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ERYTHROPOIETIN PROMOTES SCHWANN CELL MIGRATION AND ASSEMBLY OF THE PROVISIONAL EXTRACELLULAR MATRIX BY RECRUITING $\beta 1$ INTEGRIN TO THE CELL SURFACE

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Abstract

In peripheral nerve injury, Schwann cells undergo profound phenotypic modulation, adopting a migratory phenotype and remodeling the extracellular matrix so that it is permissive for axonal regrowth. Erythropoietin (Epo) and its receptor (EpoR) are expressed by Schwann cells after nerve injury; regulating inflammatory cytokine expression and minimizing the duration of neuropathic pain. The mechanism of Epo activity in the injured peripheral nerve remains incompletely understood. Herein, we demonstrate that Epo promotes Schwann cell migration *in vitro* on fibronectin (FN)-coated surfaces. Epo also rapidly recruits $\beta 1$ integrin subunit to the Schwann cell surface by a JAK-2-dependent pathway. Although $\beta 1$ integrin subunit-containing integrins were not principally responsible for Schwann cell adhesion or migration on FN under basal conditions, $\beta 1$ gene-silencing blocked the ability of Epo to promote cell migration. Epo also induced Schwann cell FN expression *in vitro* and *in vivo*. The FN was organized into insoluble fibrils by Epo-treated Schwann cells *in vitro* and into an extensive matrix surrounding Schwann cells *in vivo*. Our results support a model in which Epo promotes Schwann cell migration and assembly of the provisional extracellular matrix in the injured peripheral nerve by its effects on integrin recruitment to the cell surface and local FN production.

Keywords

peripheral nerve; neuroprotective factor; JAK-2

INTRODUCTION

Peripheral nerve injury initiates a program of events referred to as Wallerian degeneration, in which damaged axons are cleared to potentiate axonal regeneration (Griffin and Hoffman, 1993). Schwann cells play an essential role in the response to peripheral nerve injury, de-differentiating and adopting a migratory phenotype (Cheng and Zochodne, 2002; Jessen et al., 1990). Schwann cell migration is supported by provisional extracellular matrix (ECM) proteins, produced locally by Schwann cells or recruited from the plasma and organized into

new scaffolds for regenerating axons (Ide, 1996; Scherer and Salzer, 1996). Schwann cells also secrete neurotrophic growth factors, such as nerve growth factor- β (NGF- β), glial derived neurotrophic factor (GDNF), and erythropoietin (Epo) (Heumann, 1987; Höke et al., 2000; Li et al., 2005).

Cell migration requires the integrated function of integrins, cell-signaling pathways that control assembly of the actin cytoskeleton, and extracellular proteases that remodel the ECM (Lauffenburger and Horwitz, 1996). Well-documented Schwann cell migration stimulators include neurotrophin-3 (NT3) and neuregulin (NRG-1) (Yamauchi et al., 2005; 2008). Although Epo was first characterized as a hematopoietic cytokine, it is now clear that Epo is active in the injured peripheral nerve. Schwann cells express Epo receptor (EpoR) and respond to Epo, produced endogenously in injury or administered as a drug (Campana et al., 2006; Keswani et al., 2004; Li et al., 2005). Epo-binding to Schwann cell EpoR down-regulates expression of tumor necrosis factor- α (TNF- α) and reduces inflammation (Campana et al., 2006). Epo also inhibits axonal degeneration (Campana et al., 2006; Keswani et al., 2004), decreases the duration of chronic pain in animal models (Campana and Myers, 2003; Campana et al., 2006; Sekiguchi et al., 2003), and enhances regeneration of primary afferents, both morphologically and functionally (Elfar et al., 2008; Toth et al., 2008). The effects of Epo on Schwann cell migration have not been considered; however, we previously reported that Epo stimulates migration of breast cancer cells (Lester et al., 2005).

The goal of this study was to determine the effects of Epo on Schwann cell migration. We focused on how Epo regulates Schwann cell migration on fibronectin (FN) because FN expression increases in nerve regeneration and correlates with Schwann cell migration and terminal differentiation (Chernousov & Carey, 2000; Akassoglou et al., 2002). Milner et al. (1997) reported that the principal integrin responsible for Schwann cell migration on FN is α v β 8, unlike most cells, which utilize α 5 β 1 and α 4 β 1 (Carstanjen et al., 2005; Leiss et al., 2008). We report that Epo stimulates Schwann cell migration by a JAK-2-dependent mechanism that requires recruitment of β 1 integrin subunit to the cell surface. At the same time, Epo stimulates FN expression by Schwann cells *in vitro* and *in vivo*. These activities result in an increase in assembly of FN fibrils in association with Schwann cells *in vitro* and the provisional extracellular matrix in the injured peripheral nerve. We propose that the effects of Epo on cell-surface integrin activity and FN expression support peripheral nerve recovery following injury.

