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Biochimica et Biophysica Acta 1772 (2007) 543–548
Biochimica et Biophysica Acta
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Biological roles of anti-GM1 antibodies in patients with Guillain–Barré syndrome for nerve growth factor signaling

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Received 5 September 2006; received in revised form 28 December 2006; accepted 23 January 2007
Available online 28 January 2007

Abstract

To reveal the biological and pathological roles of anti-GM1 antibody in Guillain–Barré syndrome (GBS), we examined its effects on nerve growth factor (NGF) induced TrkA autophosphorylation (NGF-TrkA signaling) in PC12 cells, a sympathetic nerve cell line. The NGF-TrkA signaling is enhanced by exogenous GM1 ganglioside and this phenomenon is regarded as one of the functional aspects of GM1. The IgGs purified from patients' sera inhibited the NGF-TrkA signaling in GM1 pre-incubated PC12 cells. The degrees of inhibition by IgGs from patients paralleled their immunological reactivity to GM1. In addition, the IgGs also inhibited the neurite outgrowth of NGF-treated PC12 cells. Immunoglobulins in the rabbit sera, which were immunized by GM1, also caused a similar suppressive phenomenon. These results suggested that the anti-GM1 antibody could play roles in pathophysiology in anti-GM1 antibody positive GBS through interfering with the neurotrophic action of NGF and GM1 mediated signal modulation including NGF-TrkA signaling. It is suggested that the modulation of GM1 function is one important action of antibodies and could be one of the important mechanisms in GBS.

Keywords: Guillain–Barré syndrome; Anti-GM1 antibody; Trk A; Nerve growth factor

1. Introduction

In Guillain–Barré syndrome (GBS) and its variants there are miscellaneous antibodies against certain gangliosides. The kind of the antibody is closely related to the disease type and removal of these antibodies by plasmapheresis is an effective treatment to improve the diseases. Therefore these antibodies have been postulated as effecter molecules in these peripheral neuropathies. Particularly IgG class anti monosialogangliosides GM1 (GM1) antibodies have been implicated as potential pathogenic agents [1–3]. However the roles of these antibodies in the pathophysiology of GBS and molecular mechanisms to impair the nerve tissues are still unclear.

Recently GM1 has been known as not only a structural molecule but a functional molecule, i.e., modifier of signal transduction [4]. For example exogenous GM1 enhances the autophosphorylation of TrkA, a specific nerve growth factor (NGF) receptor, induced by NGF (NGF-TrkA signaling) and augments the neurite outgrowth in PC12 cells, a sympathetic nerve cell line [5–7]. However, little attention has been paid to the functional aspects of GM1 in the studies of GBS pathogenesis.

In this study, we adopted the potentiating effect of GM1 to NGF-TrkA signaling on PC12 cells [5–7] as the assay system for testing the biological activity of anti-GM1 antibodies, and tried to reveal the mechanisms of GBS pathogenesis caused by the anti-GM1 antibody.

2. Materials and methods

2.1. Isolation of immunoglobulin

Sera obtained from four GBS patients in the acute phase before plasmapheresis (Table 1) and four age-matched normal volunteers were tested for reactivity to GM1 [4] and *Campylobacter jejuni* by ELISA [8]. The IgG immunoglobulins were isolated from these sera using Protein-L (Nab™ Protein-

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doi:10.1016/j.bbadis.2007.01.011

Table 1
Clinical features of GBS patients

No.	Age	Sex	Diarrhea	Cranial nerve	Severity[a]	Recovery[b]	Electrophysiological diagnosis
#1	52	F	+	−	4	4	AMAN
#2	24	M	+	−	4	2.5	AMAN
#3	45	M	+	−	3	2	AMAN
#4	26	M	+	−	5	36<	AMAN

[a] Hughes functional grade [9] at the peak.
[b] Months until able to walk independently (Grade 2) from onset, AMAN: Acute motor axonal neuropathy.

L Spin Chromatography kit, Pierce) and the samples were adjusted to 1 mg/ml concentration and stored at −20 °C. In some experiments, IgG immunoglobulins were used, from which anti-GM1 antibodies were depleted with the GM1 immobilized plate.

