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좌골신경섬유 재생시 Cdc2 kinase 매개성 슈반세포 활성화의 역할 규명

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Cdc2 promotes activation of Schwann cell in regenerating axon after sciatic nerve injury in the rat.

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Cdc2 kinase is a prototypical cyclin-dependent kinase critical for G2 to M phase cell cycle transition. Yet, its function in the nervous system is largely unknown. Here, we investigated possible role of Cdc2 in axonal regeneration using sciatic nerve system in rat. Cdc2 protein levels and activity were increased in the injured sciatic nerves 3 and 7 days after crush injury and then decreased to basal level 14 days later. Administration of Cdc2 kinase inhibitor roscovitine in vivo at the time of crush injury significantly inhibited axonal regeneration when regrowing axons were analyzed using retrograde tracers. Cdc2 protein levels in cultured Schwann cells which were prepared from sciatic nerves 7 days after crush injury were much higher compared with those from uninjured sciatic nerves, suggesting that Cdc2 protein expression was primarily induced in the Schwann cells. To further investigate Cdc2 function in Schwann cell, we examined changes in cultured Schwann cell proliferation and migration in culture system. Both the number of proliferating Schwann cells and the extent of neurite outgrowth from co-cultured DRG neurons were significantly decreased by Cdc2 inhibitor roscovitine treatment in DRG culture which was prepared from animals with sciatic nerve injury for 7 days. Also, Schwann cell migration in the injured sciatic nerve explant was significantly inhibited by roscovitine treatment. Taken together, the present data suggest that Cdc2 may be involved in peripheral nerve regeneration via Schwann cell proliferation and migration.

key words : Cdc2 kinase, Schwann cell, nervous system.

I. Introduction

Axons in the peripheral nervous system (PNS) show a far greater capacity for regeneration than those in the central nervous system (CNS), which is believed to be largely due to the difference in cytological organization between the PNS and CNS (Fawcett et al., 2001). After peripheral nerve injury, its distal segment gradually degenerates, myelin sheaths break down and eventually disappear, a feature known as Wallerian degeneration (Waller, 1850). In the distal nerve stump after injury, Schwann cells, the main glial cells of the peripheral nervous system (PNS), contact with axons and transiently proliferate and then differentiate to form a complete myelination (Stoll and Muller, 1999;

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Atanasoski et al., 2004). Proliferating Schwann cells form a cell strand called Schwann cell column within basal lamina tube and increase the synthesis of adhesion molecules to induce axon-Schwann cell attachment (Vogelezang et al., 1999; Ide et al., 1983). Schwann cells at the early stage of axonal injury express neurotrophic factor such as NGF to promote the axonal regeneration (Heumann et al., 1997; Meyer et al., 1992) and accelerate the cell division in regenerating nerves.

Cell division cycle 2 (Cdc2) protein plays an essential role in promoting the G2 to M phase transition (Nurse, 1990). Recently, potential role of Cdc2 in the neural system has begun to be explored. Ekholm and Reed (2000) have reported that the control of the cell division in Schwann cells appears to be regulated by the cyclin-dependent kinase 2 (Cdk2), an essential enzyme for the transition from the G1 to S phase. In contrast, Konishi et al., (2002, 2003) have reported that Cdc2 mediates neuronal apoptosis in post-mitotic neurons in rat cerebellum and contributes to the phosphorylation of BAD leading to apoptosis of newly generated neuron during brain development (O'Hare et al., 2000; Konishi et al., 2002). Since Schwann cells are actively involved in peripheral nerve regeneration via proliferation and differentiation, it is feasible that Cdc2 mediates axonal regeneration process via Schwann cell activation. To examine this possibility, we studied the induction of Cdc2 protein in regenerating axons and its association with changes in Schwann cell activity.

We found that sciatic nerve injury increased levels of Cdc2 expression and kinase activity in the proliferating Schwann cells. We also provide evidence that the inhibition of Cdc2 activity resulted in the retardation of axonal regeneration in vivo and attenuated Schwann cell migration from the explant culture prepared from injured sciatic nerve. This implicates the functional importance of Cdc2 in peripheral nerve regeneration.

