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Short Communication

Cerebrospinal fluid cytokine levels in type 1 narcolepsy patients very close to onset ☆

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ABSTRACT

Type 1 narcolepsy is caused by a loss of hypocretin (orexin) signaling in the brain. Genetic data suggests the disorder is caused by an autoimmune attack on hypocretin producing neurons in hypothalamus. This hypothesis has however not yet been confirmed by consistent findings of autoreactive antibodies or T-cells in patient samples. One explanation for these negative results may be that the autoimmune process is no longer active when patients present to the clinic. With increasing awareness in recent years, more and more patients have been diagnosed closer and closer to disease onset. In this study, we tested whether an active immune process in the brain could be detected in these patients, as reflected by increased cytokine levels in the cerebrospinal fluid (CSF). Using multiplex analysis, we measured the levels of 51 cytokines and chemokines in the CSF of 40 type 1 narcolepsy patients having varying disease duration. For comparison, we used samples from 9 healthy controls and 9 patients with other central hypersomnia. Cytokine levels did not differ significantly between controls and patients, even in 5 patients with disease onset less than a month prior to CSF sampling.

1. Introduction

The sleep disorder type 1 narcolepsy is caused by a loss of hypocretin (hcrt, also known as orexin) in the brain (ISCD-3, 2014). In addition most of the patients also suffer from the pathognomonic symptom cataplexy (muscle atonia triggered by emotions).

Autoimmunity due to environmental triggers is considered the most likely pathogenesis (Partinen et al., 2014), an hypothesis strengthened by the report of increased numbers of cases after H1N1-vaccinations or infections (Dauvilliers et al., 2010; Han et al., 2011; Partinen et al., 2012). Despite this, experimental proof of autoimmunity in narcolepsy is still missing. One reason for this lack of evidence may be that the autoimmune process is no longer active when patients present to the clinic. Several observations suggest that this could indeed be the case. First, type 1 narcolepsy patients typically have low or even undetectable levels of hypocretin-1 (hcrt-1) neuropeptide in cerebrospinal fluid (CSF) suggesting that the autoimmune destruction of the neurons is nearly complete already at the time of diagnosis (Nishino et al., 2000). Second, cases with intermediate levels are rarely seen (Andlauer et al., 2012; Bourgin et al., 2008; Knudsen et al., 2010). Finally, observations of onset a few weeks to months following H1N1 Pandemrix vaccinations, suggest that the disease process can occur in weeks to a few months (Knudsen et al., 2012; Partinen et al., 2012). In the case of immune responses towards viral infections, the virus is typically cleared within few weeks (Carrat et al., 2008; Ennis et al., 1981), thus immune factors resulting from the autoimmune process may decrease rapidly.

In the past, diagnostic delay for narcolepsy was very long, over 10 years in most cases (Thorpy and Krieger, 2014). Thanks to increasing awareness, more and more patients are now coming to the attention of clinicians closer and closer to disease onset,

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offering new lines of investigations. In this study, we hypothesized that an active immune process could still be present in some patients close to onset, and we focused our attention on immune markers as detected in the CSF. Prior studies have found oligoclonal bands in only a small fraction of patients and normal CSF white blood cell counts (Fredrikson et al., 1990; Schuld et al., 2004) thus we focused on CSF cytokine and chemokine levels. Dauvilliers et al. (2014) examined 12 cytokines and chemokines in the CSF of narcolepsy type 1 patients, finding increased levels of IL-4. To complement this study, we extended on this observation by including patients with short (<1 year) disease duration, and by including 51 cytokines and chemokines.

Potentially, CSF cytokines could be used as a biomarker for selecting patients for studies of autoimmune mediators such as autoantibodies and autoreactive T-cells. More importantly, this could also be used as selection criteria for immunomodulatory treatment, if 1 day the disease could be stopped before hypocretin cell destruction is complete.

2. Methods

2.1. Samples

After ethical approval and informed consent, CSF samples were collected in a clinical setting at three different centers. CSF was kept cold after sampling, and transferred to −80 °C for storage. Samples from Bologna, Italy and Glostrup, Denmark were shipped to Stanford on dry-ice and stored at −80 °C until analysis. The healthy volunteers were recruited under protocol #13366 at the Stanford Sleep Clinic where the lumbar puncture was performed. None of the volunteers had any sleep disorder complaints.

CSF Hcrt-1 levels and HLA-DQB1*06:02 status were determined as described before (Kornum et al., 2011). Detection limit in the hcrt-1 assay is 10 pg/ml and for all samples with undetectable levels of hcrt-1 the level was set at 10 pg/ml for data analysis.

2.2. Cohorts

Cohort 1: 9 typical type 1 narcolepsy patients seen in the Stanford Sleep Center clinic within 20 months of disease onset. All patient samples were collected before 2009. Disease onset was defined as onset of cataplexy, as this is the most specific marker of type 1 narcolepsy. All patients had clear cut cataplexy, were HLA-DQB1*06:02 positive and had a CSF hcrt-1 level <110 pg/ml. Details can be found in Table 1.

