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자기면역성 뇌척수염 조직에서 extracellular signal regulated kinases의 발현

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Extracellular signal regulated kinases in the spinal cord of rats with experimental autoimmune encephalomyelitis

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Abstract : The phosphorylation of extracellular signal-regulated kinases (p-ERK) in the spinal cord of rats with acute monophasic experimental autoimmune encephalomyelitis (EAE) was studied using immunohistochemistry and treatment with inhibitor. P-ERK is constitutively expressed in glial cells in the normal spinal cord. In EAE, some inflammatory cells in the subarachnoid space were positive for p-ERK at the early stage, and its immunoreactivity declined when those cells infiltrated the parenchyma at the peak stage. In a blocking experiment using its inhibitor, the intravenous administration of PD98059 from day 7 to 13 post-immunization did not modulate EAE paralysis. Considering the results, we postulate that intravenous administration of PD98059 is not effective in ameliorating EAE paralysis, although many inflammatory cells express ERK in the subarachnoid space.

Key words : autoimmune encephalomyelitis, extracellular signal-regulated kinases, spinal cord

Introduction

Extracellular signal-regulated kinase (ERK) is one of three subgroups of the mitogen-activated protein (MAP) kinase family [4, 6]. The ERK pathway, which is also the 42-/44-kDa MAP kinase pathway, is activated in response to growth factors [2] and oxidative stress [1]. After phosphorylation of ERK (p-ERK), the p-ERK pathway participates in a wide range of cellular activities, including survival, proliferation, differentiation, and movement [3, 6, 7]. These events, including oxidative stress and cell proliferation, are common findings in human demyelinating diseases, such as multiple sclerosis, suggesting that ERK is involved in the development of autoimmune central nervous system (CNS) diseases.

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the CNS, mediated by $CD4^+$ T cells. EAE lesions are characterized by the infiltration of T cells and macrophages, and, at the peak stage of the disease, the activation of microglia and

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astrocytes [11], which are a major source of ERK expression *in vitro*. It is possible that ERK inhibition modulates rat EAE, because ERK inhibitors ameliorate the lesions in traumatic brain injury in some experimental paradigms [10]. Previously, we confirmed the increased expression of p-ERK in rat EAE tissues, and postulated that three forms of MAP kinase play an important role in the pathogenesis of acute monophasic EAE in rats [12].

Little is known of the expression of p-ERK in the cells that appear first in the subarachnoid space in rat EAE. As well, there is no information on whether PD98058, an inhibitor of MAP/ERK1 (MEK1), modulates rat EAE when it is administered intravenously in the course of rat autoimmune inflammation.

This study localized phosphorylated ERK in the spinal cord at the very early stage of EAE, and tested whether PD98059, an MEK1 inhibitor, modulated rat EAE paralysis.

Materials and Methods

Animals

Lewis rats were obtained from Harlan (Sprague Dawley Inc., Indianapolis, IN) and bred in our animal facility. Female rats aged 7-12 weeks and weighing 160-200 g were used in the experiments.

EAE induction

Both hind footpads of the rats in one group were injected with an emulsion (100 μl/ea) containing equal parts of myelin basic protein (1 mg/ml) and complete Freund's adjuvant (CFA) supplemented with *Mycobacterium tuberculosis* H37Ra (5 mg/ml) (Difco, Detroit, MI). The rats in the control group were immunized with CFA only. After the injections, the rats were observed daily for clinical signs of EAE.

The progress of EAE was divided into seven clinical stages: Grade 0 (G0), no signs; G1, floppy tail; G2, mild paraparesis; G3, severe paraparesis; G4, tetraparesis; G5, moribund condition or death; and R0, the recovery stage [11].

Treatment of rats with EAE with PD98059, an MEK1 inhibitor

Rats that had been immunized with myelin basic protein and adjuvant received intravenous administrations of either PD98059 (200 mg/kg/day in dimethyl sulfoxide) or vehicle (0.4%, DMSO), beginning on day 7 and continuing until day 13 PI. The inhibitor dose was based on the previously determined dose range of a specific inhibitor of MEK (U0126) used in ischemic brain injury [9]. Although PD98059 does not penetrate the blood-brain barrier [6], we administered PD98059 intravenously because a similar compound (U0126 100-200 mg/kg) effectively reduces the phosphorylation of ERK-2, a substrate for MEK, in the damaged brain with ischemia. We also found that p-ERK was intensively immunostained in vessels and astrocytes in contact with vessels in a preliminary study, and postulated that the intravenously administered drug easily contacts p-ERK in the vessels and penetrates the blood-brain barrier when inflammatory cells cross the blood-brain barrier at the effector stage of EAE (around day 9-12 PI).