II. Materials and Methods

Sciatic nerve surgery

Animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg), and sciatic nerve was exposed and crushed with a pair of forceps held tightly for 30 sec twice at 1 min intervals (Namgung et al., 2004). Animals were recovered from anesthesia and sacrificed 1 - 14 days later. Animals were deeply anesthetized with a mixture of ketamine and xylazine, and sciatic nerve was dissected, immediately frozen, and kept at -70°C until use. For some experimental purposes, the sciatic nerve was prepared by dividing into the 5 or 10 mm length segments proximal or distal to the injury site.

Western blotting and kinase assay

Nerve segments were washed with ice-cold PBS, and sonicated under 50 - 200 ㎕ of triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, pH 7.14, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM benzamidine, 0.5 mM DTT, 1 mM PMSF).

Protein (10 μg) was used for Western analysis using anti-Cdc2 antibody (p34, Santa Cruz Biotech) and anti-actin antibody (clone no. C4, ICN Biomedicals, Cleveland, USA). Electrophoresis and Western blotting were performed as described previously (Namgung et al., 2004). Primary and secondary antibodies were diluted and used as recommended by the manufacturers. For Cdc2 kinase assay, 200 μg of protein was used for each assay. The Cdc2 kinase activity was measured as described using an immune complex kinase assay and histone H1 as substrate (Namgung and Xia, 2000). Briefly, cell lysates were immuno -precipitated with anti-Cdc2 antibody (N-20, Santa Cruz Biotech) conjugated to protein sepharose A, and were subject to in vitro kinase reaction (25 mM Hepes, pH 7.4, 25 mM β-glycerophosphate, pH 7.14, 25 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.5 mM DTT, 60 μg/ml Histone H1 protein, 300 μCi/ml γ-32P ATP) for 30 min at 30℃. Samples were then boiled and the supernatants were resolved in 10% SDS-polyacrylamide gel. The gel, after drying when needed for longer exposure or strong signals, was used for an autoradiography.

Retrograde tracing techniques

For retrograde tracing of motor neurons, the sciatic nerves from anesthetized rats with ketamine and xylazine were exposed and DiI (5 ㎕ of 3% in dimethylsulfoxide) was applied to 10 mm distal to the injury site with a micropipette. The incision was sutured, and the animals were returned to their cages after they were recovered from the narcosis. Forty eight hours later, animals were sacrificed, nerve segments were embedded and frozen at -20°C. Longitudinal sections (20 μm thickness) were cut on a cryostat and mounted on positively charged slides. DiI-labeled motor neurons in the longitudinal spinal cord sections were visualized, and all the sections collected were used to count labeled motor neurons observed at T12 and L1 levels. The mean numbers of total labeled cells in individual animals were compared among groups by Student's t-test. Digitally captured images on a fluorescence microscope (Nikon model E-600, Kawasaki, Japan) were transferred to Photoshop (Adobe version 5.5) for processing and labeled appropriately.

Immunohistochemistry

For double immunofluorescence staining, cultured cells on coverslip were fixed with 4% paraformaldehyde, 4% sucrose in PBS at room temperature for 40 min, permeabilized with 0.5% nonidet P-40 in PBS, and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4 hr at room temperature. Cells were incubated with anti-βIII-tubulin antibody (TUJ1, Covance, Berkeley, USA) or anti-GAP-43 antibody (H-100, Santa Cruz Biotech) and then incubated with fluorescein-goat anti-mouse (Molecular probes, Eugene, USA) or rhodamine-goat anti-rabbit secondary antibodies (Molecular probes) in 2.5% horse serum and 2.5% bovine serum albumin for 1 hr at room temperature. After the reaction with Hoechst dye 33258 for 10 min, cells were cover-slipped with gelatin mount medium. We always included control cells treated with secondary antibody alone, which usually did not have any visible images. In cases when the nonspecific signals were high, all

the data from those experiments were not further analyzed. Sections were viewed with a Nikon fluorescence microscope and the images were captured by using Nikon camera. The merged images were produced by using layer blending mode options of the Adobe Photoshop (version 5.5).

