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Preventive and therapeutic effects of the selective Rho-kinase inhibitor fasudil on experimental autoimmune neuritis

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ABSTRACT

We studied the effects of fasudil, a selective Rho-kinase inhibitor, on experimental autoimmune neuritis (EAN). Continuous parenteral administration of fasudil prevented the development of EAN induced by P0 peptide 180–199 in Lewis rats while it also reduced EAN severity when administered after disease onset. Immunohistochemical examination disclosed a marked decrease in the amount of inflammatory cell infiltration and attenuation of demyelination and axonal degeneration. Specific proliferation of lymphocytes from fasudil-treated rats in response to P0 peptide was significantly reduced as compared with those from phosphate-buffered saline (PBS)-treated rats. Fasudil treatment was associated with a significant reduction in secretion of IFN-γ; by contrast, secretion of IL-4 was almost the same in the fasudil- and PBS-treated groups. As a result, the IFN-γ/IL-4 ratio in the supernatant was significantly deceased in fasudil-treated rats compared with PBS-treated ones. Therefore, our results indicate a beneficial effect of selective blockade of Rho-kinase in animals with autoimmune inflammation of the peripheral nerves, and may provide a rationale for the selective blockade of Rho-kinase as a new therapy for Guillain-Barré syndrome.

1. Introduction

Guillain-Barré syndrome (GBS) and its animal model experimental autoimmune neuritis (EAN) are representative of the autoimmune diseases that affect the peripheral nervous system (PNS). EAN can be induced in susceptible animals by active immunization with PNS myelin or proteins such as P2 and P0, combined with Freund's complete adjuvant (FCA) [1–3]. Blood–nerve barrier (BNB) breakdown, immunoglobulin leakage, infiltration with activated T cells and macrophages, and predominantly perivenular demyelination of nerve roots are observed in EAN [4]. The immunopathogenesis of EAN involves the integrated attack of T-cells, B-cells and macrophages [5,6]. Inflammatory cell infiltrates in the PNS of GBS patients are also composed of lymphocytes and macrophages, which exert their effects through proinflammatory cytokines, such as TNF-α [7,8] while abnormal cellular responses to P2 and P0 proteins have been reported in some patients with GBS [9]. Thus, cellular immunity may also play a pivotal role in GBS pathogenesis.

Statins, which downregulate cholesterol synthesis through inhibition of 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, have anti-inflammatory effects and are protective in animal models of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) [10–14]. Although the exact mechanism underlying this protection is still unclear, it is partly attributable to the prevention of isoprenylation of Rho GTPase, which occurs downstream of the mevalonate pathway and is required for the membrane translocation and activation of GTPase proteins [15]. The Rho family GTPases (Rho, Rac and Cdc42) act as key regulators of the actin cytoskeleton. Rho-kinase is the major effector molecule for a variety of functions of Rho GTPase [16]. Activation of Rho-kinase by GTP-bound Rho (the activated form) leads to phosphorylation of ERM, myosin light chain, collapsin response mediator protein-2 (CRMP-2), LIM kinases 1 and 2, adducin and intermediate filament [17,18]. Inhibition of Rho-kinase activity induces suppression of cell proliferation and motility. Thus, statins may inhibit the cellular function of various cell types, including immunocytes, by inducing accumulation of the inactive form of Rho in the cytosol and thereby inhibiting downstream Rho-kinase signaling. Protein prenyltransferase inhibitors and flavonoids, which down-regulate Rho GTPase, have also been shown to be protective in EAE [19,20]. Thus, blockade of the rho/rho kinase system is considered to be beneficial for CNS inflammatory demyelination.

We previously reported that fasudil, a selective Rho-kinase inhibitor, has both protective and therapeutic effects in EAE animals [21]. In EAN animals, infiltration of Rho-positive macrophages and T cells into the spinal roots has been shown, and was correlated with the clinical severity of EAN [22]. Thus, the rho/rho kinase system could also be an important therapeutic target for peripheral nerve inflammatory demyelination. We therefore aimed to extend our study to EAN to

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explore if the drug is also useful for treating peripheral nerve inflammatory demyelinating diseases, such as GBS. In this paper, we demonstrate that fasudil acts in both a preventive and therapeutic fashion in EAN, in part through inhibition of P0-specific T-cell proliferation with a marked reduction in secretion of IFN-γ and suppression of the IFN-γ/IL-4 ratio.

2. Materials and Methods

2.1. Animals

Male Lewis rats, aged 7–8 weeks, with body weights of 250–300 grams, were purchased from Charles River Japan Inc. All animal protocols were approved by the Committee on Ethics in Animal Experiments of Kyushu University and were performed according to the Guidelines for Animal Experiments of Kyushu University and the Japanese Government.

2.2. Antigen and Antibodies

The P0 peptide 180–199 (SSHRGRQTPVLYAMLDHSRS) was synthesized using a peptide synthesis system (Applied Biosystems, MA, USA), based on the 9-flourenylmethyloxycarboneyl (Fmoc) strategy, and purified by C18 reverse-phase high performance liquid chromatography (HPLC). The purity of the peptide was 95% as determined by HPLC analysis [23]. The following primary antibodies were used for immunohistochemistry and western blot analysis: anti- ezrin/radixin/moesin (ERM) antibody, anti-phospho-ERM antibody (Cell Signaling Technology, MA, USA), anti-myelin basic protein (MBP) antibody (Acris Antibodies, Herford, Germany), and anti-neurofilament heavy chain (NF-H) 200 kD antibody (Chemicon, MA, USA).

