Regulation of expression of fibronectin and its receptor, $\alpha_5\beta_1$, during development and regeneration of peripheral nerve

FRANCES LEFCORT$^{2,*}$, KRISTINE VENSTROM$^2$, JOHN A. MCDONALD$^3$ and LOUIS F. REICHARDT$^{1,2}$

$^1$Neuroscience Program, Department of Physiology and $^2$Howard Hughes Medical Institute, University of California, San Francisco, 3rd & Parnassus Avenues, U426, San Francisco, CA 94143-0724, USA
$^3$Mayo Clinic Scottsdale, 13400 E. Shea Boulevard, Scottsdale, Arizona 85259, USA

*Author for correspondence

Summary

The extracellular matrix glycoprotein, fibronectin, is a potent promoter of peripheral neurite outgrowth. Interactions of peripheral neurons with fibronectin have been shown to be primarily mediated by the $\beta_1$ class of integrin heterodimers. In the present study, we have examined the expression and regulation of fibronectin and its integrin receptor, $\alpha_5\beta_1$, in developing and regenerating chick peripheral nerve. We show that fibronectin and $\alpha_5\beta_1$ are expressed at comparatively high levels in developing nerve with $\alpha_5\beta_1$ expression on axons and non-neuronal cells. With nerve maturation, both proteins are less prominently expressed and the cellular pattern of $\alpha_5\beta_1$ expression becomes more restricted. Following lesion of mature nerve, both fibronectin and $\alpha_5\beta_1$ are strongly induced with prominent expression of $\alpha_5\beta_1$ on regenerating neurites and Schwann cells. The elevation in fibronectin levels in the regenerating nerve is highest in the vicinity of the lesion, an area undergoing extensive cellular remodeling including Schwann cell migration and growth cone extension. Our results suggest that fibronectin and its receptor, $\alpha_5\beta_1$, may mediate functionally important interactions in the development and regeneration of peripheral nerve.

Key words: fibronectin, integrin, peripheral nerve, chick.

Introduction

In the formation of the peripheral nervous system, neural crest cells migrate and neurons extend axons through areas rich in extracellular matrix (ECM; for review, Sanes, 1989). Studies in vitro have demonstrated that ECM constituents, in particular fibronectin (FN), support the attachment, spreading and migration of neural crest cells and potently promote peripheral neurite outgrowth (Rogers et al., 1983; Tomaselli et al., 1986; Humphries et al., 1988; Dufour et al., 1988). FN has been shown to be localized along the pathways of migrating neural crest cells (Newgreen and Thiery, 1980; Krotoski et al., 1986) and reagents, such as RGDS-containing peptides, which disrupt interactions of cells with fibronectin, inhibit the migration of neural crest cells (Boucaut et al., 1984).

The primary class of cellular FN receptors identified thus far are members of the integrin family of heterodimers (for review see Hynes, 1992; Hemler, 1990; Reichardt and Tomaselli, 1991). Each integrin heterodimer is composed of an $\alpha$ and $\beta$ subunit with the ligand specificity determined by the particular combination of subunits. Four heterodimers containing the $\beta_1$ subunit: $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_9\beta_1$, have been identified as FN receptors (Pytela et al., 1985; Elices et al., 1990; Takada et al., 1988; Wayner et al., 1988; Vogel et al., 1990)). $\alpha_5\beta_1$, $\alpha_\gamma\beta_1$ and possibly $\alpha_\gamma\beta_1$ interact with the RGDS-sensitive major cell attachment site of FN (Pierschbacher and Ruoslahti, 1984; Elices et al., 1991) while $\alpha_\beta_1$ appears to interact with several sites in the C terminus of FN, including two heparin-binding regions and the alternatively spliced CS1 domain (Wayner et al., 1989; Guan and Hynes, 1990; Mould and Humphries, 1991).

Dorsal root ganglion neurons in vitro have been demonstrated to interact with both of these domains, extending neurites on both the C-terminal heparin-binding fragment and the RGD-containing 75×10$^3$ $M_r$ fragment (Humphries et al., 1988; Rogers et al., 1985). Similarly neural crest cells are known to interact with each domain (Dufour et al., 1988). The interactions of both neural crest cells and peripheral neurons with FN are mediated by integrins containing $\alpha_5\beta_1$, $\alpha_\gamma\beta_1$ or $\alpha_\gamma\beta_1$ to interact with the RGDS-sensitive cell binding domain as well as $\alpha_\gamma\beta_1$ to interact with the C-terminal binding sites.

