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Protection of vincristine-induced neuropathy by Wld^S expression and the independence of the activity of Nmnat1

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Abstract

The slow Wallerian degeneration protein (Wld^S), a fusion protein containing amino-terminal E4B and full-length nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1), delays axon degeneration caused by physical damages, toxins and genetic mutations which result in patients being diagnosed with neurodegenerative diseases. It is still controversial whether the suppression of axonal degeneration by Wld^S is due to Nmnat1 or other portion. We generated Wld^S or Nmnat1-overexpressing Neuro2A cell lines, in which neuronal differentiation including neurite elongation can be induced by retinoic acid. The overexpression of Wld^S delayed the neurite degeneration by vincristine, whereas that of Nmnat1 did not delay it much. Taken together, Nmnat1 is considerably weaker than Wld^S for protection from toxic injury *in vitro*, suggesting that amino-terminal region of Wld^S is likely to be more significant for protection from axonal degeneration.

Keywords: Wld^S; Nmnat1; E4B; Wallerian degeneration; Vincristine

By the transection or crush of a peripheral nerve, distal axons undergo a fragmentation called Wallerian degeneration, which has many similar characteristics to axonal degeneration in toxin-induced degeneration or neurodegenerative diseases [3]. A spontaneous mouse strain with Wallerian degeneration-slow (*Wld^S*) mutation demonstrated the delayed Wallerian degeneration [11]. The related genes have recently been identified within the 85-kb genomic region that is tandemly triplicated in the *Wld^S* mouse [2]. The gene for ubiquitin chain assembly factor E4B and a previously undescribed gene (*D4cole1e*) that encodes a nicotinamide mononucleotide adenylyltransferase1 (Nmnat1) span the proximal and distal boundaries of the repeat unit, respectively. A chimeric mRNA that encodes an in-frame fusion protein (Wld^S protein) consisting of the amino-terminal 70 amino acids (N70) of E4B separated by an aspartic acid residue from the carboxyl-terminal 302 amino acids of Nmnat1 is abundant in the nervous system of *Wld^S* mice [5]. Distal axons of neurons of wild-type mice transfected in situ with a vector encoding the chimeric Wld^S protein survived for 2 weeks after axotomy, compared to the survival time of only 2–3 days in control animals, and Wld^S expression also protected neuromuscular junctions from injury-induced degeneration. The Wld^S protein is likely to localize predominantly in the nucleus of the neurons, suggesting that its protective action is indirect [12]. Moreover, it has been shown that Wallerian degeneration is independent of canonical apoptotic cascades. Therefore, it may be regulated by unidentified axon specific system that is distinct from that of the cell body.

To date a number of studies have reported that the expression of Wld^S dramatically delays axonal degeneration following axotomy as well as axonal loss in various neuropathic models, dysmyelination models, and toxin-induced neuropathies by taxol or vincristine [21,22]. Although studies using *Wld^S* mice have focused on peripheral nervous systems, several reports have reported that Wld^S also delays the axonal pathology in various central nervous system including the degeneration linked to the gracile axonal dystrophy (*gad*) mouse model [13]. The effect of Wld^S on mouse treated with 6-hydroxydopamine (6-OHDA), which causes Parkinson-like disease, indicated that the Wld^S protein robustly protects dopaminergic terminus [17].

It has been reported that Nmnat1 enzyme activity, but not NAD$^+$ content, is increased fourfold in the tissues of *Wld^S* mice [12]. Nmnat1 has been reported to be sufficient for axonal protection by either the association with SIRT1 or the local NAD$^+$ synthesis in neurites [1,20]. Axon protection is thus possibly

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mediated by pyridine nucleotide metabolism. However, recent report has shown that the transgenic mouse which overexpresses Nmnat1 did not delay the axonal Wallerian degeneration, suggesting that Nmnat1 does not serve the Wld^S protein [4]. These findings demonstrate that the information concerning which region or what function of Wld^S protein is sufficient for the axonal protection is controversial.

