Autoimmunity at the neuromuscular synapse: pathophysiology and disease course

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CHAPTER 2

Antibodies to active zone protein ERC1 in Lambert-Eaton myasthenic syndrome

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Abstract

Lambert-Eaton myasthenic syndrome (LEMS) is characterized by fluctuating muscle weakness and autonomic dysfunction. In 90% of the LEMS patients the disease is associated with auto-antibodies against the voltage-gated calcium channels (VGCC). Several auto-immune responses against other antigenic targets have been described to (co)-occur in LEMS patients. To identify new LEMS associated small cell lung cancer (SCLC) markers immunoprecipitation with a SCLC cell line was performed. We discovered strong immunoreactivity against the 120 kDa large ERC1 protein in one tumor-negative VGCC-positive LEMS patient. A recombinant ELISA assay and a cellular assay expressing GFP-tagged full length ERC1 were used to confirm the presence of auto-antibodies against ERC1 in this patient. Additional testing of 58 LEMS patients including 9 VGCC auto-antibody negative LEMS patients, 48 myasthenia gravis patients, 84 control patients with other diseases and 12 healthy controls revealed no other cases. ERC1 is therefore a new, but rare, antigen in LEMS.

Introduction

Lambert-Eaton myasthenic syndrome (LEMS) is an autoimmune disorder characterized by fluctuating proximal muscle weakness, loss of tendon reflexes and autonomic dysfunction [1]. In 90% of LEMS patients the disease is associated with pathogenic antibodies against the presynaptic P/Q-type voltage-gated calcium channel (VGCC) [2]. Dysfunction of these channels inhibits release of acetylcholine from the presynaptic endplate, resulting in impaired neuromuscular transmission and muscle weakness.

In 50-60% of patients small cell lung cancer (SCLC) is diagnosed [1]. To study the interaction of the immune system of LEMS patients with SCLC antigens, we performed immunoprecipitation experiments using sera of tumor-positive and –negative LEMS patients on SCLC cell lysates.

Case report

A 57-year-old man experienced trouble keeping up with his wife at recreational cycling. One year later, he noticed fluctuating ptosis, followed by mild dysarthria, diplopia and impotence. Symptoms increased during the course of the day. The patient had never smoked. Neurological examination revealed mild bilateral ptosis. Tendon reflexes were low in the arms and absent in the legs. Strength at the time of examination was normal. Anti-AChR antibodies were absent, anti-P/Q-type-VGCC antibodies were positive at 218 pmol/L (upper reference value 100 pmol/L). Electrophysiological examination showed low compound muscle action potential (3.2 mV) at rest, 21% decrement after repetitive nerve stimulation of abductor digiti minimi muscle at 3 Hz and 127% increment in the same muscle after exercise. Diagnosis of LEMS was made based on symptoms, positive anti-VGCC antibodies and electrophysiological findings. The patient had a DELTA-P tumor prediction score of 1[3] and repeated chest CT-scans and a follow-up over 7 years showed no tumor. Leg strength and ptosis improved with oral 3,4-diaminopyridine treatment. Screening for antibodies to endomysium and reticulin was positive, however no clinical signs of celiac disease were found.

Material and methods

To identify new antigenic markers in LEMS the H187 [NCI-H187], H69 [HTB-119], H82 [HTB-175] and H1607 [NCI-H1607] SCLC cell lines (ATCC, Manassas, VA, U.S.A.) were cultured according to the manufacturer's instructions. For immunoprecipitation the cells were lysed in NP-40 buffer (1% [v/v] NP-40; 150mM NaCl; 50mM Tris/HCl pH7.5) for 5 min at 4^{o}C and pre-cleared with protein A sepharose beads (Amersham, GE healthcare, Den Bosch, The Netherlands) for 60 minutes on ice. Patient IgG's were bound to protein A beads (serum diluted 1:200) for 60 min on ice, after which the beads were incubated with the pre-cleared lysate for 90 minutes at 4 ^{o}C. Bound antibody-antigen complexes were washed 5 times in NP-40 buffer, dissolved in 2x sample buffer (16% [v/v] glycerol; 0.15M DTT; 3.3% [v/v] SDS; 0.01% [w/v] bromophenol blue; 20 mM Tris/HCl pH6.8) and loaded on

a sodium dodecyl sulfate polyacrylamide gel. Unique protein bands were digested in-gel with trypsin and analysed by LC-iontrap mass spectrometry as described previously [4].

