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Detection of new anti-neutral glycosphingolipids antibodies and their effects on Trk neurotrophin receptors

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Abstract **We screened sera from patients with various neurological disorders for the presence of anti-neutral glycosphingolipids antibodies and only found them in sera from relapsing polychondritis with limbic encephalitis patients.**

Neutral glycosphingolipids are resident in membrane lipid rafts where high affinity nerve growth factor (NGF) receptor, Trk is co-localized. Therefore, we examined whether these antibodies influence the action of NGF in NGF-responsive cells. The results strongly suggest that these antibodies enhance NGF-induced Trk autophosphorylation and neurite outgrowth as well as neurofilament M expression. These data strongly indicate that these anti-neutral glycosphingolipids antibodies have an functional impact on NGF-Trk-mediated intracellular signal transduction pathway.

Keywords: Neutral glycosphingolipids; Glucosylceramide; Galactosylceramide; Trk; Tyrosine phosphorylation; Nerve growth factor

1. Introduction

There is an accumulating evidence that suggests the pathognomonic linkage between anti-gangliosides antibodies and acute motor axonal neuropathy and the cranial, bulbar and sensory variants of Guillain-Barré syndrome [1]. Inspite of so many publications at present, their real roles for the development of the neuropathies still remain to be elucidated. Gangliosides are acidic glycosphingolipids comprising a ceramide moiety embedded in the lipid bilayer and sialylated oligosaccharide core that is extracellularly displayed and capable of acting as an autoantibody target [2]. On the contrary, there is a few investigation on anti-neutral glycosphingolipids antibodies in neurological disorders. Major neutral glycosphingolipids include glucosylceramide (GlcCer), lactosylceramide (LacCer), globotriaosylceramide, globoside or galactosylceramide (GalCer). It has been only reported that anti-GalCer antibody is present in patients with GBS after *Mycoplasma pneumoniae* infection [3]. GlcCer is one of the cardinal neutral glycosphingolipids. It is located in the plasma membrane primarily in lipid rafts, a special membrane structure that also contains many signaling moieties such as receptor-type tyrosine kinases, cholesterol, sphingomyelin, and glycosphingolipids [4]. GlcCer is synthesized from ceramide through the enzymatic action of GlcCer synthase and constitutes the first step in the synthesis of all types of gangliosides. GlcCer is involved in the regulation of many biological events such as neuronal apoptosis and drug resistance to anti-cancer drugs [5,6]. Additionally, GlcCer induces neuronal growth and epidermal differentiation [7,8].

In this study, we screened sera from patients with various kinds of neurological disorders for the presence of an antibody against neutral glycosphingolipids and found novel anti-neutral glycosphingolipids antibodies against GlcCer and GalCer but not LacCer only in sera from the patients with relapsing polychondritis (RP) complicated by limbic encephalitis (LE)-like symptoms. These antibodies were not found in sera from normal controls or patients with other neurological disorders to include uncomplicated RP. Then, we tried to characterize these anti-neutral glycosphingolipid antibodies using rat pheochromocytoma cell-line, PC12 cells. Unexpectedly, these novel antibodies enhanced the action of nerve growth factor (NGF) in cultured PC12 cells. This enhancement was mediated through NGF-induced Trk, a high affinity nerve growth factor receptor exhibiting tyrosine kinase activity, autophosphorylation response. The data clearly suggests that anti-neutral glycosphingolipids antibodies have an impact on the Trk-mediated intracellular signaling pathway.

2. Materials and methods

2.1. Human sera

After obtaining informed consent, sera were taken from various neurological patients (13 patients with viral encephalitis, 5 patients with paraneoplastic neurological syndrome, 5 patients with Guillain-Barré syndrome, 4 patients with Sjögren syndrome, and 5 patients with Parkinson's disease, 3 patients with RP complicated by LE and 2 patients with RP without LE) and 13 normal controls. The samples were stored at −20 °C until use. All of the RP + LE patients exhibited psychiatric symptoms such as memory disturbance and delusions. The development of these symptoms coincided with the onset of canonical symptoms of RP such as ear swelling and erythema.