MATERIALS AND METHODS

Reagents

The JAK2 inhibitor, AG490, was purchased from Calbiochem (La Jolla, CA, USA). Recombinant human Epo was from Johnson & Johnson (San Diego, CA, USA). Soluble erythropoietin receptor (sEpoR) was from R&D Systems (Minneapolis, MN, USA). Purified FN and monoclonal antibodies targeting S100 and β -actin were from Sigma (St. Louis, MO, USA). Mouse laminin-1 (LN) was from SouthernBiotech (Birmingham, AL, USA). FN-specific polyclonal antibody was from Sigma or Dako (Glostrup, Denmark). Polyclonal antibody specific for phosphorylated EpoR was from Santa Cruz (Santa Cruz, CA, USA). β 1 integrin-specific polyclonal and monoclonal antibodies were from Chemicon (Temecula, CA, USA).

Animal surgeries

Experiments were performed using adult male Sprague-Dawley rats (200 g) from Harlan Laboratories (San Diego, CA, USA). Rats were housed in pairs with a 12 h:12 h light:dark

cycle and ad libitum access to food and water. Rats were anesthetized with 2% isoflurane (IsoSol; VedCo, St. Joseph, MO, USA). Under sterile conditions, the left sciatic nerve was crushed once for 2 sec at the sciatic nerve notch, using flat forceps (Myers et al., 2003). Chronic constriction injury (CCI) was performed as described previously (Bennett and Xie, 1988). CCI is a well-established model of neuropathic pain that induces ischemia, Wallerian degeneration, increased cytokine expression, and hyperalgesia (Campana et al., 2006). Vehicle or Epo (570 nM, 3 μ l) were injected directly into the sciatic nerve prior to crush or CCI. The muscle layer was closed using 6-0 silk sutures, followed by the skin. Twenty-four hours after injury, nerve segments (0.5 cm) immediately proximal or distal to the crush site or the entire CCI site and contralateral nerve were collected. For immunofluorescence microscopy studies, rats were perfused transcardially with 4% paraformaldehyde in phosphate buffer (0.1 mol/L, pH 7.4) prior to collecting sciatic nerve tissues.

Euthanasia was accomplished by intraperitoneal injection of an overdose of anaesthetic cocktail followed by cervical dislocation. All procedures were performed according to protocols approved by the University of California, San Diego Committee on Animal Research, and conform with NIH Guidelines for Animal Use.

Cell Culture

Schwann cells were isolated from sciatic nerves of one day-old Sprague Dawley rats, as previously described (Campana, et al., 1998a). Final preparations were 98% homogeneous, as determined by immunofluorescence for S100. Primary cultures of Schwann cells were maintained in DMEM, 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μ g/mL streptomycin, 21 μ g/mL bovine pituitary extract, and 4 μ M forskolin (complete medium) at 37°C under humidified 5% CO₂. Schwann cell cultures were passaged no more than five times.

Quantitative Real-Time PCR

DNA-free total RNA was extracted from frozen nerve tissue and cells in culture using TriZol (Invitrogen, Carlsbad, CA, USA). Samples were processed and treated with DNase. cDNA was synthesized using the ProSTAR first-strand RT-PCR kit (Stratagene, San Diego, CA, USA). Gene expression was measured by qPCR using a one-step program: 95°C, 10 min, 95°C, 30 sec, 60°C, 1 min for 50 cycles. Cyclophilin gene expression was determined as a normalizer for each sample. mRNA levels were determined by dividing normalized Cts by corresponding control Cts. Primers and probes specific for rat Epo and cyclophilin were previously reported (Bernaudo et al., 2002; Li et al., 2005; Macdonald et al., 2001). FN and β 1 integrin primers and probes were purchased from Applied BioSystems (Foster City, CA, USA). Samples without cDNA were analyzed as “no-template” controls. Samples were studied without prior treatment with reverse transcriptase to confirm the absence of contamination with genomic DNA.

Immunohistochemistry and double-labeling immunofluorescence microscopy

Distal and contralateral sciatic nerves from crush injury studies were collected from rats that were perfused transcardially. The resected tissue was immersed in 4% paraformaldehyde for 2 h and then transferred to 20% sucrose in PBS overnight. Serial 10 μ m sections were prepared using a cryostat and mounted on Superfrost Plus Micro Slides (VWR, West Chester, PA, USA). Antibodies were applied overnight at 4°C. After washing with Tris-buffered-saline/Tween (TBS-T), the sections were incubated with biotinylated secondary antibodies for 30 min, followed by ABC solution (Vector Laboratories, Burlingame, CA, USA). 3,3-diaminobenzidine (DAB) was applied for 30 sec. Nuclei were stained with 0.5% Methyl Green (Fisher scientific, Pittsburgh, PA, USA). For double-labeling immunofluorescence, primary antibodies were applied. Sections were washed and incubated

with AlexaFluor 488-conjugated goat anti-rabbit and AlexaFluor 594-conjugated goat anti-mouse IgG (5 µg/mL) for 1 h. In control studies, primary antibodies were omitted. Nuclei were stained with mounting medium containing DAPI (Invitrogen, Carlsbad, CA, USA). All slides were visualized using a Leica microscope equipped with a DFC300 digital camera and Open Laboratory software.