RT-PCR

Dorsal root ganglion (DRG) at L4-6 levels in adult rat was prepared 1, 3 and 7 days after sciatic nerve injury, and total RNA was isolated using Easy-BLUE reagent (Intron, Sungnam, Korea). Two micrograms of RNA was used as a template for reverse transcription (RT) reaction with MMLV reverse transcriptase (Promega, Madison, USA) and random primer (Bioneer, Daejeon, Korea) for 1 hr at 37℃. For PCR amplification of Cdc2 and actin cDNA, the RT reaction was diluted 4-fold in H_2O and 5 ㎕ of cDNA in 80 ㎕ of total reaction volume was used for PCR with Taq DNA polymerase (Takara, Ohtsu, Japan). The reaction was processed for 30 cycles using a thermocycler (Primus, MWG Biotech, Mü nchen, Germany). The primer sequences used for PCR were forward primer (5'ATCGGAGAAGGGACTTATGG3') and revers e primer (5'TGCAGGGATCTACTTCTGG3') for Cdc2 mRNA, and forward primer (5'CACACT GTGCCCATCTATGA3') and reverse primer (5'TACGGATGTCAACGTCACAC3') for actin mRNA.

Primary DRG sensory neuron culture

Glass coverslips were precoated with a mixture of poly-L-ornithine (0.1 mg/ml, Sigma) and laminin (0.02 mg/ml, Collaborative Research, Bedford, USA). DRG prepared from adult rats was placed in ice-cold DMEM medium (GIBCO, Grand Island, USA), and treated with DMEM containing type XI collagenase (2500 U/ml, Sigma) for 90 min at 37℃. Tissues were then washed with DMEM medium and centrifuged at 800 rpm for 1 min to remove the supernatant. After repeated washing one more time, cells were suspended in DMEM, dissociated gently with 16-20 passages through a flamed Pasteur pipette, and centrifuged at 800 rpm for 1 min to remove the supernatant. Cells were then treated with DMEM containing type SII trypsin (0.5 mg/ml, Sigma) for 10 min followed by DMEM containing trypsin inhibitor (100 μ g/ml, Sigma), EDTA (1 mM) and DNase I (80 μg/ml) for 5 min. After washing with culture medium (DMEM containing 5% heat-inactivated FBS (GIBCO), 5% horse serum, 2 mM glutamine and 1% penicillin-streptomycin), 800 - 1200 neurons were plated onto 12 mm round coverslips, cultured for 12 hr and changed with fresh culture medium. DRG neurons were further cultured for 36 hr and collected for immunofluorescence staining.

Schwann cell culture

Sciatic nerves from newborn rats (postnatal day 3) and non-injured- and injured adult rats day 7 after surgery were isolated and used for Schwann cell culture. Dissociated $1x10^6$ Schwann cells were plated onto 60 mm dish precoated with a mixture of poly-L-ornithine and laminin, cultured for 12 hr and changed with fresh culture medium. Schwann cells were further cultured for 48 hr with 2 μM forskolin and then medium was changed with 10% serum plus 2

μM forskolin or 0.5% serum in the absence of forskolin and cultured for 48 hr. Cells were harvested and used for Western analysis.

Explant culture

Sciatic nerves prepared from adult rats were placed in ice-cold DMEM medium (GIBCO, Grand Island, USA), and placed on glass coverslips precoated with a mixture of poly-L-ornithine (0.1 mg/ml, Sigma) and laminin (0.02 mg/ml, Collaborative Research, Bedford, USA). Tissue was cultured with 200 ㎕ culture medium (DMEM containing 5% heat-inactivated FBS (GIBCO), 5% horse serum, 2 mM glutamine and 1% penicillin-streptomycin) for 1 hr and added with 500 ㎕ culture medium. After 36 hr culture, medium was changed with fresh medium containig roscovitine or vehicle. Explant were further cultured for 24 hr and fixed for immunofluorescence staining.

III. Results

To examine whether Cdc2 was involved in axonal regeneration, Cdc2 protein levels in both sciatic nerves and dorsal root ganglion (DRG) were measured by Western blot analysis. Sciatic nerves and DRG at L4, L5 and L6 levels were dissected 3-14 days after sciatic nerve crush injury. As shown in Fig. 1A, Cdc2 protein levels were increased in the ipsilateral segment compared with the contralateral sides 3 and 7 days after injury and then decreased to the basal level 14 days after injury. Cdc2 expression was not detected in the DRG with or without nerve injury (Fig 1B).