Fibronectin expression is regulated both during embryogenesis (Roman and McDonal, 1992) and in wound repair in adult mammalian skin (ffrench-Constant and Hynes, 1989; ffrench-Constant et al., 1989; Clark, 1990). During embryogenesis, the pattern of alternative splicing of FN is
spatially and temporally regulated with inclusion of the alternatively spliced EIIIA and EIIIB regions only during the early stages of embryogenesis (ffrench-Constant and Hynes, 1989). During cutaneous wound healing in adult skin, these two embryonic splice forms are reexpressed by the cells at the wound base (ffrench-Constant et al., 1989).

The pronounced elevation in FN expression following skin injury is thought to be a critical component of the wound response as it provides a provisional matrix that facilitates the migration of several cell types into the wound region (for review, see Clark, 1990).

Previous work has shown that the responsiveness of sensory neurons to FN, assayed in vitro, is down regulated during embryogenesis (Kawasaki et al., 1986; Millaruelo et al., 1988). Similarly, recent work suggests that the tissue distribution of the α5β1 integrin receptor becomes more restricted during embryogenesis (Muschler and Horwitz, 1991; Roman and McDonald, 1992). However, the expression and function of FN receptors has been shown to increase in epidermal cells isolated from healing wounds (Takashima et al., 1986; Grinnell et al., 1987). Several neuronal cell surface adhesion molecules whose expression decreases during development have been shown to be upregulated following nerve injury (Daniloff et al., 1986; Martini and Schachter, 1988).

The aim of the present study was to characterize the expression and regulation of FN and one of its putative neuronal integrin receptors, α5β1, in the peripheral nerve. We have focused on the α5β1 heterodimer because preliminary examinations of the other β1-containing FN receptors did not reveal appropriate cellular expression (αc), or obvious expression changes (α3) or adequate reagents (αv) to prompt their further study (data not shown). Our results show that FN and α5β1 are more prominently expressed in the developing chick peripheral nerve than in the mature nerve and, following transection of a mature nerve, the levels of both fibronectin and the α5 integrin subunit are strongly increased.

**Materials and methods**

**Surgery**

Three-week-old White Leghorn chickens were anaesthetized with ketamine/xylazine and the medial-ulnar nerve innervating the right wing was transected. The proximal and distal nerve stumps were juxtaposed minimizing their separation to facilitate regeneration; the maximal gap distance between the two stumps was no greater than 1 mm. At various time points following nerve transection (3 days, 1 week, 2 weeks, 4 weeks), the animals were killed by an overdose of the same anaesthetics followed by CO2 asphyxiation. The regenerated nerve was removed from the animal and divided into equal length segments (2-3 mm) proximal and distal to the transection site (Fig. 1). An equal length segment from the contralateral unoperated nerve was removed and served as control.

All tissues were frozen immediately and stored at −80°C usually overnight before homogenization. For each experiment, nerves from 4-7 animals were transected and homogenized together. This procedure was repeated 2-4 times for each experimental time point.  

**Antibodies**

The anti-fibronectin-CS1 monoclonal antibody (mAb FN-kv1) was generated using E10 cultured chick brain glial cells as the immunogen (Harlow and Lane, 1988). Cultured glial cells, scraped off in phosphate-buffered saline (PBS: 0.2g/l KCl, 0.2g/l KH2PO4, 2.16 g/l Na2HPO4, 8 g/l NaCl, 0.1mM CaCl2, 0.1mM MgCl2) with 1 mM PMSF, chymotrypsin, leupeptin, antipain and pepstatin were injected with RIBI adjuvant (Hamilton, Montana) into Balb/C mice. Hybridoma supernatants were screened for their ability to block chick E6 retinal cell adhesion to the ‘CS1’ domain of fibronectin (peptide sequence: DELPQLVTLPHPHPNLHGPEILDVPSTC). The hybridoma secreting FN-kv1 was subcloned by limiting dilution, grown in RPMI with 4% fetal calf serum and 1% Nutridoma (Boehringer-Manheim Biochemicals, Indianapolis, IN) and injected into Balb-c mice to obtain ascites. The allotype of the FN-kv1 mAb is mouse IgG1 (Calbiochem Hybridoma Subtyping kit, San Diego, CA). To purify the ascites Trizma (pH 8-9) was added to 100 mM and NaCl to 2.5 M. The ascites was then fractionated on protein-A Sepharose-CL-4B (Pharmacia, Uppsala Sweden) according to Harlow and Lane (1988). The IgG was dialyzed against PBS (Ca2+- and Mg2+-free) and stored at −20°C. A second monoclonal antibody against fibronectin, VA13, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA (under contract NO1-HD-6-2915 from the NICHD). VA13 recognizes intact FN as well as the 140/160×10^3 Mr cell-binding fragments generated by elastase digestion. Both mAbs gave identical staining patterns on immunoblots and immunocytochemistry and were thus used interchangeably.