Extraction of FN

Soluble and insoluble FN were extracted as previously described (Aguirre-Ghiso et al., 2001). Briefly, cells and nerve tissue were sonicated and extracted with 1 mL of 3% Triton X-100, 10 mM EDTA in PBS containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Extracts were centrifuged for 15 min at 14,000 rpm. Supernatants were stored as the soluble fraction. The insoluble pellet was treated with 100 mg/mL DNase (Ambion, Austin, TX, USA) for 20 min and centrifuged at 14,000 rpm. Next, 4% deoxycholate in 50 mM Tris-HCl (pH 8.8) and 20 mM EDTA were added to the pellet. The mixture was centrifuged for 5 min. The remaining pellet was solubilized in Laemmli buffer (Laemmli, 1970).

Cell-surface labeling

Cell surface proteins were labeled with biotin as previously described (Li et al., 2005). Briefly, cells were labeled with Sulfo-NHS-LC-Biotin (1 mg/mL) (Pierce Biotechnology, Rockford, IL, USA) at 4°C for 15 min. Labeling was terminated by washing the cells in PBS containing 100 mM glycine. Cells extracts were prepared in RIPA buffer (1% Triton X-100, 0.5% sodium de-oxycholate, 0.1% SDS, 1 mM sodium orthovanadate). To determine protein content, bicinchoninic acid assays were performed (BCA; Pierce, Rockford, IL, USA). Cell extracts (100 µg) were affinity-precipitated with streptavidin-Sepharose® overnight at 4°C. The streptavidin-Sepharose® beads were washed with RIPA buffer and then transferred to Laemmli sample buffer. SDS-PAGE and immunoblot analysis were performed.

SDS-PAGE and Immunoblotting

Schwann cells and nerve tissues were extracted in RIPA buffer. Protein content was determined by BCA assay. Equal amounts of protein were subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% non-fat milk and incubated overnight at 4°C with primary antibodies. Blots were rinsed again, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:2,000, Cell Signaling Technology, Danvers MA,) and visualized with ECL solution (GE Healthcare, Bio-Science Corp, Piscataway, NJ) and Hyperfilm™ (Amersham Biosciences, Piscataway, NJ, USA). Blots were re-probed for β-actin as a loading control.

β1 integrin gene-silencing

Rat β1 integrin-specific siRNAs (#1 sense: CGACAUCAUCCCAAUUGUAUU; antisense: 5'-PUACAAUUGGGAUGAUGUCGUU; #2 sense: AAUGAAAUGAGGAGGAUUAUU; antisense : 5'-PUAAUCCUCCUCAUUUCAUUUU), and non-targeting control-2 (NTC) siRNA were from Dharmacon (Chicago, IL, USA). Primary cultures of Schwann cells (1×10^6) were transfected with β1 integrin-specific or NTC siRNA (25 nM) by electroporation using the rat neuron nucleofector kit (Amaxa, Gaithersburg, MD, USA) as previously described (Mantuano et al., 2008). β1 integrin gene-silencing, 48 h post electroporation, was determined by qPCR and immunoblot analysis.

Cell adhesion assays

Adhesion of Schwann cells was studied using 96 well plates (Falcon, Franklin Lakes, NJ, USA), as described by Salcioni et al. (2004). Surfaces were coated with FN or LN (5 µg/mL) overnight at 4°C, and blocked with 2% bovine serum albumin (BSA) for 2 h. Schwann cells were treated with vehicle or Epo (0.17–17 nM) in Sato media (Bottenstein and Sato, 1980) 15 min prior to plating. Cells (1.0×10^5) were introduced into each well in 100 µL complete media and allowed to adhere for 60 min at 37°C. The cells were then washed, fixed with 4% paraformaldehyde, and stained with crystal violet (Sigma, St. Louis, MO, USA). Extracts were prepared in 1% SDS. The absorbance was determined at 595 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell migration

Migration of Schwann cells was studied using 6.5-mm Transwell chambers with 8 µm pores (Corning Costar, Corning, NY, USA), as previously described (Mantuano et al., 2008). The bottom surface of each membrane was coated with FN (5 µg/mL). Schwann cells (100,000) in Sato medium (Bottenstein and Sato, 1980) supplemented with 1 µg/mL BSA were added. Epo was added to the top chamber, the bottom chamber, or both chambers. In some studies, Epo was added after the cells were allowed to adhere for 1 h, excluding possible effects of Epo on Schwann cell migration that may have resulted from altered adhesion. In some studies, 10% FBS was added to the lower chamber. Cells were allowed to migrate for 4 h at 37° C. The upper surface of each membrane was cleaned with a cotton swab. The membranes then were stained with Diff-Quik (Dade-Behring, Deerfield, IL, USA). The number of cells on the bottom surface of each membrane was counted.