2.2. Cell cultures

PC12 cells (Human Science Research Resource Bank, Osaka) were maintained in RPMI medium (RPMI1640, GIBCO BRL) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. To detect phosphorylated proteins, cells were cultured on a collagen-coated 12-well plate for 1 day, and then cultured in serum-free RPMI medium, when indicated, with 50 μM GM1 (Sigma) for 12 h at 37 °C. After exposure to GM1, cells were washed by serum-free RPMI medium and preincubated with immunoglobulin isolated from patients' or control serum, or rabbit anti-GM1 IgG (Calbiochem) at the indicated concentration (25–100 μg/ml) for 30 min at 37 °C. Then cells were treated with NGF (7S NGF, Sigma) at 50 ng/ml for 30 min [5]. For morphological studies, the cells were cultured on 6-well plates at 5×10^4 cells/well and treated with GM1, immunoglobulins and NGF in the same conditions described above. After an additional 72 h of culturing, ten different microscope fields were selected blindly, photographs taken, and the neurite outgrowth was quantitated by comparing the neurite length and the somal diameter. The neurite index was calculated as the ratio of the number of cells with neurites (longer than 2× somal diameter) to those without neurites [4].

2.3. Immunoprecipitation and immunoblotting

After NGF treatment, the cells were immediately washed by ice-cold PBS with 1 mM Na_3VO_4 and lysed in lyses buffer (20 mM Tris–HCl, pH 8.0/137 mM NaCl/1% NP-40/10% glycerol/50 mM NaF/1 mM Na_3VO_4/1 mM PMSF/1 μg/ml leupeptine/10 μg/ml aprotinin) for 30 min on ice with agitation. High-speed centrifuge (10,000×*g*) was performed to obtain cell free lysate and the lysates were normalized for proteins. To detect TrkA phosphorylation, the cell-free lysates were immunoprecipitated with anti-TrkA antibody agarose conjugated (Santa-Cruz) at 4 °C over night. The resultant immunoprecipitates were electrophoretically transferred to PVDF membranes after SDS-PAGE with 8% gels and were probed with anti-phosphotyrosine antibody (PY20, Zymed Laboratories Inc). Detection was performed according to the manufacturer's direction (ECL plus, Amersham). The membrane was reprobed with anti-TrkA monoclonal antibody (Santa-Cruz) using manufacturer-specified reprobing protocols (ECL manual, Amersham) [5–7]. To quantify the signals on the films, densitometry was performed on personal computer using NIH Image and the ratio of the phosphorylated proteins (p-TrkA) to the total amount of the proteins (total TrkA) was calculated.

3. Results

3.1. Clinical and Immunological profiles of the patients

All four patients had diarrhea before the onset of GBS symptoms and three patients showed positive anti-*Campylobacter jejuni* antibodies. They showed grade 3 to 5 severity (Hughes functional grade [9]) and were electrophysiologically diagnosed as acute motor axonal neuropathy (AMAN) on the grounds that they showed fibrillation voltage in electromyography and a decrease of compound muscle action potential with normal conduction velocity. All patients had IgG type anti-GM1 antibodies (Tables 1, 2).

Table 2
Relative reactivities of sera from patients and controls against the GM1

	GM1 ganglioside		
	IgG	IgM	IgA
Patients			
#1	+	+	+
#2	+	++	+
#3	++	+	−
#4	+++	−	−
Controls			
#1	−	−	−
#2	−	−	−
#3	−	−	−
#4	−	−	−

(−), (+), (++) and (+++) mean <×160, ×160, ×320, ×640 respectively against the indicated antigens using ELISA. Cut-off value is an average of healthy control [8].