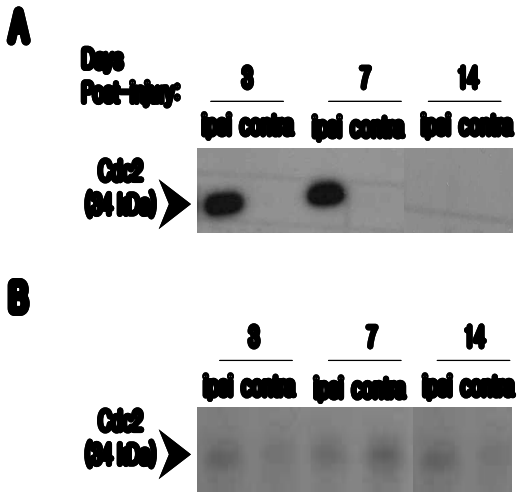

Fig. 1. Induction of Cdc2 protein in the injured sciatic nerve. (A) Sciatic nerves were prepared from adult rats 3, 7 and 14 days after injury. Tissues from ipsilateral (ipsi) and contralateral sides (contra) were separately prepared. Protein extract was used for Western analysis with anti-Cdc2 antibody. Cdc2 protein was strongly induced at day 3 and 7 after crush injury compared with the contralateral side, and then was undetectable 14 days later. (B) Any significant changes in Cdc2 protein levels in DRG were not observed among samples with different treatments.

We then investigated Cdc2 mRNA expression by RT-PCR. As shown in Fig 2A, Cdc2 mRNA levels were increased in the sciatic nerves 3 days after injury compared with non-injured control (0 day) and further increased up to 7 days after surgery (Fig. 2A). To further investigate the local expression pattern of Cdc2 mRNA from the proximal to the distal stumps of nerves, the tissue lysates were prepared from four divided nerve segments with 5-mm length each (Fig. 2B). Cdc2 mRNA showed a pattern of distal shift as the injury periods were extended.

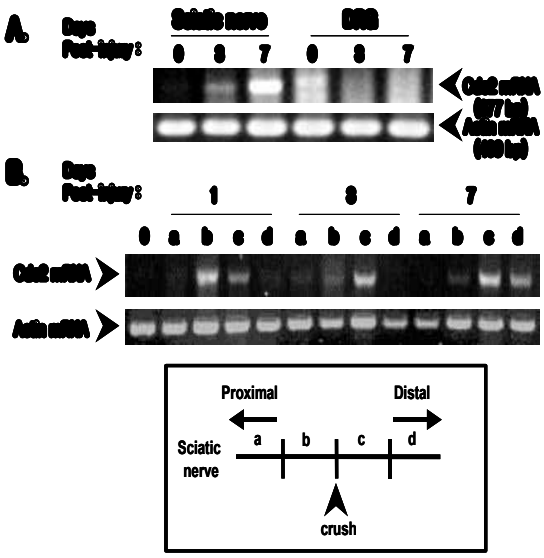

Fig. 2. Induction of Cdc2 mRNA.
(A) Sciatic nerves were prepared from adult rats 3 and 7 days after injury. Day 0 is the control from non-injured rat. Total RNA extract was used for reverse transcriptation reaction and then processed PCR analysis with Cdc2 or actin primers. Cdc2 mRNA expression was gradually increased from day 3 to day 7 in sciatic nerve after injury, but not detected in DRG. (B) Nerve segments (0.5 cm long) as shown in the lower panel were used for RT-PCR. Cdc2 mRNA levels were shifted toward the distal end. PCR reaction for actin mRNA was used as an internal loading control.

