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Autoimmunity to Gephyrin in Stiff-Man Syndrome

Case Study

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Summary

Stiff-Man syndrome (SMS) is a rare disease of the central nervous system (CNS) characterized by chronic rigidity, spasms, and autoimmunity directed against synaptic antigens, most often the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD). In a subset of cases, SMS has an autoimmune paraneoplastic origin. We report here the identification of high-titer autoantibodies directed against gephyrin in a patient with clinical features of SMS and mediastinal cancer. Gephyrin is a cytosolic protein selectively concentrated at the postsynaptic membrane of inhibitory synapses, where it is associated with $GABA_A$ and glycine receptors. Our findings provide new evidence for a close link between autoimmunity directed against components of inhibitory synapses and neurological conditions characterized by chronic rigidity and spasms.

Introduction

Paraneoplastic neurological syndromes include a variety of disorders of the peripheral (PNS) or the central (CNS) nervous system that occur in association with cancer but are not the result of metastasis or tumor compression (Dalmau and Posner, 1999; Darnell, 1999). There is increasing evidence that many of these disorders, including paraneoplastic Stiff-Man syndrome (SMS), are associated with autoimmunity against neuronal antigens. The prevalent hypothesis is that cancer tissues may express neuronal antigens and that in some patients, an immune response directed against these antigens spreads to the nervous system, thus inducing neurological symptoms (Anderson et al., 1987).

SMS (also known as Stiff-Person syndrome) is an acquired rare disorder of the CNS characterized by rigidity and spasms of the body musculature (Moersch and Woltman, 1956; Lorish et al., 1989; Blum and Jankovic, 1991; Stayer and Meinck, 1998). The disease resembles a chronic form of tetanus and affects mostly women. Clinical and pharmacological evidence suggests that SMS results from an impairment of inhibitory pathways that control the activity of motoneurons in the brainstem and spinal cord (Meinck et al., 1984; Floeter et al., 1998). Electromyography shows a persistent motoneuron activity that is indistinguishable from voluntary muscle activity, except that the patients are not able to relax. Most patients respond to high doses of drugs that enhance the action of GABA, such as benzodiazepines, baclofen, or sodium valproate. These treatments, however, do not affect the slowly progressive course of the disease. Patients are eventually bedridden, and respiratory failure or sudden death may occur (Mitsumoto et al., 1991). In the majority of cases, an autoimmune pathogenesis appears likely (Solimena and De Camilli, 1991).

More than 60% of patients with SMS have high titer autoantibodies directed against glutamic acid decarboxylase (GAD) (Solimena et al., 1988, 1990), the cytosolic enzyme that synthesizes the inhibitory neurotransmitter GABA in the nerve endings of GABA-secreting neurons (Erlander and Tobin, 1991). This finding supports the hypothesis that SMS results from an impairment of GABAergic transmission. Patients positive for anti-GAD autoantibodies represent an autoimmunity-prone population, as they frequently suffer from other organ-specific autoimmune diseases, most frequently type I diabetes (Solimena et al., 1990, 1995). Notably, pancreatic β cells, the targets of autoimmunity in type I diabetes, are among the few cell types outside the CNS expressing detectable levels of GAD (Reetz et al., 1991). Furthermore, anti-GAD autoantibodies are found in the majority of pre- and newly diagnosed diabetic subjects, albeit with lower titer than in patients with SMS (Baekkeskov et al., 1990; Solimena and De Camilli, 1991; Verge et al., 1998).

In ~5% of patients, SMS is associated with cancer, generally breast cancer, and these patients typically are positive for autoantibodies directed against amphiphysin I (De Camilli et al., 1993; Folli et al., 1993; Floyd et al., 1998). Amphiphysin I is a cytosolic presynaptic protein that is widely distributed at synapses, including inhibitory synapses (Lichte et al., 1992; David et al., 1996; Wigge and McMahon, 1998; Takei et al., 1999). This protein is present at only very low concentration in other tissues but is often overexpressed in breast cancer cells (Floyd et al., 1998). Strikingly, removal of the cancer and immunotherapy are accompanied by a significant improvement of the neurological symptoms (Folli et al., 1993; David et al., 1994), suggesting that in these patients, SMS has a paraneoplastic origin.

