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Original article

Melatonin alterations and brain acetylcholine lesions in sleep disorders in Cockayne syndrome

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Abstract

Background: Cockayne syndrome (CS) is a genetic disorder caused by deficient nucleotide excision repair. Patients with CS exhibit progeroid features, developmental delay, and various neurological disorders; they are also known to suffer from sleep problems, which have never been investigated in detail. *Objective:* The aim of this study is to investigate the pathogenesis of sleep disorders in patients with CS. *Methods:* We performed a questionnaire survey of the families of patients with CS, enzyme-linked immunosorbent analyses of the melatonin metabolite, 6-sulphatoxymelatonin (6-SM), in the patients' urine, and immunohistochemistry in the hypothalamus, the basal nucleus of Meynert (NbM), and the pedunculopontine tegmental nucleus (PPN) in four autopsy cases. *Results:* Sleep–wakefulness rhythms were disturbed in patients with CS, and these disturbances seemed to be related to a reduced urinary excretion of 6-SM. In addition, although the hypothalamic nuclei were comparatively preserved, acetylcholine neurons (AchNs) were severely decreased in the NbM and PPN. *Conclusions:* AchNs modulate both arousal and rapid eye movement sleep, and selective lesions of AchNs in the PPN and/or NbM in combination with disturbed melatonin metabolism might be involved in the sleep disorders in CS.

Keywords: Cockayne syndrome; Sleep disorders; Melatonin; Immunohistochemistry; Hypothalamus; Acetylcholine

1. Introduction

Cockayne syndrome (CS) is a rare genetic disorder that is caused by deficient nucleotide excision repair [1], and postnatal growth failure with a loss of fat; psychomotor developmental delays become evident in patients with CS in infancy or early childhood. Affected children have characteristic facial features, such as sunken eyes, sharp noses, and carious teeth, and they sunburn easily [2]. In addition to developmental delay, patients with CS suffer from neuropathy, visual impairments, neural deafness, cerebellar ataxia, and spasticity, all of which interfere with their quality of life. Autopsies of these patients have demonstrated various pathological changes throughout the peripheral and central nervous systems, including demyelinating peripheral neuropathy, pigmentary retinopathy, optic atrophy, a small brain, patchy demyelination and atrophy of the white matter, calcification predominating in the basal ganglia, and cerebellar atrophy with neuronal loss and gliosis [3,4]. Nancy and Berry [5] have divided 140 published CS cases into the following three types: type I, which is the most prevalent classical childhood disorder; type II, which is the severe congenital or infantile variant of the disorder; and type III, which has an atypical

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late onset of the disorder with prolonged survival. CS patients in Japan are known to exhibit sleep abnormalities and thermoregulation problems, which have rarely been examined in detail. We conducted a preliminary enzyme-linked immunosorbent analysis (ELISA) of the urinary secretion of melatonin metabolites and found a possible impairment of melatonin metabolism in patients with severe motor and intellectual disabilities [6]. In addition, we found a selective reduction of acetylcholine neurons (AchNs) in the basal nucleus of Meynert (NbM) and the pedunculopontine tegmental nucleus (PPN) in autopsy cases with xeroderma pigmentosum group A (XP-A), which is also caused by a hereditary nucleotide excision repair deficiency and which results in cutaneous photosensitivity and various neurodegenerative symptoms but not severe sleep problems [7].

In order to investigate the pathogenesis of sleep disorders in patients with CS, we performed a questionnaire survey in the families of patients with CS, a comprehensive ELISA measurement of 6-sulphatoxymelatonin (6-SM), which is a predominant melatonin metabolite, in the patients' urine, and immunohistochemistry in the hypothalamus, NbM, and PPN in four autopsy cases. Sleep problems were observed in patients with CS, and the disturbed metabolism of melatonin and/or the selective reduction of AchNs may be involved in sleep disorders.

2. Materials and methods

2.1. Questionnaire survey

We made a paper-and-pencil questionnaire that was specific for CS. In collaboration with the Japan Cockayne Syndrome Network, the questionnaires were distributed to family members of patients in the network. The questionnaire was written in Japanese and contained 13 questions. It assessed the current motor and mental abilities, presence or absence of auditory and visual impairments, bedtimes and rising times, naps, the use of hypnotics, and the presence or absence of sleep problems and thermal dysregulation in the patients.

2.2. Measurement of 6-SM and data analysis

Melatonin has a 24-h rhythm that peaks during the night in normally entrained individuals. This has been examined with the plasma and saliva levels of melatonin *per se* or the urinary secretion of 6-SM [8]. Urine samples were collected in the morning, which is when the urinary 6-SM secretion shows its maximum level, and these samples thus reflect the peak melatonin production during the previous night [9]. We measured the urinary excretion of 6-SM early in the morning in 11 samples from nine patients with CS aged from 4 to 31 years (4 males and 7 females), eight samples of eight patients with XP-A with ages from 7 to 29 years (5 males and 3 females), and nine samples of nine age-matched controls who did not have any chronic disorders with ages from 4 to 32 years (4 males and 5 females). The analysis of the urine 6-SM was performed with an ELISA, using an assay kit from GenWay Biotech, Inc. (San Diego, CA, USA). The urine samples were diluted prior to the assay. The results were revised by the creatinine (Cre) values, and we obtained a corrected urinary value of 6-SM (ng/mg Cre).

All of the data are presented as the mean [standard deviation (SD)]. The Stat Flex statistical program, version 6 (Artech Co., Ltd., Osaka, Japan), was used for the data analysis [10]. Bartlett's test was used to confirm whether the samples had equal variances, and they were judged to have inhomogeneous variances. Independent samples were examined by a Kruskal–Wallis test in order to compare the nonparametric data among controls, patients with CS, and patients with XP-A. A Mann–Whitney *U*-test was used to compare the nonparametric variables between each pair of groups.

2.3. Immunohistochemical analyses of the autopsy brains

The subjects included four cases of clinically and genetically confirmed CS who were aged from 7 to 35 years and six controls who did not have any pathological changes in the central nervous system and who were aged from 4 months to 38 years. Coronal sections of each formalin-fixed brain sample were cut and embedded in paraffin. Serial 6-μm-thick sections were cut from the diencephalon, including the hypothalamus and the NbM, and the lower midbrain, including the PPN. After microwave antigen retrieval, each section was treated with mouse monoclonal antibodies to microtubule-associated protein 2 (MAP2; EMD Millipore Corporation, Billerica, MA, USA), glial acidic fibrillary protein (GFAP; Nichirei Biosciences Inc., Tokyo, Japan), acetylcholinesterase (AchE; Thermo Fisher Scientific Inc., Rockford, IL, USA), tyrosine hydroxylase (TH; Thermo Fisher Scientific Inc.), calbindin-D28K (CD; Leica Microsystems Ltd., Milton Keynes, UK), vasopressin (VP; Biomeda Corporation, Foster City, CA, USA), and orexin A (OxA; Nuclea Diagnostic Laboratories LLC, Cambridge, MA, USA) at the following concentrations: 1:1 (GFAP), 1:100 (MAP2, CD, VP, and OxA), 1:250 (AchE), and 1:400 (TH). Antibody binding was visualized with the avidin–biotin–immunoperoxidase complex method (Nichirei Biosciences Inc.) according to the manufacturer's protocol. No staining was confirmed in the sections in the absence of antibody.

2.4. Quantitative evaluation and data analysis

The location of NbM was determined by immunohistochemistry for CD and was ventral to the globus