Patient serum reactivity to ELKS/RAB6-interacting/CAST family member 1 (ERC1) was confirmed with recombinant ERC1 fragments (ERC1/ELKS/Rab6-interacting/CAST family member 1; Isoform 1 **Uniprot: Q8IUD2-1**) in ELISA. Overlapping recombinant ERC1 fragments were amplified from RNA from the SCLC cell line NCI-H187 by RT-PCR using the following primers: ERC1 part 1 (aa 1-403) forward primer: 5'- CATATGTATGGAAGTGCCCG- 3'and reverse primer: 5'- CTCTTCCAGGTCTCGAAGC -3' ERC1 part 2 (aa 384-783) forward primer: 5'- CCTCTATGGAGCGTGGGC -3' and reverse primer 5'- TTCCAACTCAGCTATCTTCTTATCT -3' ERC1 part 3 (aa 764-1116) forward primer: 5'- GGAGGTGGAAAATGAGAAGAA -3'and reverse primer: 5'- CTCGAGAGAGGACTCTTCCA -3' and ligated into the pET28A vector using the XhoI and NdeI restriction sites. Recombinant protein production and purification of ERC1 fragments as well as the ELISA, were performed as reported [5]. To investigate the occurrence of ERC1 autoimmunity, 12 healthy controls and 189 additional patients with LEMS, myasthenia gravis and other autoimmune or neuromuscular diseases were tested (table 1). All patients were included after obtaining informed consent and approval by the Medical Ethical Committee of the LUMC.

Eukaryotic GFP-tagged flERC1 expression in U2OS was enabled by first cloning flERC-1 from the three overlapping fragments with the forward primer of ERC1 part 1 and the reverse primer of ERC1 part 3 into the pCR-Blunt II-TOPO vector, after which the sequence was subcloned into p-EGFP-N2 vector via the EcoRI sites provided by the TOPO vector. Immunoreactivity against flERC1 was established by immunostaining according to a previously described protocol [6] and imaged with a Leica DM 5500 fluorescent microscope.

Results

Western blot analysis of four different SCLC cell lines (H187, H69, H82 and H1607) using LEMS patient sera revealed strong reactivity of one patient serum against an approximately 120 kDa large protein (Figure 1A). The protein was immunoprecipitated from the H187 cell line with the serum IgG of this patient and mass spectrometry analysis identified the protein as being ERC1 (Mascot protein score 364, 16 unique peptides). ERC1 immunoreactivity was confirmed on ELISA with recombinant ERC1 fragments as well as eukaryotically expressed flERC1 (Figure 1B). ELISA experiments revealed that most of the immunoreactivity was directed against the amino-terminal residues. Subsequent testing of 12 healthy controls, 132 patients with different autoimmune diseases as well as 57 LEMS patients, including nine seronegative LEMS patients (Table 1), did not identify more patients with auto-antibodies against this protein.