2.3. Induction and clinical evaluation of experimental autoimmune neuritis in Lewis rats

Experimental autoimmune neuritis (EAN) was induced in Lewis rats by immunization with 200 μg of P0 peptide 180–199 emulsified in an equal volume of complete Freund's adjuvant containing 4 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco, KS, USA). The P0 emulsion (0.1 ml) was injected subcutaneously in both sides of a tail base. Every day, the rats were weighed and examined for clinical signs of EAN and scored as follows: 0, normal; 1, limp tails; 2, impaired righting reflex; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, moribund or dead.

2.4. Fasudil treatment using ALZET Mini Osmotic Pump

Fasudil (Asahi Chemical Industries, Tokyo, Japan) was administered continuously via a subcutaneously implanted ALZET mini-osmotic pump (DURECT Corporation, CA) with a dose of 100 mg/kg/day according to our study on experimental autoimmune encephalomyelitis (EAE) [21]. Briefly, in the preventive study, rats immunized with 200 μg of P0 peptide 180–199 were continuously administered fasudil from day −2, while in the therapeutic study, fasudil was started at the onset of neurological illness. Control animals were given phosphate-buffered solution (PBS) using the same osmotic pumps.

2.5. Antigen specific T-cell proliferation assays

Splenocytes were harvested and processed into single cell suspensions. Cells (2×10^5 cells/well) were distributed into 96-well round bottom plates (Falcon, Becton Dickinson, NJ, USA) and cultured with P0 peptide 180–199 (0.1, 0.5, 1, 5, 10 μM), phytohemagglutinin (PHA; 10 μg/ml), or medium alone. After 48 h of culture, 1 μCi of [^{3}H] thymidine was added to each well and cultures were harvested 18 h later and assessed for incorporation of [^{3}H] thymidine. All assays were performed in triplicate.

2.6. Cytokine analysis

The supernatants from cultures of splenocytes were harvested at 72 h and 120 h: 72 h for IFN-γ assays and 120 h for IL-4 assays. Both cytokines were analyzed using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions, as described previously [24]. All assays were performed in triplicate.

2.7. Western blot analysis for ERM phosphorylation

To quantify Rho-kinase activity in the liver (day 10) and lymph nodes (LNs) (day 10), western blot analysis of phosphorylated ERM (ezrin T567, radixin T564 and moesin T558) and total ERM was performed as described previously [25]. ERM is phosphorylated by Rho-kinase at T567 (ezrin), T564 (radixin) and T558 (moesin). Equal amounts of extracted proteins were separated by SDS-PAGE and subjected to immunoblot analysis. The regions containing ERM family proteins were visualized by electrochemiluminescence. Band intensities from western blots were quantified densitometrically by ImageJ 1.34 s downloaded from http://rsb.info.nih.gov/ij. The extent of ERM phosphorylation was normalized to the levels of total ERM.

2.8. Histopathology and immunohistochemistry

Rats were anesthetized and perfused with PBS and 4% buffered paraformaldehyde. Sciatic nerves were collected on day 18 after antigen immunization in the preventive study, and on day 35 in the therapeutic study. The tissues were dissected and post-fixed in 4% buffered paraformaldehyde solution and embedded in paraffin. After embedding, 6-μm-thick sections were prepared. For routine neuropathological evaluation, sections were stained with hematoxylin-eosin (H-E) stain. Because the function of NF-H is to maintain axonal structural integrity and disruption of axonal membrane integrity results in neurofilament proteins being released into the extracellular space [26], immunohistochemistry for NF-H was used for evaluation of axonal damage. MBP immunohistochemistry was used for evaluation of demyelination because it is a highly abundant protein in the PNS myelin. For immunohistochemical analysis, sections were deparaffinized in xylene, hydrated in ethanol, and incubated in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase. After rinsing in tap water, the sections were completely immersed in distilled water and autoclaved for 15 min to enhance the immunoreactivity of MBP and NF-H. Subsequently, sections were incubated with primary antibody diluted in 5% non-fat milk in 25 mM Tris-HCl pH 7.6 containing 0.5 M NaCl, 0.05% NaN_3 and 0.05% Tween 20 (TBST) for 1 h at room temperature. As a secondary antibody, peroxidase-labeled anti-rabbit IgG (Vector Laboratories, CA, USA) was used. The colored reaction product was developed using Simple Stain DAB solution (Nichirei, Tokyo, Japan). The sections were counterstained lightly with hematoxylin. Inflammatory cell infiltrates were graded by hematoxylin and eosin (H-E) stain as: 0, no abnormality; 1, cellular infiltration adjacent to a vessel; 2, cellular infiltration in immediate proximity to a vessel; 3, cellular infiltration around a vessel and in more distant sites [27]. The severity of demyelination was graded by MBP immunostaining as: 0, none; 1, isolated demyelinated axons perivascular or scattered; 2, many foci of perivascular demyelination; 3, extensive demyelination, perivascular and confluent [28].

2.9. Statistical analysis

Disease frequency was compared using Fisher's exact probability test. Ratios of phosphorylated ERM against total ERM, proliferation of T cells and cytokine production were compared using the Student's *t* test. All other statistics were analyzed using the Mann-Whitney *U* test. A value of $p<0.05$ was considered significant.