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2.2. Anti-neutral glycosphingolipids antibodies

To evaluate the patients' sera for the presence of anti-neutral glycosphingolipids antibodies, purified GlcCer (a generous gift from Dr. Hirabayashi Y. Brain science Institute, RIKEN, Wako, Japan), GalCer (a generous gift from Dr. Furukawa K., Nagoya University School of Medicine, Nagoya, Japan), and LacCer (BioMol, USA) were processed by thin-layer chromatography (using a solvent of chloroform, methanol, and distilled water in a 55:20:3 vol/vol/vol ratio) and blotted onto a polyvinylidene difluoride membrane by an electrothermal blotter (ATTO Co. Ltd., Tokyo, Japan) as described previously [9]. This polyvinylidene difluoride (PVDF) membrane was probed with human sera (500× dilution) in blocking buffer (2% non-fat milk in the wash buffer phosphate-buffered saline (PBS) containing 1% Triton X-100). Following application of the second antibody, a positive band was detected with an enhanced chemiluminescence reagent (Perkin–Elmer Inc., Boston, MA). In some case, we also examined the presence of anti-GlcCer antibody using chemically synthesized pure GlcCer (from Dr. Y. Hirabayashi).

2.3. Cell culture and treatment

Rat pheochromocytoma-derived PC12 cells and stable transfectants of human *trk*-complimentary DNA (PCtrk cells) were cultured as described [10]. The cells were cultured as monolayers in DMEM containing 7.5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin [11]. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. For morphological observation, cells were cultured in 24-well culture plates and examined using a phase contrast microscope [12]. Following photography, the cells were lysed in sodium dodecyl sulfate (SDS)-sample buffer and evaluated by immunoblot analysis using an anti-neurofilament M antibody for neuronal differentiation as described previously [11].

2.4. Immunoprecipitation and immunoblot analysis

Because both neutral glycosphingolipids and Trk are located in the membrane lipid raft, a membrane structure that is also the site of transmembrane signaling, we examined the effect of anti-neutral glycosphingolipids antibodies on the high affinity NGF receptor, Trk. PCtrk cells were preincubated with sera (500× dilution) for 30 min and then stimulated with 50 ng/ml nerve growth factor (NGF) for 5 min. To evaluate receptor activity, Trk was immunoprecipitated with an anti-Trk antibody (α-Trk; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and subjected to immunoblot analysis using either an anti-phosphotyrosine antibody (α-PY, 4G10, Upstate Biotechnol. Inc., Lake Placid, NY) or α-Trk antibody as described [10–12]. In some case, antibody-positive sera were precleared with protein-L agarose to remove all immunoglobulins for 4 h in the cold room with agitation.

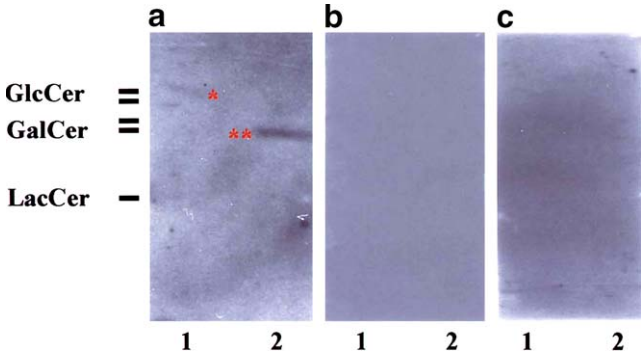

Fig. 1. Immunoblot analysis for neutral glycosphingolipids. Neutral glycosphingolipids (GlcCer and LacCer (lanes a(1), b(1) and c(1)), GalCer (lanes a(2), b(2) and c(2)) were electrothermally blotted onto a polyvinylidene difluoride membrane. The membrane was probed with sera from the patients with RP + LE obtained before (a) and after (c) steroid hormone therapy and with sera from patients with only RP (b). The position of each neutral glycosphingolipids was determined on a high-performance thin-layer chromatography plate developed simultaneously without electrothermal blotting. Red asterisks indicate the positive bands corresponding to GlcCer (∗) and to GalCer (∗∗). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