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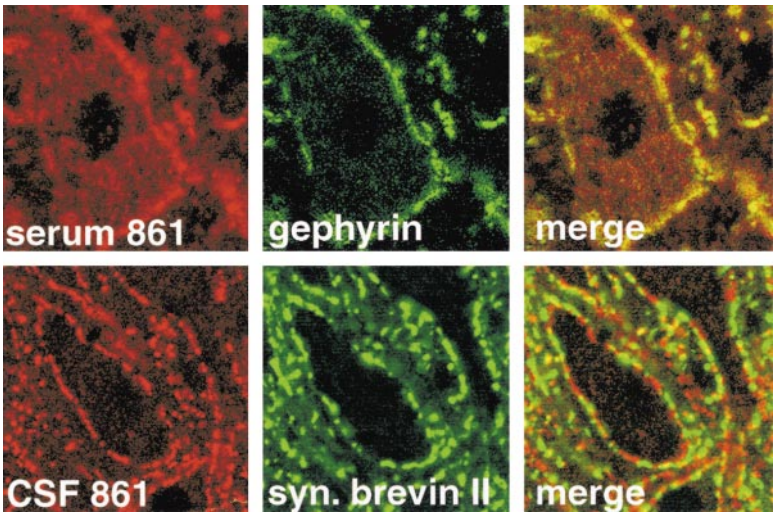

Figure 1. Autoantibodies of SMS Patient 861 React with Inhibitory Synapses of the CNS

Confocal microscopy of motoneurons in sections of the rat brainstem labeled with the serum (top left panel) or the CSF (bottom left panel) of SMS patient 861. Sections were double immunostained with a mouse anti-synaptobrevin II antibody (bottom middle panel)—a marker of a subpopulation of presynaptic terminals—or a mouse monoclonal anti-gephyrin antibody (top middle panel). The antigen recognized by the patient's autoantibodies is enriched in a subpopulation of synapses on the soma of large motoneurons.

We report here the case of a patient with clinical features of SMS, mediastinic cancer and autoantibodies against gephyrin.

Results

Clinical Findings

A 58-year-old male (patient 861 of our caseload) was admitted in June of 1998 with a 2 month history of progressive gait disturbance, dysarthria, and dysphagia due to muscle stiffness and spasms. There was no past history of illness, including diabetes mellitus, and the family history was unremarkable. On neurological examination, there was muscle stiffness of the neck, trunk, shoulders, and legs. Muscle cramps that disappeared during sleep were present on the left half of the face and in both legs. Stiffness of the tongue muscles was associated with slow movements of the tongue, as well as dysarthria and dysphagia with no abnormal pharyngeal reflex, and alterations of the esophagus by endoscopic examination. Ocular movements were normal, and opsoclonus was absent. Spasms occurred spontaneously or were precipitated by active and passive movements, acoustic stimulation, or touch. Presence of the spasms prevented the examination of jaw jerk and deep tendon reflexes in the legs. A reflex myoclonus associated with tonic spasms in the leg was elicited by cutaneous stimulation of the foot. Likewise, a startle response, such as head retraction reflex, was triggered by cutaneous stimulation of the face. Sensory functions were normal. Electromyography showed a low-firing frequency of normal units at rest. Renshaw's recurrent inhibition could not be estimated because the ratio between the H and the M waves (Kimura, 1989) was very low on his soleus. Brain, cervical, and lumbar MRIs were also normal. Routine laboratory investigations were normal, with the exception of the presence of anti-nuclear antibody (speckled type, 1:160) and of the tumor marker NCC-ST 439 (28.9 U/ml; normal range, $<$4.5 U/ml). No abnormalities in cell count, protein, or IgG levels were found in the cerebrospinal fluid (CSF). Gallium scintigraphy and chest CT revealed a mass in the mediastinum, while additional investigations were negative for the presence of metastasis elsewhere. Oral diazepam was initiated before surgery (20 mg/day). Both the stiffness and spasms lessened markedly, and the patient was able to walk and speak smoothly. After the removal of the mediastinal cancer, his symptoms further improved, and the patient was discharged. Histological investigation of the mediastinal tumor demonstrated an undifferentiated carcinoma of undetermined origin. A detailed clinical report of this patient has been reported elsewhere (Harada et al., 1999).

