transcription factors, DEC1 and DEC2, after a serum shock. It has been reported that Bmal1/2 and vascular endothelial growth factor expression by serum shock are altered as DEC (24). Although the differential expression pattern of DEC1 and DEC2 at 24 and 48 h remain unclear, most of the clock and clock-controlled genes are altered as DEC pattern. The potential role for the circadian clock within the oral cancer cells depends upon the circadian expression of *DEC1* and *DEC2*, suggest that the serum-induced transcription of these molecules does necessitate translation.

Chronotherapy aims at determining optimal dosing schedules based mostly upon physiological circadian rhythms (35, 36). Core circadian genes rhythmic expression is present in most peripheral tissues (37). The rhythmic expression is induced in cultured fibroblasts upon entrainment by serum shock (38). DEC1 overexpression decreased the expression level of DEC2, whereas DEC1 deficiency increased the expression of DEC2 mRNA (23). Thus,

overexpression and deficiency of DEC1 had virtually opposite effects on the phase and expression levels of DEC2. The double knockdown of DEC1 and DEC2 markedly decreased the amplitude of the circadian expression of several clock genes, emphasizing the essential role of the DEC loop in the molecular clock system. We found that serum-shocked HSC-3 cells show circadian gene expression of DEC1 and DEC2. Our results on the circadian rhythm of oral cancer cells provide a useful framework for drug chronotherapy. We hypothesize that the circadian rhythmicity of oral cancer can play a marked role in identifying the timing of medication administration and targeting the molecular clock for the development of novel therapeutics.

In conclusion, gene expression profiles of *DEC1* and *DEC2* in synchronized HSC-3 cells upon entrainment by serum shock offer important new insights into studying the maintenance of circadian rhythms at the cellular level. Since circadian rhythm regulates many physiological processes of living beings, this will help us to create a better understand-

ing of DEC1/DEC2 functions. Furthermore, our results indicate that oral cancer cells are synchronized with serum shock, suggesting a possible relationship between circadian oscillations and cancer cell characteristics. It may be useful to synchronize the circadian clock in cancer cells in order to synchronize the expression of transcription factors that can regulate cancer cells.

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Conflicts of Interest

The authors declare no conflict of interest.

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