Statistical analysis

In all experiments, replicates refer to separate experiments, typically performed with internal duplicates or triplicates. Results of cell adhesion, migration, and qPCR experiments were subjected to analysis of variance (ANOVA). Tukey's or Student's Neuman Keuls *post hoc* analysis was used to assess differences between treatment groups. For comparison of two groups, a Student's T-test was performed.

RESULTS

Epo promotes Schwann cell migration on FN

In the injured peripheral nerve, Schwann cells express significantly increased levels of Epo and EpoR (Li et al., 2005). To test the effects of Epo on Schwann cell migration, we established primary cultures of rat Schwann cells and transferred these cells to Transwell units in which the underside of the membrane was coated with FN. The cells were added to the Transwell chambers in Sato medium supplemented with 1 µg/mL BSA, to optimize cell survival (Bottenstein and Sato, 1980). Sato media with or without FBS (10%) was added to the bottom compartment. Epo (17 nM) or vehicle was added to the top chamber, bottom chamber, or both chambers. Fig. 1A shows representative images of Schwann cells that migrated to the underside of the Transwell membrane, with and without Epo treatment. Epo significantly increased Schwann cell migration ($p < 0.01$) when added to the top or bottom chamber (Fig. 1B). The magnitude of the increase was similar to that observed with MMP-9 or NRG-1, our positive controls (Mantuano et al., 2008). Next, we performed dose-response studies. Epo dose-dependently increased Schwann cell migration. The effect was statistically significant with 0.17 nM Epo.

Epo recruits $\beta 1$ integrin to the cell surface

Because $\beta 1$ -subunit-containing integrins are essential for extending and maintaining normal Schwann cell processes (Feltri et al., 2002), we studied the effects of Epo on $\beta 1$ integrin levels in Schwann cells in culture. To detect cell-surface $\beta 1$ integrin, we treated cells with the impermeable biotinylation reagent, sulfo-NHS-LC-biotin. Cell-surface proteins were recovered by affinity-precipitation with streptavidin-Sepharose®. $\beta 1$ integrin in the precipitates was detected by immunoblot analysis. Fig. 2A shows that the level of biotinylated $\beta 1$ integrin was very low in untreated cells. Epo caused rapid and robust re-localization of $\beta 1$ integrin into the NHS-LC-biotin-accessible pool, which we assumed was the cell surface. Epo failed to regulate total cellular $\beta 1$ integrin over the same time course; the mature form of $\beta 1$ integrin (125-kDa) and the immature form (105 kDa), which is restricted mainly to the secretory pathway, were unchanged. These results suggested that the principal activity of Epo is to redistribute $\beta 1$ integrin to the cell surface in Schwann cells.

Fig. 2B demonstrates that in cells treated with Epo for 5 min, the increase in cell-surface $\beta 1$ integrin subunit correlated with an increase in the level of phosphorylated EpoR. When Schwann cells were pretreated with the JAK-2 inhibitor, AG490, Epo-induced $\beta 1$ integrin relocalization to the cell surface was blocked, suggesting that EpoR-initiated cell-signaling is necessary (Fig. 2C). JAK2, a tyrosine kinase, is activated in Schwann cells following binding of Epo to EpoR (Li et al., 2005). To test whether Epo regulates $\beta 1$ integrin mRNA expression, Schwann cells were treated with Epo for 5 h. Fig. 2D shows that $\beta 1$ integrin mRNA was not significantly increased.

Epo promotes Schwann cell adhesion to FN

The $\beta 1$ integrin subunit associates with α subunits to form adhesion receptors from diverse ECM proteins (Wu et al., 1993). To test whether Epo regulates the activity of FN-binding integrins, we examined Schwann cell adhesion to immobilized FN. Adhesion to LN was compared as a control. Cells were treated with Epo or vehicle for 15 min and then allowed to adhere for 1 h. As shown in Fig. 3A–B, Epo promoted Schwann cell adhesion selectively to FN ($p < 0.01$) and had no effect on Schwann cell adhesion to LN.

To demonstrate that Epo promoted Schwann cell migration independently of its effect on adhesion, migration studies were performed in which the cells were allowed to attach to the Transwell membrane for 1 h in the absence of Epo. The membranes then were washed and the medium was replaced with fresh medium containing Epo or vehicle. Fig. 3C demonstrates that under these conditions, Epo still promoted cell migration.

$\beta 1$ integrin is required for Epo-promoted Schwann cell adhesion and migration

The $\beta 1$ integrin gene was silenced in Schwann cells using siRNA. Schwann cell primary cultures were transfected with a battery of candidate rat $\beta 1$ integrin specific siRNAs or with NTC siRNA. As shown in Fig. 4A, $\beta 1$ integrin siRNA #1 decreased $\beta 1$ integrin mRNA by 85–95% within 24–48 hours. $\beta 1$ integrin siRNA #2 reduced $\beta 1$ integrin mRNA by approximately 50%. No change in $\beta 1$ integrin mRNA was observed in cells transfected with NTC siRNA compared with untransfected cells (data not shown). To confirm that $\beta 1$ integrin silencing was achieved at the protein level, we studied cultures transfected with $\beta 1$ integrin siRNA #1, $\beta 1$ integrin siRNA #2, or with NTC by immunoblot analysis. Fig. 4B demonstrates that both $\beta 1$ integrin-specific siRNAs reduced levels of $\beta 1$ integrin subunit.