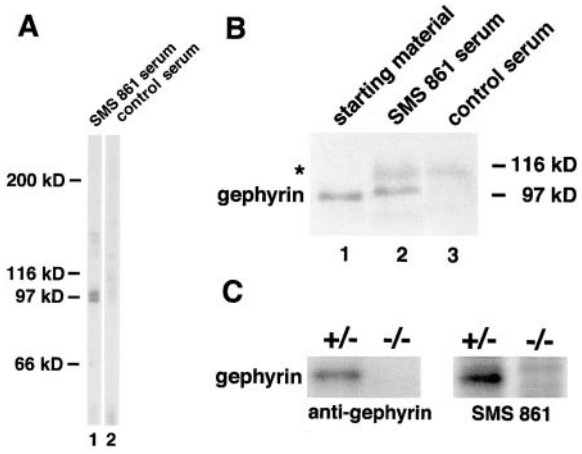

Figure 2. Autoantibodies of SMS Patient 861 Recognize Gephyrin in Rodent Tissues

(A) Postnuclear supernatant of rat brain homogenates immunoblotted with either the serum 861 or a control human serum. Serum 861 reacts with a ~97 kDa protein doublet.

(B) Immunoprecipitation from Triton X-100 extracts of rat brain postnuclear supernatants with either serum 861 (lane 2) or a control human serum (lane 3) followed by Western blotting with an anti-gephyrin antibody. An aliquot of the starting material for immunoprecipitation was loaded in lane 1. Serum 861, but not the control serum, immunoprecipitates gephyrin. An asterisk indicates an unrelated protein.

(C) Western blotting on total spinal cord homogenates from heterozygous (+/−) or homozygous (−/−) gephyrin null mice with an anti-gephyrin antibody or serum 861.

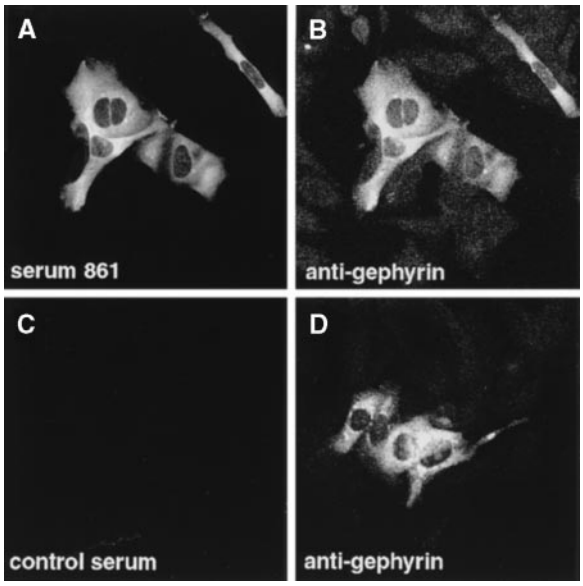

Figure 3. Autoantibodies of SMS Patient 861 Recognize Human Gephyrin in Transfected Fibroblasts

Double immunofluorescence and confocal microscopy of CHO cells transiently transfected with human gephyrin cDNA. Cells were immunostained either with the serum of patient 861 (A) or with a control serum (C), and with a monoclonal antibody directed against gephyrin (B and D).