Tissue sampling

Tissues were sampled on day 9-10 postimmunization (PI), which matches the early stages of EAE. The spinal cords of normal and CFA-immunized (control) animals were sampled on matching days in the EAE experiments. Three rats in each group were sacrificed under ether anesthesia. Pieces of the spinal cords were either snap frozen for frozen sectioning, or processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). We focused on the early stage spinal cord of rat EAE (day 10 pi), because we have already studied rat EAE at the peak stage [12].

Immunohistochemistry

After deparffinization and hydration, the sections were treated with 0.3% H_2O_2 in distilled water for 30 minutes to block endogenous peroxidase. In frozen sections, air dried sections were fixed in ethyl ether for 10 minutes prior to blocking of endogenous peroxidase. After three washes with PBS, the sections were exposed to 10% normal goat serum, and then incubated with primary antisera including rabbit polyclonal anti-p-ERK (diluted 1:200) antibodies for 1 hour at RT. To detect T cells or macrophages, R73 or ED1 (Serotec, London, UK) was applied, respectively. After three washes, the appropriate biotinylated secondary antibody and the avidin-biotin-peroxidase complex (ABC) from the Elite kit (Vector, Burlingame, CA) were added sequentially. Peroxidase was developed with either a diaminobenzidine substrate kit (Vector, Burlingame, CA) or amino ethyl carbazole substrate kit(Zymed). The sections were counterstained with hematoxylin before

being mounted.

Results

Constitutive expression of p-ERK in the spinal cord

Immunohistochemistry was used to visualize the phenotypes of cells in the subarachnoid space at the early stage of EAE. As shown previously, they were mainly T cells and macrophages that were positive for R73 or ED1, respectively [11].

The immunohistochemistry of the spinal cord of complete Freunds' adjuvant immunized control rats showed that some astrocytes (Fig. 1A) and neurons (Fig. 1B) constitutively expressed p-ERK, with weak immunoreactivity. This means that p-ERK is constitutively expressed in some glial cells and neurons in the spinal cord. In EAE, some inflammatory cells expressed p-ERK in the subarachnoid space (Fig. 1C), suggesting that some inflammatory cells in the subarachnoid space still required the phosphorylation of ERK. With the progression of inflammation at the peak stage (day 13), vascular endothelial cells and astrocytes showed increased p-ERK immunoreactivity (Fig. 1D). The immunohistochemical findings imply that phosphorylation of ERK occurs continuously in the normal spinal cord, and is increased when inflammatory cells expressing p-ERK infiltrate the subarachnoid space.