Next, we performed adhesion studies with Schwann cells in which the $\beta 1$ integrin subunit was silenced with either specific siRNA and with cells that were transfected with NTC siRNA. In the absence of Epo, $\beta 1$ integrin siRNA #1 and #2 did not significantly inhibit cellular adhesion to FN (Fig. 4C), consistent with the results of prior studies indicating that

$\beta 1$ integrins play a minor role in Schwann cell adhesion to FN (Milner et al., 1997). Epo promoted adhesion of control Schwann cells, transfected with NTC siRNA to FN in a dose-dependent manner; the optimal Epo concentration increased adhesion about 2-fold ($p < 0.01$). $\beta 1$ integrin silencing with either siRNA #1 or #2, significantly inhibited Epo promoted Schwann cell adhesion to FN. The efficacy of siRNA #1 was slightly improved. These results suggest that the effects of Epo on Schwann cell adhesion require $\beta 1$ integrin.

Next, we tested whether $\beta 1$ integrin is necessary for Epo-promoted Schwann cell migration. As shown in Fig. 5, Epo significantly increased migration of cells that were transfected with NTC siRNA ($p < 0.01$), as anticipated. Specific silencing of $\beta 1$ integrin with siRNA #1 reduced the basal rate of Schwann cell migration by 25% in the absence of agonists ($p < 0.01$). However, more importantly, in these cells, Epo failed to promote cell migration. These results suggest that Epo promotes Schwann cell migration by a mechanism that requires $\beta 1$ integrin.

Epo facilitates FN fibril formation

A major function of $\beta 1$ subunit-containing integrins is to seed formation of FN fibrils that extend throughout the provisional ECM (Wu et al., 1993). These fibrils may be distinguished from intracellular FN and plasma FN based on solubility in detergent. Because $\beta 1$ integrin was up-regulated on the surface of Epo-treated Schwann cells, we undertook studies to determine whether Epo promotes generation of detergent-insoluble FN fibrils. Schwann cells were cultured in serum-free Sato medium for 15 h. Established methods were applied to extract insoluble and soluble FN from the cultures (Aguirre-Ghiso et al., 2001). As shown in Fig. 6A, Epo substantially increased the amount of detergent-insoluble FN fibrils recovered in association with the Schwann cells; however, surprisingly, Epo also increased the amount of FN recovered in detergent-soluble fractions.

To test whether the increase in FN recovery in both detergent fractions was due to increased FN expression, we studied the effects of Epo on FN mRNA expression. Schwann cells were treated with Epo for 4 or 8 h. Fig. 6B shows that Epo significantly increased FN mRNA expression. We also studied the effects of hypoxia (1% oxygen) on FN expression by Schwann cells because hypoxia induces endogenous Epo expression (Digicaylioglu et al., 1995). Epo mRNA expression was substantially increased ($p < 0.01$) in Schwann cells cultured in 1.0% oxygen for 8 h as anticipated (Fig. 6C). FN mRNA expression also was significantly ($p < 0.05$) increased (Fig. 6D). This effect was blocked when the cells were cultured in the presence of soluble EpoR (1 $\mu\text{g/mL}$), demonstrating that endogenously-produced Epo drives expression of FN in hypoxia.

Epo induces FN expression in injured sciatic nerve

In rodent models of peripheral nerve injury, Schwann cells account for 90% of the mRNA during the first 24 h (Asbury and Johnson, 1978). To test whether Epo regulates FN expression by Schwann cells *in vivo*, rat sciatic nerves were subjected to chronic constriction injury (CCI). Epo or vehicle was injected intraneurally into the CCI site at the time of surgery. After 24 h, nerve tissue was collected from the CCI site for qPCR analysis. Fig. 7 shows that Epo significantly increased FN mRNA present at the CCI site ($p < 0.01$), compared with vehicle-treated injured nerves. In sham-operated nerves, FN mRNA was not detected (results not shown).

Next, we performed immunohistochemistry studies to detect FN protein in crush-injured sciatic rat nerves. At the time of injury, nerves were injected intraneurally with Epo or vehicle at the crush injury site. Sciatic nerve tissue (distal to the injury site and contralateral side) was collected 24 h later. As shown in Fig. 8A–D, FN immunoreactivity was barely

detectable in the uninjured contralateral nerve, as anticipated (Akassoglou et al., 2002). In crush-injured nerves treated with vehicle, FN immunoreactivity was increased distal to the injury site. Epo treatment further increased FN immunoreactivity distal to the injury site compared with vehicle-treated nerves. The effects of Epo on FN fibril deposition in injured nerves were confirmed by subjecting whole nerve extracts (distal and proximal nerve segments) to immunoblot analysis (Fig. 8E). Immunoblotting for β -actin confirmed equal loading in each lane. Taken together, these immunohistochemistry and immunoblotting results and our qPCR data demonstrate that Epo promotes FN production by Schwann cells both *in vitro* and *in vivo*.