Autoantibodies to Gephyrin

Evidence of autoimmunity in previous cases of SMS prompted the search for autoantibodies against neuronal antigens in this patient. Indirect immunocytochemistry on sections of brain and spinal cord using the serum of the patient as the source of primary antibodies revealed the presence of autoantibodies that specifically recognized a subset of synapses (Figure 1, bottom row). An identical immunostaining was obtained when the patient's CSF was used in place of the serum (Figure 1, bottom row, left panel). The staining pattern was clearly distinct from that produced by antibodies directed against GAD or amphiphysin I but was highly suggestive of inhibitory synapses. It was therefore compared with the staining produced by antibodies directed against gephyrin, a postsynaptic cytoplasmic protein associated with receptors for the inhibitory neurotransmitters glycine and GABA (Prior et al., 1992; Kirsch et al., 1993; Betz, 1998) (Figure 1, top row). The two staining patterns were identical, pointing to gephyrin as the putative autoantigen.

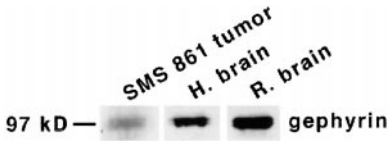

Figure 4. The Tumor Tissue of SMS Patient 861 Expresses Gephyrin

Protein extract from the tumor tissue of patient 861 (100 μg, lane 1), human brain (10 μg, lane 2), and rat brain (5 μg, lane 3) were immunoblotted with a monoclonal antibody directed against gephyrin. The gephyrin antibody recognizes a band of identical mobility in the tumor tissue and in human and rat brain.

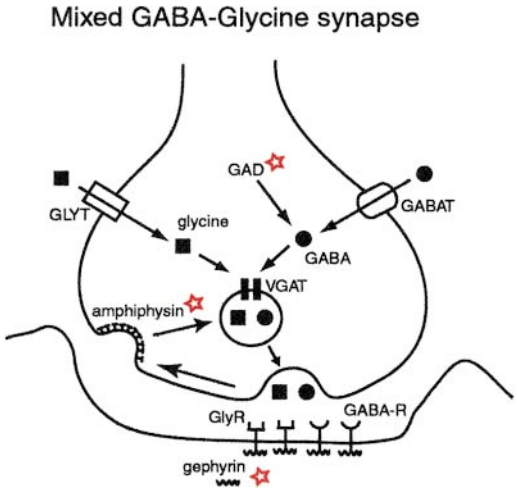

Figure 5. Mixed GABA/Glycine Synapses as the Putative Targets of Autoimmunity in SMS

Red stars indicate pre- and postsynaptic autoantigens of SMS. Abbreviations: GAD, glutamic acid decarboxylase; GABAT, GABA transporter; GABA-R, GABA receptor; GLYT, glycine transporter; GlyR, glycine receptor; VGAT, vesicular GABA/glycine carrier.

When tested against mammalian brain extracts by Western blotting, the autoantibodies of the patient did not react with GAD or amphiphysin I but selectively recognized a protein doublet with an apparent electrophoretic mobility of ~97 kDa, i.e., the same molecular weight as gephyrin (Figure 2A). In addition, the ~97 kDa autoantigen immunoprecipitated by the patient's autoantibodies comigrated with gephyrin and was recognized by an anti-gephyrin antibody (Figure 2B). The 97 kDa autoantigen was absent in brain extracts from gephyrin knockout mice (Feng et al., 1998) (Figure 2C). Finally, the patient autoantibodies selectively labeled CHO cells transiently transfected with the cDNA of human gephyrin but not the surrounding, untransfected CHO cells (Figures 3A and 3B). On the contrary, no staining was present in gephyrin-transfected CHO cells incubated with the serum of a control subject (Figures 3C and 3D). Taken together, these results conclusively prove that the serum and the CSF of the patient contained high-titer autoantibodies directed against gephyrin.