3.2. Effects of GM1 incorporation on TrkA phosphorylation

It is reported that exogenous GM1 enhances TrkA autophosphorylation caused by NGF [5–7]. To confirm this phenomenon in our experimental system, we examined the

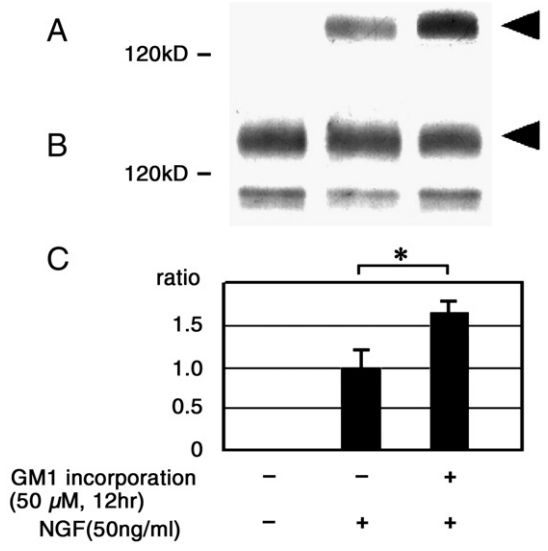

Fig. 1. Effects of GM1 incorporation to the NGF-TrkA signaling. (A) TrkA phosphorylation was assessed by immunoblot analysis using anti-phosphotyrosine antibody. Arrowheads indicate TrkA. (B) Total TrkA levels were determined using anti-Total TrkA antibody. (C) The ratio of phosphorylated TrkA to total TrkA were normalized to net intensity values for GM1 untreated controls and represented the mean with S.E. (error bar) of three replicate experiments. The asterisk shows significant ($P<0.05$) difference between indicated data pair.

effects of GM1 incorporation, 50 μM for 12 h, on TrkA phosphorylation in PC12 cells. We observed about a 2-fold increase in NGF-induced TrkA autophosphorylation in GM1-treated PC12 cells compared with that in GM1-untreated cells (Fig. 1).

3.3. Influence of GM1-immunized rabbits' sera and immunoglobulins derived from normal controls and patients with GBS on NGF-TrkA signaling

To ascertain that anti-GM1 antibody inhibits the potentiating effects of GM1 on NGF-TrkA signaling we performed examinations using sera from GM1-immunized rabbits. It suppressed the exogenous GM1 enhanced NGF-TrkA signaling on GM1-treated PC12 cells with dose dependency (Fig. 2A–C) and GM1 non-immunized rabbit's sera didn't show such depressive effects (Fig. 2D).

Immunoglobulins from the four normal controls that did not react to GM1 (Table. 2) showed no effects on the TrkA autophosphorylation (Fig. 3). Immunoglobulins derived from GBS patients inhibited the TrkA autophosphorylation dose-dependently (25 to 100 μg/ml) (Fig. 4) and proportionally to their reactivity to GM1 (Table 2 and Fig. 5A–C). The anti-GM1 antibody-depleted immunoglobulins could no longer depress the TrkA autophosphorylation (Fig. 5D).

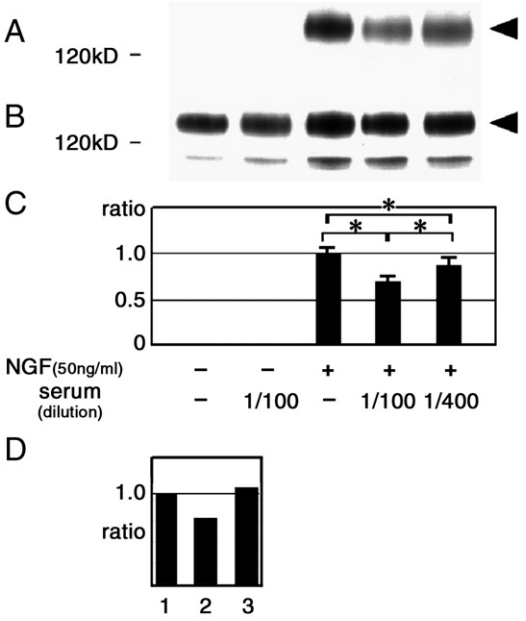

Fig. 2. Effects of antibody from GM1-immunized rabbit to the NGF-TrkA signaling on GM1-incorporated PC12 cells. (A) TrkA phosphorylation was assessed by immunoblot analysis using anti-phospho-tyrosine antibody. Arrowheads indicate TrkA. (B) Total TrkA levels were determined using anti-Total TrkA antibody. (C) The ratio of phosphorylated TrkA to total TrkA were normalized to net intensity values for NGF treated without the antibody controls and represented the mean with S.E. (error bar) of three replicate experiments. The asterisks show significant ($P<0.05$) differences between indicated data pairs. (D) The ratio of phosphorylated TrkA to total TrkA. Cells were treated by 50 ng/ml NGF without rabbit's serum (1), with serum from GM1 immunized rabbit (2) and with serum from unimmunized rabbit (3).