FN in the injured sciatic nerve may be derived from the plasma or produced locally. In either case, we hypothesized that the FN would associate with Schwann cells due to expression of FN-binding integrins. Fig. 8F–K shows the results of double-labeling immunofluorescence microscopy studies in which sections of Epo-treated crush-injured sciatic nerves were immunostained to detect FN and β 1 integrin or FN and S100, a specific Schwann cell marker. β 1 integrin co-localized entirely with FN immunoreactivity, whereas FN also dispersed independently of the integrin subunit. Similarly, S100 and FN partially co-localized. These results support our model in which the FN matrix is assembled initially in association with Schwann cells in the Epo-treated injured peripheral nerve.

DISCUSSION

Exposure of injured peripheral nerves to Epo may promote regeneration (Elfar et al., 2008; Grasso et al., 2007; Keswani et al., 2004; Toth et al., 2008). In rodent models, Epo facilitates recovery from neuropathic pain (Campana and Myers, 2003; Campana et al., 2006; Sekiguchi et al., 2003); however, the mechanism underlying Epo-mediated recovery is incompletely understood. In this study, we demonstrated that Epo stimulates Schwann cell migration on FN. Because Epo promoted migration when added to the bottom or top chamber, it appears that Epo acts as a chemokinetic factor for Schwann cells. This effect was associated with an apparent rapid redistribution of β 1 integrin to the Schwann cell surface. Although the β 1 chain-associated α -chains were not identified, Epo-treated Schwann cells demonstrated increased adhesion to FN, suggesting that α 5 β 1 or α 4 β 1 were formed. β 1 integrin was necessary for the effects of Epo on Schwann cell migration, suggesting that relocation of β 1 integrin to the cell surface and increased cell migration may be mechanistically linked. However, these data do not rule out other simultaneous mechanisms by which Epo may promote Schwann cell migration. For example, in breast cancer cells, Epo promotes cell migration by activating ERK/MAP kinase (Lester et al., 2005). Epo activates ERK/MAP kinase in Schwann cells as well (Li et al., 2005).

Under basal conditions, Schwann cell migration on FN is mediated mostly by α v β 8 (Milner et al., 1997). This is supported by the findings by Feltri et al., (2002) who showed that conditional deletion of β 1 integrin in Schwann cells did not affect migration, survival, or proliferation in immature (E17.5-P5) Schwann cells. We propose that Epo, by relocating β 1 integrins to the cell surface, may mobilize a second battery of FN receptors, supplementing the activity of constitutively-expressed α v β 8. These integrins then work in a complementary manner to promote Schwann cell association with the provisional ECM.

Treatment of injured nerves or Schwann cells in culture with Epo increased FN expression. The FN was assembled into detergent-insoluble fibrils in culture and into an extensive ECM *in vivo*. FN is an essential component of the provisional ECM, which is important for anchorage of cells and for guiding cell migration in embryonic development and wound healing (Urbich et al., 2002). Simultaneous regulation of FN expression and Schwann cell

$\beta 1$ integrin localization, at the subcellular level, is consistent with a model in which ECM remodeling is a major function of Epo in the injured peripheral nerve.

The function of FN in peripheral nerve injury has been characterized (Lefcort et al., 1992). FN is largely undetectable in normal sciatic nerve. After injury, FN accumulation may reflect local synthesis or transfer from the bloodstream. Dissociated adult rat DRG neurons extend long axons within degenerating myelin along FN-enriched host astrocytes (Tom et al., 2004). Blockade of FN function inhibits axonal extension. Our immunohistochemistry studies do not allow us to determine whether the increase in intraneural FN, observed after Epo treatment, is derived from the plasma or local synthesis; however, our results suggest that FN expression by Schwann cells is at least partially involved. Schwann cells in culture expressed increased levels of FN mRNA in response to Epo. FN mRNA also was increased in Epo-treated injured sciatic nerves. In double-labeling immunofluorescence microscopy studies, FN was co-localized with Schwann cell bodies and $\beta 1$ integrin in Epo-treated injured nerves. FN has been previously localized to Schwann cell endoneurial tubes, suggesting that whether Schwann cells synthesize FN *in vivo* or the FN is derived from plasma, the FN may be polymerized in association with Schwann cell surfaces (Baron-Van Evercooren et al., 1986).