Neuronal autoantigens of paraneoplastic disorders are often detected in the cancer tissue (Furneaux et al., 1990), supporting the autoimmune origin of the neurological symptoms. Western blotting of the patient's mediastinal tumor with a monoclonal antibody directed against gephyrin detected a 97 kDa protein with an electrophoretic mobility identical to that of the gephyrin band present in human and rat brain (Figure 4). Thus, gephyrin was expressed in the patient's cancer.

The detection of autoantibodies against gephyrin in this patient is of considerable interest, considering that exaggerated muscle rigidity in response to sensorial or emotional stimuli, i.e., a common characteristic of SMS and a clinical feature of this patient, is present both in gephyrin knockout mice (Feng et al., 1998) and in hereditary hyperekplexia (Shiang et al., 1993; Saul et al., 1999). Hereditary hyperekplexia is a genetic disorder

associated with mutations in the glycine receptor, a main binding partner of gephyrin. This disease, also known as stiff-baby syndrome or startle syndrome, is characterized by the presence at birth of hypertonia, exaggerated startle reflex, and pronounced brainstem reflexes, including head retraction reflex (Kok and Bruyn, 1962; Andrew and Owen, 1997). The muscle stiffness usually recedes during the first year of life, but marked startle reflex, sometimes accompanied by transient hypertonia, can persist throughout adult life. Similar to patients with hyperekplexia, our patient had a pronounced head retraction reflex. Notably, some cases of hyperekplexia had originally been reported as congenital forms of SMS (Sander et al., 1980). A close relationship between these two disorders is further suggested by the observation that, in addition to GABA, glycine neurotransmission may also be affected in SMS (Floeter et al., 1998).

Discussion

Our data indicate that patients with clinical features of SMS can have autoantibodies directed against either pre- or postsynaptic components of inhibitory synapses (Figure 5). Spinal cord interneurons that control motoneuron activity have been shown to cosecrete both GABA and glycine (Jonas et al., 1998). We suggest that these mixed GABA/glycine synapses are the primary targets of autoimmunity in SMS. The postsynaptic hyperpolarization associated with the activation of $GABA_A$ receptors is slower but more prolonged than the inhibition mediated by glycine receptors (Jonas et al., 1998). Thus, the relative prevalence of chronic rigidity or startle responses in different SMS patients may be attributed to the variable degree of involvement of GABA or glycine neurotransmission.

The pathogenesis of paraneoplastic neurological syndromes of the CNS is still unclear. Each of these conditions is accompanied by the presence of high-titer autoantibodies directed against one or a few intracellular antigens shared by neurons and cancer cells, such as gephyrin in the patient described here. The question remains as to how autoantibodies arise and whether and how these autoantibodies can gain access to the cytosolic compartment of neurons and perturb the activity of their target antigens (Vincent et al., 1999; Whitney and McNamara, 1999). Alternatively, T cells or other associated antibodies directed against surface antigens may mediate the disease. There is evidence that T cell–mediated cytotoxicity, rather than humoral autoimmunity, mediates Purkinje cell death in the paraneoplastic cerebellar degeneration associated with anti-cdr2 autoantibodies (Albert et al., 1998). A variety of paraneoplastic syndromes affecting the neuromuscular junction, such as myasthenia gravis, Lambert-Eaton myasthenic syndrome, and Isaac's syndrome (Drachman, 1994; Lennon et al., 1995; Newsom-Davis, 1999), are caused by autoantibodies against surface antigens. At least two disorders of the CNS, Rasmussen's encephalitis and paraneoplastic cerebellar ataxia, can apparently be mediated by autoantibodies directed against the glutamate receptor 3 or the metabotrobic glutamate receptor 1, respectively (Rogers et al., 1994; Sillevis Smitt et al., 2000). Thus, autoantibodies directed against as yet unknown surface antigens may coexist with antibodies to dominant cytosolic antigens in the serum of patients with paraneoplastic neurological syndromes of the CNS but not be easily detectable in the assays routinely used for autoantibody screening. These autoantibodies could be generated by the spread of an autoimmune response against macromolecular complexes, including both surface antigens and associated intracellular proteins (Vincent et al., 1999; Whitney and McNamara, 1999).