In this study, we demonstrate the protective activity of Wld^S or Nmnat1 in neuronal cell line Neuro2A by the neurite-toxic neuropathy. We established the Neuro2A cell lines which overexpress Wld^S, Nmnat1, or E4B. These cell lines differentiated (neurite elongation) by retinoic acid, were exposed with vincristine, and then the effects of axonal degeneration were evaluated. Wld^S delayed the neurite degeneration by vincristine as previously reported, whereas that of Nmnat1 did not delay as much as Wld^S did. These findings may reveal that amino-terminal region of Wld^S is more significant for protection from axonal degeneration than the activity of Nmnat1.

Neuro2A cell line were cultured under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). For neurite elongation, Neuro2A cells were cultured with all-trans retinoic acid (20 μM; Sigma) for 72 h.

Mouse Nmnat1 cDNA were amplified from Wld^S cDNA provided from Dr. George Wilmot (Emory University School of Medicine), by the polymerase chain reaction (PCR) with ExTaq (TAKARA, Tokyo, Japan) using primers; 5′-ATGGACTCATCCAAGAAGACAGAG-3′ (sense), and 5′-GTGTCACAGAGTGGAATGGTTGTG-3′ (antisense). The amplified fragments were subcloned into pBluescript II SK^+ (Stratagene, La Jolla, CA, USA), and their sequences were verified. The FLAG-tagged Nmnat1, E4B and Wld^S cDNAs were then subcloned into pCR (Invitrogen).

The retroviral expression vectors for FLAG-tagged Nmnat1, E4B and Wld^S, were constructed with pMX-puro which was obtained from Dr. T. Kitamura (University of Tokyo). For retrovirus-mediated gene expression, Neuro2A cells were infected with retroviruses produced by Plat-E packaging cells and then cultured in the presence of puromycin (0.2 μg/ml; Sigma).

For Immunoblot analysis, the cells were lysed in a solution containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. Immunoblot analysis was performed with anti-FLAG mAb (1 μg/ml; M2, Sigma), and then detected with horseradish peroxidase-conjugated antibodies to mouse immunoglobulin G (1:10,000 dilution, Promega) and an enhanced chemiluminescence system (ECL, GE Healthcare).

To evaluate the protection from axonal degeneration by vincristine, cells were cultured with retinoic acid (20 μM) for 72 h, and then further treated with vincristine (5 nM) for the indicated times. The cells that have longer neurites than the diameter of their cell body were then counted, and the ratio between cell number with or without neurites was calculated. The percentage

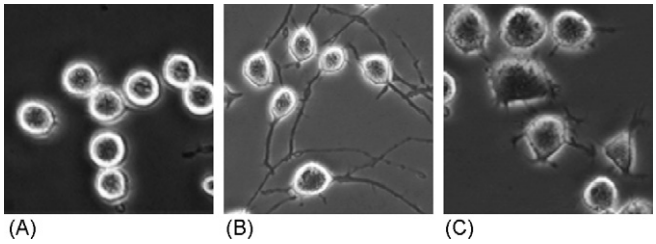

Fig. 1. Neurite extension induced by retinoic acid and vincristine-induced neuropathy. (A) Neuro2A cells. (B) Neuro2A cells cultured with retinoic acid (20 μM) for 72 h. (C) Neuro2A cells were cultured with retinoic acid (20 μM) for 72 h, and then further cultured with vincristine (5 nM) for 8 h.

of cells with neurites is shown as mean ± S.D. of values from five independent experiments.

The Neuro2A cell line known as a neuronal differentiation model *in vitro*, is morphologically amoeboid-like or neuron-like shapes with short spines without retinoic acid (Fig. 1A). We performed the cell culture with all-trans retinoic acid for neurite elongation, and used vincristine as neurotoxin to inflict an injury on elongated neurites. The culture with retinoic acid for 72 h caused the formation of neurite-like elongation on Neuro2A cells (Fig. 1B). Further culture with retinoic acid and vincristine sustained vigorous damages on neurites, and the culture of 8 h with vincristine changed almost all cells to round- or amoeboid-like shapes (Fig. 1C). Thus, the combination of retinoic acid and vincristine on Neuro2A cells was ascertained to be available for the axonal degeneration model *in vitro*.