To determine whether Cdc2 activity is indeed involved in axonal regeneration, we investigated axonal regeneration in the presence of Cdc2 kinase inhibitor. We first found that nerve injury increased strongly Cdc2 kinase activity in the distal portion of sciatic nerve (DMSO treated group in Fig 3). When the Cdc2 inhibitor roscovitine (2 $\mu\ell$ of 100 mM) was injected into the site of nerve crush at the time of nerve injury, Cdc2 kinase activity was decreased in the distal stump of the sciatic nerves prepared 7 days after injury. The inhibitory activity of roscovitine for Cdc2 kinase was further confirmed by decreased phosphorylation of histone H1 protein by treating in vitro kinase reaction mixture with roscovitine (labled Dist + rosco in the Fig 3). Together, these results show that induced kinase activity in the regenerating sciatic nerves was effectively inhibited by roscovitine in vitro as well as in vivo conditions.

To determine whether increased Cdc2

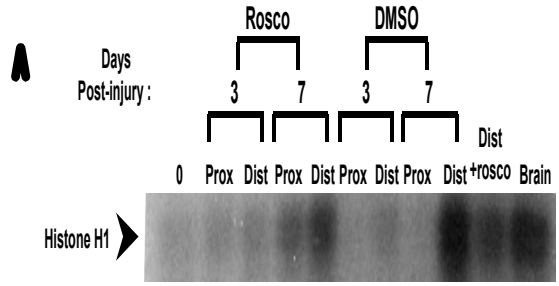

Fig. 3. Inhibition of Cdc2 kinase activity in lesioned nerve by Cdc2 inhibitor roscovitine (Rosco). Two hundred nanomoles of roscovitine or an equilivalent volume of DMSO were injected into the injured site immediately after injury. Nerves were removed 3 or 7 days after injury and used for kinase assay. (A) Cdc2 kinase activity was separately examined in the proximal and distal stump. Also, roscovitine was treated in the in vitro kinase reaction buffer (Dist + rosco).

protein level was expressed in Schwann cells, we analyzed Cdc2 in the cultured Schwann cells. Sciatic nerve was used from adult and postnatal day 3 and Schwann cells were harvested 3 days after culture. As shown in Fig. 4, Cdc2 protein was strongly induced in proliferating cells prepared from sciatic nerves which had undergone sciatic nerve injury and similar levels of Cdc2 protein induction was observed in the proliferating cells prepared from newborn

animals. These data suggest that Cdc2 activity in the Schwann cells correlated well with cell proliferation activity.

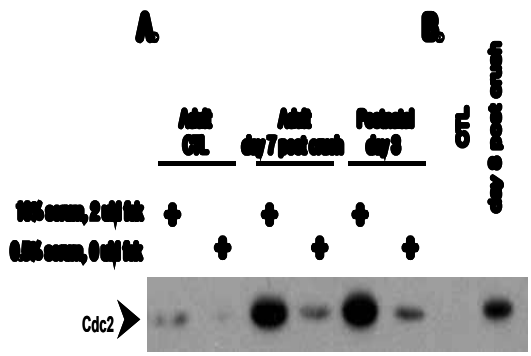

Fig. 4. Cdc2 protein was up-regulated in proliferating Schwann cell after injury. (A) Primary Schwann cells in the sciatic nerves isolated from adult rats or newborn rats were prepared (see Materials and Methods for details). Dissociated sciatic nerve was pre-cultured for 48 hr in 10% serum plus 2 μM forskolin (fsk) and then culture media were changed with 10% serum plus 2 μM forskolin or 0.5% serum plus no forskolin and further incubated for 48 hr. Cells were harvested and used for Western analysis. Cdc2 protein levels were strongly induced in proliferating Schwann cells, which were similar to those prepared from newborn animals.

We then examined whether the inhibition of Cdc2 activity would affect neurite outgrowth of the DRG neurons. Primary culture of DRG at L4, 5 and L6 was prepared and treated with roscovitine. DRG neurite was immunostained with anti-TUJ antibody and anti-GAP-43 antibody to detect neuron-specific marker protein tubulin and axonal growth associated protein GAP-43 respectively. Preconditioned DRG neurons (labeled 'Vehicle' in the Fig 5)