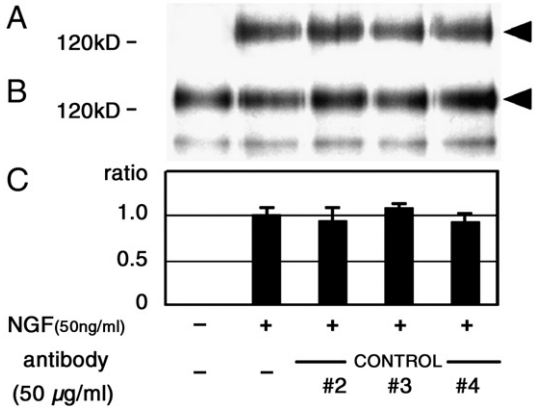

Fig. 3. Effects of IgG isolated from normal controls to the NGF-TrkA signaling on GM1 incorporated PC12 cells. (A) TrkA phosphorylation was assessed by immunoblot analysis using anti- phospho-tyrosine antibody. Arrowheads indicate TrkA. (B) Total TrkA levels were determined using anti-Total TrkA antibody. (C) The ratio of phosphorylated TrkA to total TrkA were normalized to net intensity values for NGF treated without the antibody controls and represented the mean with S.E. (error bar) of three replicate experiments. There were no significant differences between each data pairs.

3.4. Effects of immunoglobulins on NGF induced neurite outgrowth

Photomicrographs of GM1-incorporated PC12 cells treated with or without NGF and immunoglobulins are shown in Fig. 6. There is no neurite formation in the absence of NGF (Fig. 6A–D). NGF-induced neurite outgrowth is arrested by the addition of immunoglobulins only from GBS patients (Fig. 6E–H). The neurite index indicates the almost complete

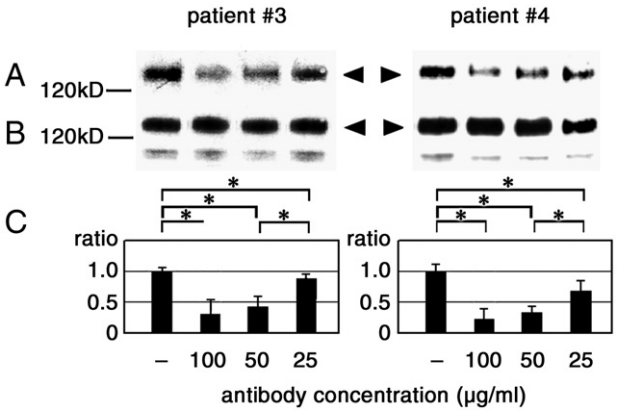

Fig. 4. Dose dependency of the IgG isolated from GBS patients to the NGF-TrkA signaling on GM1-incorporated PC12 cells. Cells were stimulated by 50 ng/ml NGF. (A) TrkA phosphorylation was assessed by immunoblot analysis using anti- phospho-tyrosine antibody. Arrowheads indicate TrkA. (B) Total TrkA levels were determined using anti-Total TrkA antibody. (C) The ratio of phosphorylated TrkA to total TrkA were normalized to net intensity values for the antibody untreated controls and represented the mean with S.E. (error bar) of three replicate experiments. The asterisks show significant ($P<0.05$) differences between indicated data pairs.