A number of reports have indicated that the expression of Wld^S noticeably delays axonal degeneration following axotomy as well as axonal injury by physical damages or toxin-induced neuropathy. However, it is still controversial which region is indeed significant to protect neurite degeneration, amino-terminal region of E4B, Nmnat1, or both. To clarify the contentious aspects, we generated Neuro2A cell lines, which stably overexpress FLAG-tagged Wld^S, FLAG-tagged E4B, or FLAG-tagged Nmnat1 by using retroviral expression system. To examine the expression of each proteins, Immunoblot analysis was performed using anti-FLAG antibody, indicating that each stable cell lines expressed the proteins introduced by

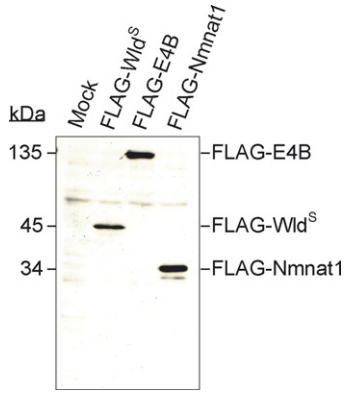

Fig. 2. Establishment of Neuro2A cell lines which stably express FLAG-tagged E4B, Wld^S, or Nmnat1. The cell lysates (20 μg) of Neuro2A cell lines expressing FLAG-tagged E4B, FLAG-tagged Wld^S, FLAG-tagged Nmnat1 or mock were subjected to immunoblot analysis with antibodies to FLAG.

retroviral expression system, and that the expression levels of exogenous proteins from each stable cell lines are almost same (Fig. 2).

Next, each stable cell lines were cultured with retinoic acid for 72 h, and all the cell lines were susceptible to neuronal differentiation by retinoic acid (Fig. 3A, upper panels). After the induction of neurite elongation, cells were further cultured with vincristine for 8 h to determine the protective activities against toxin-induced neuropathy. The differences in the protective activities by vincristine were observed, and Wld^S overexpressing cell line showed protective activity against the neurite damages by vincristine, whereas other cell lines showed weak or none compared to Wld^S overexpressing cell line (Fig. 3A lower panel).

To further quantitatively verify the neurite-toxicity by vincristine, the number of cells that maintain its neurites was counted at the indicated points after the addition of vincristine (Fig. 3B). The effect of Neuro2A cell lines overexpressing FLAG-tagged E4B is similar to that of the mock transfectant. More than 80% of Neuro2A cells overexpressing FLAG-tagged