The response to PNS injury is regulated not only by Epo, administered therapeutically, but also by endogenously-produced Epo (Li et al., 2005). Thus, endogenously produced Epo may contribute to the deposition of FN observed under basal conditions. The ability of endogenously-produced Epo to function as a regulator of FN expression and assembly was demonstrated in our hypoxia experiments. The extent to which endogenously-produced Epo controls FN production may be influenced by the oxygen tension in the injured nerve. Hypoxia favors Epo expression by Schwann cells and FN expression as a secondary event. Others have demonstrated regulation of Schwann cell FN expression by various compounds that control cell signaling. For example, treatment of Schwann cells with cAMP analogues *in vitro* increases deposition of FN into the extracellular matrix and FN fibril assembly (Baron-Van Evercooren et al., 1986). Ascorbate also may promote assembly of FN fibrils on Schwann cell surfaces in association with type IV collagen (Chernousov et al., 1998).

Although FN is encoded as a single gene, alternative splicing of the FN mRNA may generate over ten variants of the mature protein (Vogelezang et al., 1999; White et al., 2008). How alternative FN splicing contributes to the function of FN remains incompletely understood. Intraneural FN production is substantially increased in the latter stages of nerve injury when regeneration dominates (Ide et al., 1983; Scherer and Salzer, 1996). Antibodies that block interaction of FN with cells inhibit axon elongation and nerve repair (Wang et al., 1992). Our PCR primers and probes were designed to amplify all the potential variants of FN. Using this strategy, our methods were optimized to detect gene expression. Nevertheless, potential effects of Epo on FN splice variant production may be important and thus, a topic for future investigation.

Conditional deletion of $\beta 1$ integrin in Schwann cells inhibits radial sorting (Feltri et al., 2002), which refers to the extension of Schwann cell processes within bundles to segregate out axons destined for myelination. Because of the apparent enhancement of $\beta 1$ integrin function by Epo, we hypothesize that Epo may improve radial sorting in developing nerves. The ability of Epo to simultaneously induce FN accumulation enhances the possible importance of this candidate mechanism.

Clinically, Epo is used as an FDA-approved drug for the treating anemia. However, Epo also has received attention for its activity in the nervous system. Epo has been shown to enhance cognition in both normal as well as diseased human subjects (Ehrenreich et al., 2007;

Miskowiak et al., 2007). Epo also improved sensory neuropathy in kidney failure patients (Hassan et al., 2003). Derivatives of Epo with neurotrophic, but not hematopoietic activity represent potential therapeutics for treating neurological dysfunction (Brines et al., 2008; Campana et al., 1998b; Leist et al., 2004). Future studies to determine whether Epo derivatives mimic the activity of Epo in regulating formation of the provisional extracellular matrix in PNS injury may be justified.

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Fig. 1. Epo promotes Schwann cell migration on FN-coated surfaces

(A) Images of Schwann cells which migrated to the underside surface of Transwell membranes. Cells were treated with Epo (17 nM) or vehicle. 10% FBS was added to the bottom chamber as indicated. Migration was allowed to proceed for 4 h. (B) Quantification of Schwann cell migration results, * $p < 0.01$ compared with respective vehicle controls. (C) Effects of various Epo concentrations added to both chambers on Schwann cell migration. All data are expressed as “mean $\pm$ SEM” (n=3).

Fig. 2. Epo recruits $\beta 1$ integrin to the cell surface in Schwann cells

(A) Primary cultures of Schwann cells were treated with Epo (1.7 nM) or vehicle for the indicated times. Biotinylated cell-surface proteins were streptavidin affinity-precipitated and subjected to immunoblot analysis for $\beta 1$ integrin (upper panel). (B) Primary Schwann cells were treated with Epo (1.7 nM) or vehicle for 5 min. Biotinylated cell-surface proteins were streptavidin affinity-precipitated and subjected to immunoblot analysis for $\beta 1$ integrin or phosphorylated EpoR. (C) Primary Schwann cells were pretreated with AG490 (20 μ M), the pharmacological inhibitor of JAK2, 15 min prior to Epo (1.7 nM) treatment for 5 min. Biotinylated cell-surface proteins were streptavidin affinity-precipitated and subjected to immunoblot analysis for $\beta 1$ integrin (upper panel). For all panels, total cell extracts were

also immunoblotted for $\beta 1$ integrin as a loading control (lower panel). Equal amounts of cellular protein were loaded into each lane, subjected to immunoblot. Each blot represents at least 5 independent studies. (D) Primary Schwann cells were treated with Epo (1.7 nM) or vehicle for 5 h. Total RNA was extracted and real time qPCR for $\beta 1$ integrin mRNA was performed (n=3).

Fig. 3. Epo-stimulated Schwann cells demonstrate increased adhesion to immobilized FN (A) Images of Schwann cell adhering to FN or LN (5 μ g/mL). (B) Quantification of adhesion results. Schwann cells treated with Epo (1.7 nM) or vehicle which adhered to FN or LN coated plates after 1 h. Data are expressed as “mean $\pm$ SEM” (n=6), * p <0.05 compared to vehicle control on FN. (C) Quantification of Schwann cell migration results. Cells were plated for 1 h prior to treatment with Epo (17 nM) or vehicle. Migration proceeded for 3 h. Data are expressed as “mean $\pm$ SEM” (n=3), ** p <0.01 compared to vehicle control.