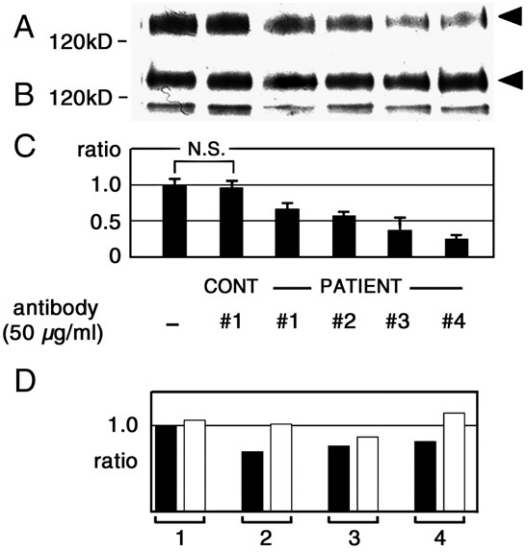

Fig. 5. Effects of IgG isolated from GBS patients and a control to the NGF-TrkA signaling on GM1 incorporated PC12 cells. Cells were stimulated by 50 ng/ml NGF. (A) TrkA phosphorylation was assessed by immunoblot analysis using anti- phospho-tyrosine antibody. Arrowheads indicate TrkA. (B) Total TrkA levels were determined using anti-Total TrkA antibody. (C) The ratio of phosphorylated TrkA to total TrkA were normalized to net intensity values for the antibodies untreated controls and represented the mean with S.E. (error bar) of three replicate experiments. There are significant ($P<0.05$) differences between all data pairs without indicated by N.S. (D) The ratio of phosphorylated TrkA to total TrkA. The black/white bars indicate the ratios obtained from GBS patient's immunoglobulin before/after treatment by GM1 absorption column. 1: Control #1, 2: Patient #4, 3: Patient #3, 4: Patient #1.

inhibition of NGF-induced neurite outgrowth by patients' immunoglobulins (Fig. 6E–H).

4. Discussion

In this paper we showed that the GBS (AMAN) patients' IgG, which was reactive to GM1, inhibited the potentiating effect of GM1 to NGF-TrkA signaling dose-dependently and interfered with the NGF induced neurite outgrowth in PC12 cells. It is reasonable to suppose that this inhibition was due to the IgG reacting directly to GM1, because sera from rabbits immunized by GM1 had similar suppressing effects with dose-dependency (Fig. 2), the degree of the inhibition was in proportion to the reactivity to GM1 of incubated IgG (Fig. 4), and the anti-GM1 antibody-absorbed immunoglobulins didn't have such depressive effects. On the other hand, O'Hanlon et al. reported that monoclonal anti-GM1 IgM antibody from human neuropathy induced small neuritogenic effects on PC12 cells [10]. Although it appears to conflict with our results, this difference may arise from the difference between IgG and IgM, in that anti-GM1 IgM antibody relates to multifocal motor neuropathy rather than to AMAN. These results suggested that the interaction between GM1 on plasma membranes and anti-GM1 antibodies affected the *in vivo* nervous system in the patients with anti-GM1–antibody-positive GBS, particularly with AMAN in which mainly affected on the axon of neuron, since the PC12 cell is not a model of Schwann cell but nerve cell.

Ten to 42% of patients with GBS have high titer of anti-GM1 antibody [11–13] and it has been suggested that IgG antibodies against GM1 were strongly associated both with axonal degeneration and reversible conduction failure in GBS [14,15]. The effects of anti-GM1 antibodies on the electrical activity of nerve tissues have been reported and it was suggested that the antibodies could cause damages to neural membranes or interfere with nerve conduction, or both [16–19]. Nevertheless it is still difficult to explain, for example, why there are many autoantibody-negative patients, or why damage to the nerve conduction is reversible. These facts suggest that we have to consider another functional aspect of anti-GM1 antibody in GBS pathogenesis. Our results indicated the functional significance of anti-GM1 antibody, which is the interference with the biological function of GM1.