Cohort 2 and 3: 16 typical type 1 narcolepsy patients within 1 year from onset of cataplexy and 15 typical patients more than 1 year from onset. 14 of these were collected after the winter of 2009–10, none were vaccinated with the Pandemrix H1N1 vaccine. All had clear cut cataplexy, CSF hcrt-1 levels <110 pg/ml, and where DQB1*06:02 positive. 9 patients with other central hypersomnias (type 2 narcolepsy and idiopathic hypersomnia) were also included, all had hcrt-1 levels >250 pg/ml (normal levels). None of these had cataplexy and all were DQB1*06:02 positive. For this control group, disease onset was determined as onset of subjective excessive daytime sleepiness.

5 narcolepsy patients from cohort 2 had been lumbar punctured very close to an abrupt, well defined onset, and these were used in a subanalysis comparing to carefully matched patients. The five patients close to onset had an age span of 7.5–14.4 years and a BMI span of 15.6–23.3. For the comparison we therefore included all type 1 narcolepsy patients in cohort 2 within the age interval 5–15 years and BMI interval 15–23.5. Using these criteria we got a very well matched control group (Table 1).

2.3. Luminex assay for cerebrospinal cytokines

CSF samples were processed by the Stanford core facility: Human Immune Monitoring Center. CSF samples were spun 10 min. at 300 g, and run undiluted in technical duplicates using human 51-plex Luminex kits (Luminex 200 IS System, Affymetrix). Briefly, beads conjugated with capture antibodies for each cytokine, CSF, and cytokine standards were added to pre-wetted 96-well filter-bottom plates and incubated 2 h at room temperature plus 18 h at 4 °C. After washing, the plates were incubated with a biotin-labeled detector antibody cocktail for 2 h at room temperature, washed again, incubated with streptavidin-PE for 40 min, and washed. Samples were acquired in reading buffer on the Luminex MAP200 instrument, with collection criteria set for 100 beads per analyte (2000 beads total). Data were analyzed using MasterPlex software (Hitachi Software Engineering America Ltd., MiraiBio Group).

Concentrations of the following cytokines were determined: CD40 ligand, CXCL5/ENA78, CCL11/Eotaxin, FGFβ, CSF3/G-CSF, CSF2/GM-CSF, CXCL1/GRO alpha, HGF, sICAM-1, IFNα, IFNβ, IFNγ, IL-10, IL-12A/IL-12p70, IL-12B/IL-12p40, IL-13, IL-15, IL-17, IL-17F, IL-1A, IL-1B, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, CXCL10/IP10, Leptin, LIF, CSF1/M-CSF, CCL2/MCP-1, CCL7/MCP-3, CXCL9/MIG, CCL3/MIP-1A, CCL4/MIP-1B, NGF, PAI-1, PDGFB, CCL5/Rantes, Resistin, SCF, TNFL6/sFAS ligand, TGFα, TGFβ, TNF/TNFα, LXα/TNFβ, TNF10/Trail, sVCAM-1, VEGF.

The coefficient of variation (CV) was determined for each measurement (performed in duplicates). Only 4% of 2550 cytokine concentration measurements had CV >25, most of these were samples where the value fell below the detection limit. Lower limit of detection (LOD) level was set as the value measured in the blank plus 3 times the standard deviation of the blank (Table S1 and S2). Detectable samples with CV >25 were excluded from the data analysis.

Table 1
Clinical characteristics of patients and controls.

Group	Cohort 1			Cohort 2		Cohort 3	
	Narcolepsy type 1	Healthy controls	Narcolepsy < 1 year	Narcolepsy > 1 year	Other hypersomnias	Narcolepsy < 1 month	Narcolepsy 3 month–3 years
N	9	9	16	15	9	5	12
0602 positive	9	5	16	15	9	5	12
Age	22.4 [6–62]	34.3 [18–42]	10.2 ± 0.3	11.2 ± 2.0	10.9 ± 2.6	10.4 ± 2.7	10.2 ± 2.6
BMI[a]	23.1 [18–31]	23.2 [20–27]	22.2 ± 6.2[a]	24.4 ± 5.3[a]	20.4 ± 5.7[a]	19.6 ± 3.1	19.7 ± 2.5
Sex (M/F)	3/6	3/6	7/9	7/8	5/4	4/1	6/6
Months from onset	10.2 [2–20]	–	3.5 ± 2.5	23.8 ± 7.7	31.4 ± 16.8	0.6 ± 0.2	14.5 ± 9.7
CSF Hcrt-1	47.3 ± 33.9	249.9 ± 28.7	25 ± 29	21 ± 26	364 ± 93	33 ± 23	33 ± 26

[a] Data from 1 patient is missing in the three marked groups.