In conclusion, the precise role of autoantibodies to cytosolic neuronal proteins in the pathogenesis of autoimmune neurological diseases deserves further investigation. However, the identification of gephyrin as the autoantigen of an autoimmune CNS disorder whose clinical presentation resembles that of humans and mice with genetic alteration of inhibitory synapses adds new evidence to the hypothesis that the autoantibodies of SMS patients are closely linked to pathogenic mechanisms.

Experimental Procedures

Antibodies

The serum and CSF of patient 861 and healthy control subjects were collected after informed consent and stored at −20°C until used. A mouse monoclonal antibody directed against synaptobrevin II was kindly provided by Dr. R. Jahn (Goettingen, Germany). A mouse monoclonal antibody directed against gephyrin was purchased from Transduction Laboratories (Lexington, KY). Cy3-conjugated goat anti-human IgG (Jackson Immunoresearch, West Grove, PA) or Alexa488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) was used for immunocytochemistry at a dilution of 1:50 or 1:100, respectively.

Cell Transfection and Immunocytochemistry

The full-length cDNA of human gephyrin (Z. Nikolic, C. Villman, C.-M. Becker) was subcloned as a ClaI-PstI insert into the mammalian expression vector pRK5. Transient transfection and immunostaining of CHO cells were performed as previously described (Dirkx et al., 1995). Indirect immunofluorescence staining on frozen sections of paraformaldehyde-fixed rat brain and spinal cord was performed as previously described (De Camilli et al., 1983). For the staining of gephyrin-transfected CHO cells, the serum of patient 861 and the control serum were used at a dilution of 1:100. For the staining of rat brain sections, the serum and CSF of patient 861 and control human sera were used at dilutions of 1:8 and 1:2, respectively. The monoclonal antibodies against gephyrin or synaptobrevin were used at a dilution of 1:100. Confocal microscopy was performed using a BioRad MRC 1024 (Bio-Rad, Hercules, CA).

Western Blotting

Postnuclear supernatants from rat brain and spinal cord of gephyrin$^{+/-}$ and gephyrin$^{-/-}$ mice were prepared as previously described (Reetz et al., 1991; Feng et al., 1998). Protein (100 μg) was loaded in each lane, separated by SDS gel electrophoresis, and then transferred onto nitrocellulose. For Western blotting, human sera and anti-gephyrin antibody were used at dilutions of 1:1000 and 1:200, respectively. Mouse and human IgG were detected with rabbit anti-mouse or rabbit anti-human IgG followed by ^{125}I-protein A (Solimena et al., 1990).

Tumor tissue from SMS patient 861 was pulverized under liquid nitrogen and resuspended (4 μl/μg tissue) in 150 mM NaCl, 10 mM HEPES (pH 7.5) containing a protease inhibitor cocktail (Complete, Roche, Indianapolis, IN). Next, the tissue powder was homogenized using a motorized pellet pestle and centrifuged at 600 × g. The pellet was discarded, and the supernatant was analyzed for protein content with the BCA assay (Pierce, Rockford, IL). Extracts from human brain in sample buffer (10 mg/ml) were purchased from Clontech (Palo Alto, CA). Protein from the tumor tissue and from human

and rat brain was separated by SDS gel electrophoresis and immunoblotted with the anti-gephyrin antibody followed by peroxidase-conjugated goat anti-mouse IgG and Supersignal chemiluminescence reagent (Pierce).

Immunoprecipitation

Immunoprecipitation with human sera from Triton X-100 rat brain extracts was performed as previously described (De Camilli et al., 1993). Immunoprecipites were separated by SDS gel electrophoresis and immunoblotted with the anti-gephyrin antibody as described above.

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