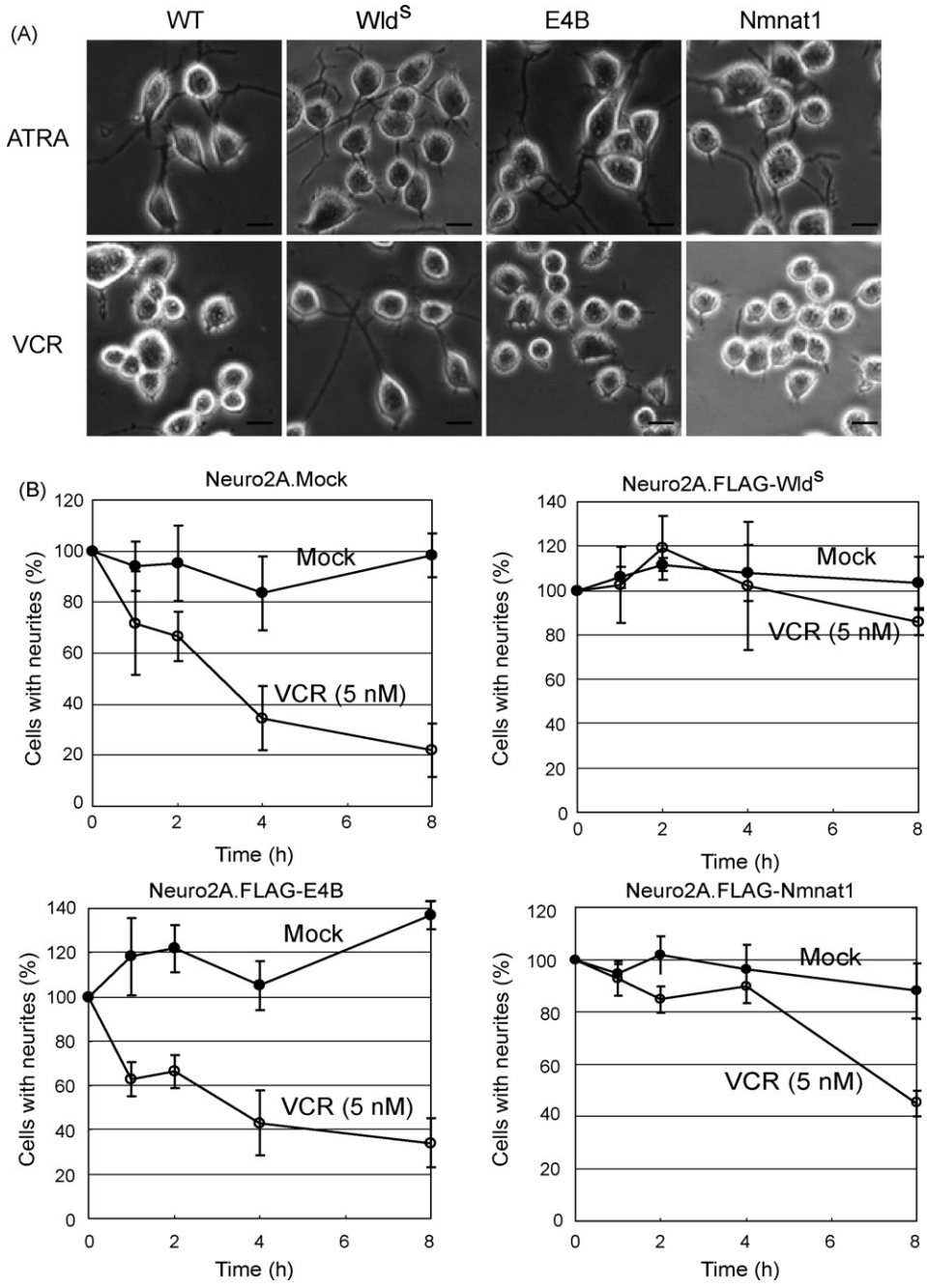

Fig. 3. Protection of vincristine-induced neuropathy by overexpression of Wld^S. (A) Neuro2A cell lines expressing FLAG-tagged E4B, FLAG-tagged Wld^S, FLAG-tagged Nmnat1 or mock cultured with retinoic acid (20 μM) for 72 h, and then further cultured with vincristine (5 nM) for 8 h. Scale bar: 10 μm. (B) Stable Neuro2A cell lines are treated with vincristine for the indicated times, and the cells which have longer neurites than the diameter of its cell body, were counted. The percentage of cells with neurites is shown as means ± S.D. of values from five independent experiments.

Wld^S maintained the neurites after treatment with vincristine for 8 h. Thus, Wld^S showed considerable protective activity against vincristine, as previously described. However, less than 50% of Neuro2A overexpressing FLAG-tagged Nmnat1 kept the neurite after the treatment with vincristine for 8 h, indicating that Nmnat1 demonstrates weaker protective activity than Wld^S. These findings demonstrate that Nmnat1 is likely to be partially related to the protection from axonal degeneration, thus amino-terminal 70 amino acids is presumably more significant than Nmnat1 in terms of the rescue from neuropathy via vincristine.