In some peripheral neuropathies neurotrophic factors and their receptors' expression increased at peripheral nerve tissue [20,21]. Nerve growth factors have various effects on neuronal

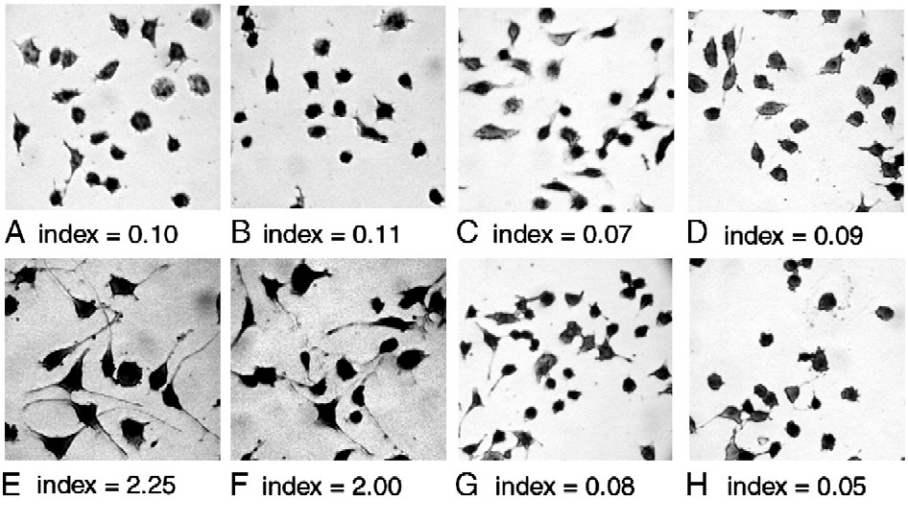

Fig. 6. Morphological examinations of neurite outgrowth of GM1-incorporated PC12 cells at 72 h after NGF stimulation. A–D. NGF (−), E–H. 50 ng/ml NGF, A and E: IgG (−), B and F: 100 μg/ml IgG (control), C and G: 100 μg/ml IgG (patient #2), D and H 100 μg/ml IgG (patient #4). The neurite index was shown (see Materials and methods).

cells including regeneration and survival. Although their pathophysiological roles in GBS were unclear, the fact of increased expression suggests their functional significance in GBS pathophysiology. For example, it is well known that NGF plays important roles in nerve regeneration and repair. In addition, anti-GM1 antibodies and its subclass pattern were tightly related to the slow recovery of GBS [22]. Owing to these facts, our results suggested the association between the blocking of neurotrophic factor signaling by anti-GM1 antibodies and the prolonged recovery of GBS.

GM1 has been implicated in neuronal development and differentiation [23]. Exogenous gangliosides promote neurite outgrowth in primary cultures of neuron and cell lines, and facilitate the repair of damaged neuronal cells. It was suggested that the exogenous ganglioside exert these effects by modulation of the function of growth factor receptors [6,7,24–26]. Therefore gangliosides could be understood as signaling modulators for some types of receptors [27]. Mutoh et al. have shown that the exogenously added GM1 gets tightly associated with TrkA receptor protein and enhances the autophosphorylation of the receptor [5]. It is still unclear how GM1 enhances the NGF-TrkA signaling but it is suggested that GM1 must be membrane bound to be effective, that is incorporated into the cell membrane [27,28]. Recently GM1 has been known as one of the structural and functional molecules in the micro domain of the cell membrane, the so-called "functional lipid raft" especially in immune cells [29,30]. PC12 cells also have this micro domain which contain TrkA [31]. Using confocal microscopy, we observed that anti-GM1 antibodies caused the aggregation of GM1 on the cell membrane of PC12 cells (preliminary unpublished observation). It can be speculated that anti-GM1 antibodies interfere with the NGF-TrkA signaling by functional modification of the micro domain. If this speculation is true, anti-GM1 antibody could affect other signal transduction not only in neural cells but also in immune cells, for example brain derived nerve growth factor (BDNF) signaling in motor neurons and T cell receptor signaling in lymphocytes. These modulations could contribute to the immunological pathophysiology in GBS.

In conclusion, the anti-GM1 antibody may have effects on the pathophysiology in anti-GM1–antibody-positive GBS through disturbing neurotrophic action. It is important to regard the antibody as a modifier to GM1 function and, from this point of view, further investigation is needed to elucidate the role of the "GM1–anti-GM1 interaction" for understanding the GBS pathogenesis.

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