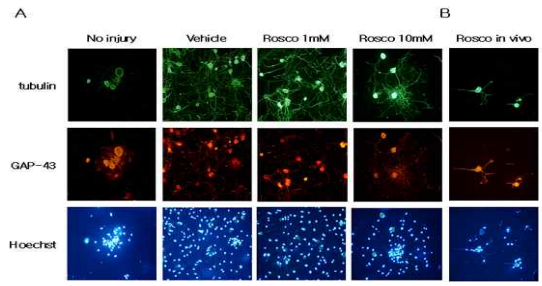

Fig. 5. Suppression of neurite outgrowth and Schwann cell proliferation by Cdc2 inhibitor treatment. (A) Neurons from L4, L5 and L6 DRG of adult rats were prepared 7 days after sciatic nerve injury. DRG neurons were dissociated and cultured in the presence of 1 μM or 10 μM of roscovitine (Rosco). Increased neurite outgrowth of DRG sensory neurons prepared from a rat which had undergone sciatic nerve injury for 7 days (labled 'Vehicle') was inhibited by roscovitine treatment at 10 μM in vitro. (B) In vivo roscovitine treatment was performed by injecting 2 $\mu\ell$ of 100 mM of roscovitine at the injury site. Neurite outgrowth and Schwann cell proliferation were inhibited by roscovitine treatment.

showed long neurite outgrowth compared with controls. Then, DRG neurons, when treated with 10 mM roscovitine, showed retarded neurite outgrowth, and decreased GAP-43 immunostaining intensity. We further found that the number of cell nuclei including non-neuronal Schwann cells, which was visualized by Hoechst staining, was significantly decreased by roscovitine treatment.

To examine whether Cdc2 kinase activity is involved in axon regeneration, we analysed regenerating motor neurons located in the spinal cord. Retrograde tracer DiI was injected at the 10 mm distal from the injury site 2 days before sacrificing the animals. Analysis of DiI-labeled motor neurons in the spinal cord showed that the number of DiI

labeled motor neurons was much lower 2 days after injury compared with non-injury group, then were increased 7 - 14 days post crush, showing a similar level as those in non-injury group. In animals treated with roscovitine, DiI labeled motor neurons were significantly lower than the vehicle treated groups at 2 and 7 days post crush. (Fig. 6)

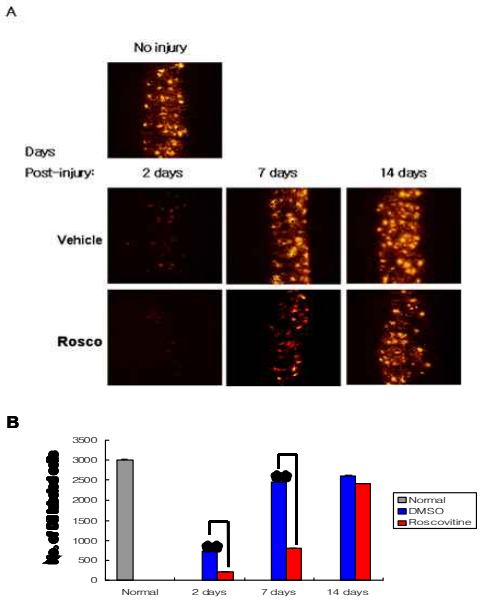

Fig. 6. Decreases in the number of DiI-labeled motor neurons in the spinal cord by Cdc2 inhibitor. (A, B) Two nanomoles of roscovitine were administered into the injury site, and DiI was injected 1 cm distal to the injury site immediately, 5, or 12 days after crush (labled 2 days, 7 days and 14 days in the Figure respectively). Spinal cord sections (20 μm thickness) between T12 and L1 levels were prepared 2 days later. The number of DiI-labeled motor neurons on individual sections was counted and summated. Comparison between DMSO- and roscovitine treated groups was made by Student's t-test (**, $p < 0.01$, $n = 4$). Error bars denote standard error of mean (SEM).

Previous studies have shown that during peripheral nerve regeneration, Schwann cells in the injury area actively proliferated and migrated distally. Here, we investigated whether induced Cdc2 activity in Schwann cells were involved in cell migration. Sciatic nerve segments both proximal and distal to the crush point were isolated and used the explant culture in the DMEM containing 10% serum. Culture was treated with 10 mM of roscovitine 24 hr later and further cultivated for another 24 hr. It was observed that both Schwann cell proliferation and migration in roscovitine-treated explant group were significantly inhibited in comparison with the injury group with no roscovitine treatment (Fig. 7). In non-injury group, no detectable proliferation and migration of Schwann cells was observed.