Fig. 4. β 1 integrin facilitates adherence to FN in Epo-stimulated Schwann cells

(A) Primary Schwann cells were transfected with β 1 integrin-specific siRNAs or with NTC siRNA. β 1 integrin mRNA levels were determined by real time qPCR, 24 and 48 h after transfection (n=4/group). (B) Schwann cells were transfected with NTC siRNA, β 1 integrin siRNA #1 or β 1 integrin siRNA #2. Equal amounts of cell extract (35 μ g of protein) were subjected to immunoblot analysis for β 1 integrin. The same blots were probed for β -actin as a loading control. Blots represent three independent studies. (C) Schwann cells, which were transfected with β 1 integrin siRNA #1, β 1 integrin siRNA #2 or NTC siRNA, were pretreated with Epo (0–17 nM) or vehicle for 15 minutes. Adhesion was allowed to proceed

for 1 h. Adhesion results are expressed as “mean $\pm$ SEM” (n=4), * p <0.05 and ** p <0.01 compared to vehicle-treated NTC cells.

Fig. 5. Epo induced Schwann cell migration is $\beta 1$ integrin dependent

(A) Images of Schwann cells transfected with NTC siRNA or $\beta 1$ integrin siRNA #1 that migrated to the underside surface of Transwell membranes. Cells were treated with Epo (1.7 or 17 nM) or vehicle and placed on FN-coated plate. Migration was allowed to proceed for 4 h. (B) Quantification of cell migration results. Data are expressed as “mean $\pm$ SEM” (n=6), ** $p < 0.01$ compared with vehicle-treated NTC cells.

Fig. 6. Epo stimulates the expression and assembly of FN in Schwann cells

(A) Soluble and insoluble FN levels in Epo (1.7 nM) or vehicle treated Schwann cells for 15 h. Equal amounts of protein (2.5 μg) were analyzed by immunoblotting using an anti-FN polyclonal antibody. Blots were probed for β-actin as a loading control. Blots represent four independent experiments. (B) FN mRNA expression in Epo (1.7 nM) or vehicle treated Schwann cells for 4 and 8 h. Data are expressed as “mean ± SEM” (n=5), * $p<0.05$; ** $p<0.01$. (C) Epo mRNA expression in Schwann cells placed under normoxia or hypoxia for 8 h. Results are expressed as fold increase compared to cells under normoxia. Data are expressed as “mean ± SEM” (n=4–6), ** $p<0.01$ compared to normoxia controls. (D) FN mRNA expression in Schwann cells placed under hypoxia for 8 h with or without a neutralizing soluble Epo receptor (sEpoR, 1 μg). Results are expressed as fold increase compared to cells under normoxia without sEpoR. Data are expressed as “mean ± SEM” (n=4–6), * $p<0.05$ compared to cells under normoxia without sEpoR.

Fig. 7. Intraneural injection of Epo into rat sciatic nerve increases FN mRNA

Nerves with chronic constriction injury (CCI) were injected with Epo (570 nM) or vehicle at the time of surgery. Twenty-four hours later nerves were collected at the injury site. FN mRNA was analyzed by qPCR (n=4–6/group). Data are expressed as “mean $\pm$ SEM”, ** p <0.01 compared to respective vehicle controls.

Fig. 8. Epo stimulates FN assembly in injured peripheral nerve *in vivo*

Images of (A) uninjured contralateral rat sciatic nerve from vehicle-treated rats immunostained for FN after 24 h; (B) uninjured contralateral rat sciatic nerve from Epo-treated rats immunostained for FN after 24 h; (C) crush injured rat sciatic nerve (distal segment) from vehicle-treated rats immunostained for FN after 24 h; (D) crush injured rat sciatic nerve (distal segment) from Epo-treated rats immunostained for FN after 24 h. Nuclei are marked by methyl green. Magnification 500 $\times$, scale bar 50 μ m. (E) Immunoblot of FN from injured sciatic nerves treated with Epo or vehicle after 24 h. Rat sciatic nerves were injected with Epo or vehicle at the time of crush injury. Twenty-four hours later, detergent soluble and insoluble fractions were collected. Nerve lysates (0.5 μ g of protein) were subjected to SDS-PAGE and immunoblot analysis using an anti-FN specific polyclonal antibody. Purified FN (25 ng) was used as a positive control. β -actin was used as a loading control. (F–H) Double-labeling immunofluorescence for FN (green) and β 1 integrin (red). (I–K) Double-labeling immunofluorescence for FN (green) and S100 (red) in Epo-treated crush injured nerve after 24 h. Nuclei are marked with DAPI (blue). Merged images are shown on right. Photomicrographs have 630 $\times$ magnification. Scale bar is 30 μ m. Images are representative of n=3–4/group.