Here we showed that the amino-terminal region of the Wld^S protein is likely to be significant for the protection of neurotoxicity by vincristine and that Nmnat1 is not as valuable. In *Wld*^{S} mice, injury-induced Wallerian degeneration is delayed tenfold (for 2–3 weeks) by a dominant mutation compared to wild-type mouse [11,15]. It has been shown that in crosses with progressive motor neuronopathy (*pmn*) mice or myelin protein zero (P0) null mutants, Wld^S significantly delayed axonal degeneration [7,18]. Similar reports on taxol toxicity suggested that Wld^S apparently protects degeneration against taxol-induced neuropathy as well [21]. In the CNS, Wld^S also protects against both genetic and toxic insults. In *Wld*^{S} mouse, some nigrostriatal axons remain functional after 6-hydroxydopamine lesions in *Wld*^{S} mice, and the number of axonal spheroids is reduced in the gracile tract of mice with gracile axonal dystrophy (*gad*), in which ubiquitin carboxyterminal hydrolase-L1 (UCH-L1) is defective [13,17]. It has been shown that acute axon degeneration (AAD) in transected spinal cord axons has recently been linked to Wallerian degeneration, and that AAD is also delayed by Wld^S. However, all axon degeneration is not delayed by Wld^S. Superoxide dismutase 1 (*SOD1*) transgenic mouse models of amyotrophic lateral sclerosis (ALS), the proteolipid protein (*Plp*) null model of hereditary spastic paraplegia and neurite degeneration caused by botulinum neurotoxin C1 are not compensated by Wld^S [19]. Wld^S is likely to be more efficient in early-onset or acute disorders. The differences of axonal insults which are protective by Wld^S, may give us answers to determine the active domain of Wld^S.

To date, it is still controversial which region of the Wld^S sequence is required for axon protection and which kind of molecules are implicated. Nmnat1 has been reported to be sufficient for axon protection through the SIRT1 or through local NAD^+ synthesis in neurites [1,20]. However, these results are contradictory with reports using transgenic mice that overexpress Nmnat1 [4]. This Nmnat1 transgenic mouse is not similar to the *Wld*^{S} mutant mouse. Our present results using Neuro2A overexpressing Nmnat1 is similar to that of Nmnat1 transgenic mouse. These findings suggest that Nmnat1 is not an active domain for Wld^S protein. The requirement for the E4B-derived amino-terminal 70 sequence or the junctional region between E4B-derived N70 sequence and Nmnat1 sequence may be required for axonal protection. Recently, several candidates who physically or functionally associate with Wld^S were reported. Amino-terminal 70 amino acids of Wld^S binds to valosin-containing protein (VCP, also known as p97) and the transcriptional level of pituitary tumour transforming gene 1 (PTTG1) is altered in *Wld*^{S} mice [9,10]. The analysis to clarify the relation between Wld^S and these proteins still remains to be investigated.

The linkage between Wallerian degeneration and apoptotic pathways has not been reported. The *Wld*^{S} gene is not likely to link to anti-apoptotic factors such as bcl-2 [16]. The activation of the apoptotic effector caspase 3 and products of caspase-mediated degradation are not detectable in injured axons, and caspase inhibition does not block Wallerian degeneration [8]. Wld^S does not block apoptotic death of neuronal cells [6]. Hence, the identity of the functional linkage with *Wld*^{S} gene or protein will be imperative in helping to elucidate the mechanism of Wallerian degeneration, and besides to discover unidentified cell maintenance system.

We have previously reported that E4B interacts with and regulates fasciculation and elongation protein zeta 1(FEZ1), a protein implicated in neurite extension, indicating the potency of E4B in neuritogenesis [14]. In this case, carboxy-terminal U-box domain, but not an amino-terminal 70 animo acids sequence of E4B, is necessary for neuritogenesis. These findings may demonstrate that E4B has dual functional domains for the neurite elongation and for the protection from axonal degeneration. Further functional analysis of Wld^S at the molecular level would prove to provide therapeutic benefits not only for the suppression in axonal injury but also for neurodegenerative diseases.

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