Fig. 7. Reduction of Schwann cell proliferation and migration by Cdc2 inhibitor. Sciatic nerve fragments both proximal and distal to the injury site were isolated and used for explant preparation. The culture was maintained in serum containing DMEM medium for 24 hr, roscovitine (10 mM) or DMSO vehicle was treated, and further cultured for 24 hr. Cell nuclei were visualized with Hoechst dye staining.

IV. Discussion

Our data clearly show that Cdc2 mRNA and protein levels are induced in regenerating nerves after injury and that

Cdc2 activity was similarly induced in the distal stump of the regenerating axon which was inhibited by roscovitine. We further demonstrate that Cdc2 protein which was expressed in the proliferating Schwann cells is involved in cell proliferation and migration. These observation suggest that Cdc2 activity may be involved in peripheral nerve regeneration via the activation of Schwann cell.

Cyclin and cyclin-dependent kinase are a key regulator of the eukaryotic cell cycle and especially cyclin B1 and Cdc2 are particularly important for the entry G2 to M phase (Norbury and Nurse, 1990; Dunphy et al., 1988). However, the role and mechanism of Cdc2 in nerve regeneration remain unknown. In this study, we found that Cdc2 was expressed in the regenerating nerves 3 and 7 days after sciatic nerve crush injury. Cdc2 mRNA was detected 3 days after crush injury and upregulated by 7 days after injury. Our data further showed that Cdc2 mRNA induction was shifted from the proximal stump to the distal stump between 1 day and 7 days post injury. Interestingly, both Cdc2 mRNA and protein were not detected in the DRG neuron. These data indicate that Cdc2 expression is localized in non-neuronal cells such as Schwann cells after nerve injury.

Phosphorylated Cdc2 has kinase activity in the cells and then actively involved in the mitogenic activity (Connell-Crowley et al., 1993; Solomon et al., 1992). To examine whether Cdc2 kinase activity was required in the nerve regeneration, we performed kinase assay after roscovitine treatment in vivo and in vitro. Strong Cdc2 kinase activity, as determined by Histone H1 phosphorylation in vitro was detected in the distal stump of sciatic nerves 7 days after injury. Roscovitine treatment at the injury site significantly inhibited Cdc2 activity particularly at the distal stump. We also found that in the Schwann cell culture, DRG neurite outgrowth and non-neuronal cell proliferation were inversely affected by roscovitine treatment. These data suggest that Schwann cell proliferation is of great importance in neurite outgrowth and may be controlled by Cdc2 activity.

We have previously shown that Cdc2 protein is colocalized with Schwann cell specific marker protein S100β in the sciatic nerve (Seo et al., 2004). Here, we showed strong induction of Cdc2 expression in cultured Schwann cell prepared from sciatic nerve which had undergone nerve injury for 7 days, and the levels of Cdc2 induction were similar in proliferating Schwann cells prepared from newborn animals. These results suggest that Cdc2 may be a key regulator for the functional involvement of Schwann cells in an area of regenerating sciatic nerves, and thus reinforces the importance of physiological role of Schwann cells in nerve regeneration as has been demonstrated previously (Chan et al., 2001; Cosgaya et al., 2002; Yamauchi et al., 2003, 2004).

Functional regulation of Cdc2 kinase activity in relation to peripheral nerve regeneration is a research area under active investigation in this laboratory. We have recently found that treadmill training of rat enhanced levels of Cdc2 activity in regenerating nerves (Seo et al., manuscript in preparation). We also found that oriental medicinal drug 'Hominis placenta' augmented sciatic nerve regeneration after injury (Seo et al., manuscript submitted), raising the

possibility that herbal drugs may be useful for inducing nerve regeneration after injury. We are currently examining the effect of herbal drugs and functional involvement of Cdc2 kinase activity in nerves after injury in the spinal cord as well as peripheral nerves. These and other approaches would be helpful to clarify detailed molecular mechanism underlying the role of Cdc2 in Schwann cell proliferation and migration.

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