Effects of PD98059 in rats with EAE

By immunohistochemistry, a temporal increase in p-

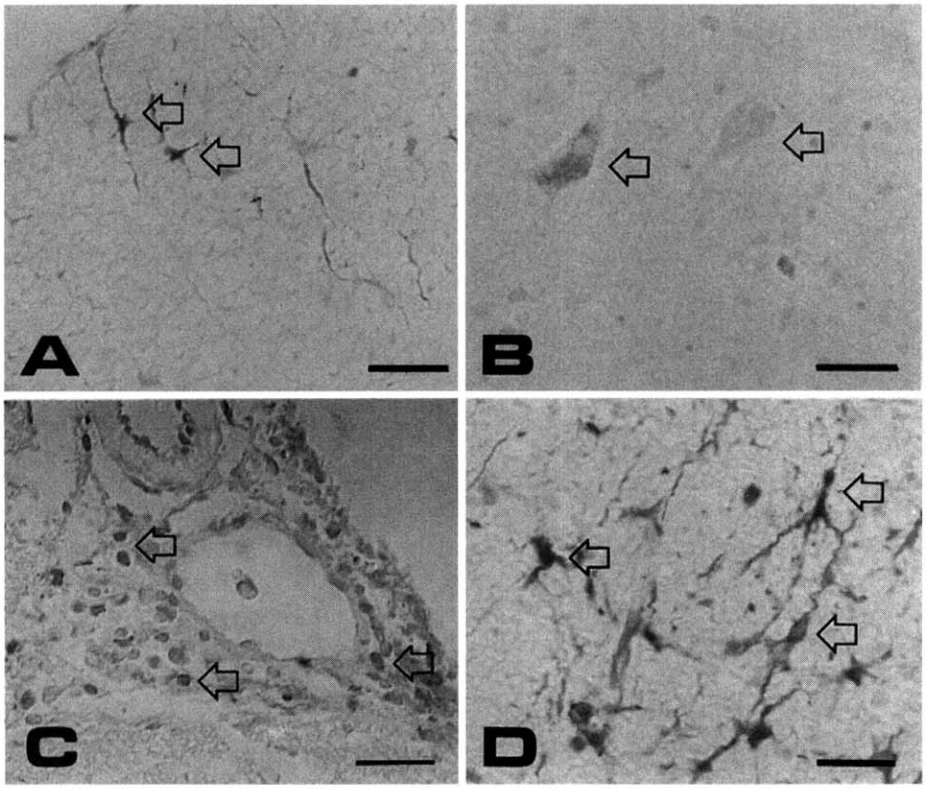

Fig. 1. Immunohistochemistry of phosphorylated ERK in the spinal cords of complete Freund's adjuvant immunized control rats (A, B) and those with EAE (C, D). P-ERK was constitutively expressed in some glial cells (A, arrows) and neurons (B, arrows) in the normal control spinal cord. With the infiltration of inflammatory cells in EAE at day 10, p-ERK was immunostained in some inflammatory cells (C, arrows) in the subarachnoid space, and increased p-ERK immunoreactivity in astrocytes was recognized at the peak stage(day 13) of EAE(D, arrows). A, B and D: paraffin embedded sections, Color was developed using DAB substrate kit. C : frozen section. Color was developed using AEC kit. Counterstained with hematoxylin. The scale bar represents 30 μm.

Table 1. Effects of intravenous PD98059 injection on the clinical parameters of EAE in rats

	First onset	Duration of paralysis	Maximum grade
Vehicle (DMSO)*	13.3±0.66 (n=5)	4.4±0.87 (n=5)	G3
PD98059 *	12.6±0.5 (n=5)	4.4±0.24 (n=5)	G3

*Lewis rats immunized with myelin basic protein and adjuvant were given PD98059 (200 mg/kg/day in dimethyl sulfoxide) or vehicle (0.4%, DMSO) intravenously beginning on day 7 and ending on day 13 PI.

ERK was detected in the inflammatory cells in the subarachnoid space in early EAE as well as the constitutive expression of p-ERK in the normal spinal cord. We asked whether the temporal inhibition of ERK activity modulates EAE paralysis. To study the effect of PD98059, an MEK inhibitor, in the course of EAE, we compared EAE with or without PD98059 treatment. The first onset of EAE paralysis in PD98059 treatment (12.6±0.5 days pi) was not different from that in vehicle-treated control rats (13.3±0.66 days pi) (Table 1). As well, the duration of paralysis in PD98059 treatment (4.4±0.24 days) was not significantly different from the controls (4.4±0.87 days).

Discussion

This study confirmed that the majority of inflammatory cells in the subarachnoid space in EAE express p-ERK, and suggested that autoreactive T cells and some bystander macrophages use p-ERK at the early stage of EAE. Although it is not clear when these cells begin to express p-ERK, it is highly possible that autoreactive T cells begin to express p-ERK during the proliferation process in peripheral lymph nodes, because T cell proliferation is associated with p-ERK activation *in vitro* [5]. It is postulated that early T cell proliferation in SAS is associated with the activation of p-ERK in rat EAE.

This is the first study to test the effect of an inhibitor of MEK1, PD98059, on the course of EAE. PD98059 was administered to EAE-affected rats intravenously. Unexpectedly, PD98059 did not change the clinical parameters of EAE. We postulate that PD98059 is insufficient to inhibit p-ERK activity in this model, or that the pathogenic mechanism of rat EAE is different from that of traumatic brain injury [8]. It is unlikely that the route of PD98059 administration in this study was inadequate for the suppression of MEK in this EAE model, because intrathecal injection of PD98059 (2 μl/ 200 μmol/liter) in a previous study [8] produced results similar to the PD98059-treated and vehicle-treated groups in this study (data not shown).

In summary, this report provides the first evidence that ERK pathways contribute to the activation of inflammatory cells in the early stage of rat EAE. The manipulation of MEK1 with intravenous PD98059 was insufficient to suppress its target enzyme, which might be involved in the devastating progression of tissue damage in autoimmune CNS inflammation.

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