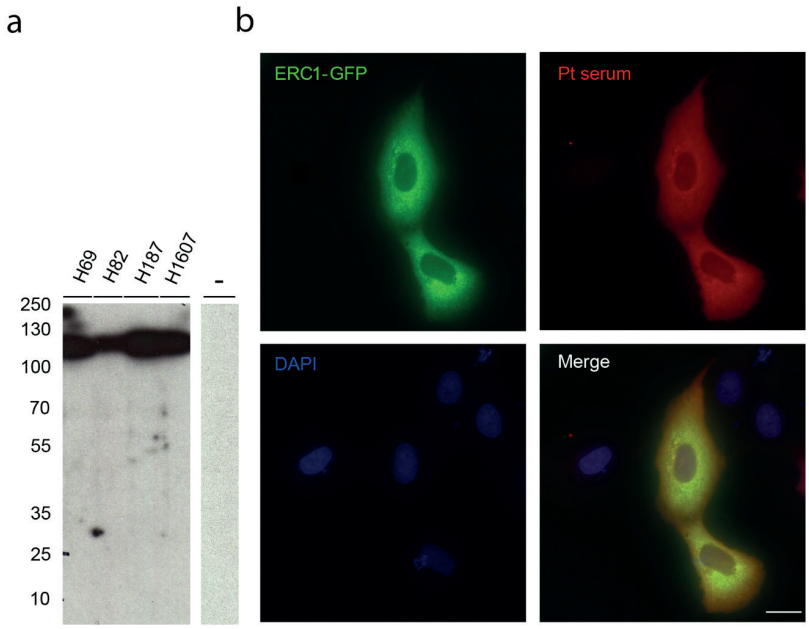

Figure 1. Patient serum immunoreactivity to eukaryotic fl ERC1
a) Western blot showing that the patient's serum recognized a 120 kDa protein from four different SCLC cell lines. The protein was identified as ERC1. Serum from a healthy control patient (-) did not show immunoreactivity against this protein.
b) Patient's serum only showed immunoreactivity (red) against ERC1-GFP transfected U2OS cells and not to the non-transfected cells, visualized with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining, confirming immunoreactivity of the auto-antibodies to ERC1. Scale bar 25 µm

	Nr of patients
Lambert Eaton myasthenic syndrome (total)	57
With SCLC	15
Anti-VGCC antibody negative	9
Myasthenia gravis (total)	48
AChR myasthenia gravis	22
MuSK myasthenia gravis	13
Seronegative myasthenia gravis	13
Other (total)	84
SCLC without LEMS	7
Anti-Hu syndrome	3
Polyneuropathy	39
Systemic lupus erythematosus	25
Celiac disease	10
Healthy controls	12

Table 1. Overview of patient populations tested for ERC1 immunoreactivity Patients N=201

Discussion

We identified ERC1 as an antigen in LEMS. Testing of additional sera of patients with LEMS or other autoimmune and neuromuscular disorders rendered no other positive cases, suggesting the presence of antibodies against ERC1 to be rare.

ERC1 is involved in several cellular functions. It can act as an oncoprotein following gene rearrangement [7] and is involved in activation of the NF-κB pathway, a transcription factor playing an important role in inflammation, development and cancer [8]. Furthermore, ERC1 contributes to the formation of presynaptic active zones and has a supportive but non-essential role in synaptic transmission, promoting calcium-dependent acetylcholine (ACh) exocytosis [7]. ERC1 was also indicated to directly associate with the b4 subunit of the VGCC in the cerebellum of mice [9].

We cannot exclude that the ERC1 antibodies are just a coincidental finding, due to the use of sensitive screening assays. However, we think that this is unlikely because ERC1 is involved in neuromuscular transmission, which is the target of the immune response in LEMS. Antibodies to the intracellular protein ERC1 could be a consequence of a disintegrating presynaptic axon due to the immune response against the VGCC's. Structural damage of the presynaptic axon would be in contrast to current knowledge about the pathophysiological mechanism. LEMS IgG is thought to induce functional loss of VGCC, without involvement of complement deposition and membrane lysis [10]. Alternatively, ERC1 antibodies could be part of an anti-tumor immune response against SCLC, which is known to express proteins necessary for exocytosis like VGCC and ERC1 [11]. However, this explanation remains implausible as this patient has no smoking history and a DELTA-P tumor prediction score of 1[1,12]. One could postulate that the ERC1 antibodies cause a false-positive VGCC radio-immunoprecipitation assay, as ERC1 is closely associated with VGCC. This phenomenon has been described for potassium channels [6]. However, ERC1 is an intracellular antigen, thus it is unlikely that antibodies to ERC1 are pathogenic. For the same reason ERC1 will not be able to induce epitope spreading causing secondary VGCC autoimmunity.

In parallel to ERC1, auto-antibodies to other presynaptic proteins involved in ACh release, such as synaptotagmin I, laminin β2 and the presynaptic muscarinic acetylcholine receptor, have been described in LEMS [1,12]. Synaptotagmin I, like ERC1, is present in presynaptic active zones and in SCLC cells. Immunizing rats with segments of Synaptotagmin I induced electrophysiological abnormalities reminiscent of presynaptic dysfunction as seen in LEMS [12]. We specifically screened sera of seronegative LEMS and MG patients for immunoreactivity against ERC1. Since no reactivity to ERC1 was found in these sera, antibodies to ERC1 are not a marker for seronegative LEMS or MG.

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