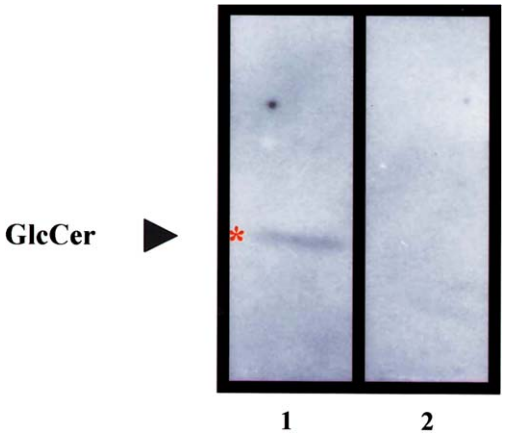

Fig. 2. Immunoreactivity against chemically synthesized pure GlcCer. Chemically synthesized pure GlcCer were electrothermally blotted onto a polyvinylidene difluoride membrane. The membrane was probed with sera from patients with RP + LE (1) and from disease controls (2). Red asterisks indicate the positive bands corresponding to GlcCer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

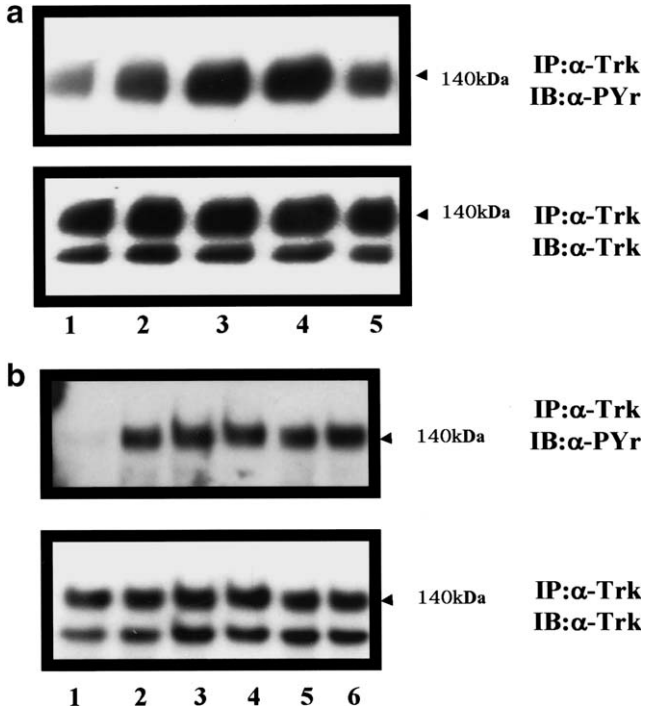

Fig. 3. Effect of sera on Trk-tyrosine kinase activity in response to NGF. PCtrk cells were cultured on 15 cm in diameter culture dishes and were pretreated with serum-free medium for 1 h. (a) Then, cells were treated with sera (×200 diluted in final) (lane 3, patient with RP + LE; lane 4, another patient with RP + LE; same patient with RP + LE obtained after steroid hormone treatment) for 30 min and stimulated with 50 ng/ml NGF (lanes 2–5) or without (lane 1) for 5 min. (b) Cells were treated with sera (×200 diluted in final) (lanes 3, 4, patients with RP only; lane 5, disease control (Parkinson's disease); lane 6, a normal control) for 30 min and stimulated with 50 ng/ml NGF (lanes 2–6) or without (lane 1) for 5 min. After stimulation, cells were collected with chilled PBS and subjected to the immunoprecipitation as described in "Section 2". These were performed at least three times with identical results. Arrows indicate position of the Trk protein.

These precleared sera were subjected to Trk-immunoprecipitation. Detection of positive bands was performed using Western blot Chemiluminescence Reagent Plus (Perkin–Elmer Inc.) [8,10].