Two antibodies were used to recognize the integrin α5 subunit: (1) A2F7, a monoclonal antibody from Drs J. Muschler and A. F. Horwitz (University of Illinois, Urbana; Muschler and Horwitz, 1991) and (2) α5-47, a polyclonal antibody (affinity-purified IgG) generated against a 20mer peptide corresponding to the C terminus of the human α5 sequence (LPYGTMEMQKLPPATSDA; prepared as in Roman et al., 1989). Since the polyclonal antibody, α5-47, provided a much stronger signal for immunocytochemistry than the A2F7 monoclonal antibody, it alone was used for all immunocytochemistry.

To identify axons, two neural specific mAbs were used: (1) a mAb generated against a β-tubulin isoform (c34) specific for neurons (Tuji, from Dr A. Frankfurter, University of Virginia; Yaginuma et al., 1990)) and (2) a cocktail of mAbs against the 68, 160, 200×10^3 Mr neurofilament subunits (Boehringer-Manheim). To label Schwann cells, a polyclonal antibody against the S-100 protein (Dakopatts) was used. A polyclonal affinity-purified antibody raised against mouse laminin (LN) was used to visualize LN isoforms (JW2; Lander et al., 1985).

**Immunoblots**

Nerve segments were homogenized in 10 mM Hepes, pH 7.4 in 0.15 M NaCl, 0.32 M sucrose, 2 mM PMSF, 1 mM chymotrypsin, leupeptin, aprotinin and pepstatin, and centrifuged at 10,000 g.
Expression of fibronectin in chick peripheral nerve

For immunocytochemical comparisons of expression levels of a particular antigen, sections of the two nerves being compared (e.g. immature vs. mature nerve; control vs. regenerating nerve) were collected on the same slide. After immunolabeling, photographic exposures of equal duration were taken and all negatives were processed equally.

Results

To identify the chicken \(\alpha_5\) subunit, we used two different antibodies: an affinity-purified polyclonal antibody generated against the C terminus of the human \(\alpha_5\) sequence (\(\alpha_5\)-47) and a monoclonal antibody (A2F7) generated against the 150×10^3 \(M_r\) band (‘band 1’; Hyenes et al., 1989) immunoprecipitated by the CSAT antibody (Boyzczko and Horwitz, 1986). Previous work has confirmed that this 150×10^3 \(M_r\) protein from chick fibroblasts binds to a FN column and is a chick homolog of the human \(\alpha_5\) subunit (Hyenes et al., 1989; Muschler and Horwitz, 1991). Both antibodies recognized a single band of 150×10^3 \(M_r\) in extracts of chick peripheral nerve (Fig. 2A, lane 1 and 2). Since it was essential to determine that the \(\alpha_5\)-47 polyclonal antibody was specifically recognizing a chick homolog of the human \(\alpha_5\) subunit, we tested whether A2F7 would recognize the 150×10^3 \(M_r\) protein immunoprecipitated from a chick peripheral nerve extract by the \(\alpha_5\)-47 affinity-purified IgG. A single band was recognized by the mAb A2F7 (Fig. 2B, lane 1, which was not recognized in a control immunoprecipitation, Fig. 2B, lane 2) indicating that the two antibodies interact with the same antigen and that the \(\alpha_5\)-47 IgG recognizes a chick homolog of the human \(\alpha_5\) integrin subunit. Additional evidence that the \(\alpha_5\)-47 polyclonal antibody recognizes a chick fibronectin receptor is that \(\alpha_5\)-47 immunohistochemically stains fibrillar apparent ECM contact sites and focal contacts (Roman et al., 1989) in chick fibroblasts cultured overnight on fibronectin (data not shown; see Methods for details on additional control experiments).

Two different monoclonal antibodies against FN were used in this study: mAb Va13, which recognizes intact FN and the RGD-cell binding domain, and mAb FN-kv1, which recognizes the CS1 domain and intact FN. Both antibodies recognized purified bovine FN on a reducing gel (Fig. 2C, lanes 1 and 2) and recognized the same bands, around 220×10^3 \(M_r\), on an immunoblot of chick peripheral nerve extract (Fig. 2C, lanes 3 and 4).

Distribution of \(\alpha_5\) and FN in normal adult nerve

The \(\alpha_5\) subunit associates with the integrin \(\beta_1\) subunit to form the functional FN receptor, \(\alpha_5\beta_1\); it has not been observed associated with any \(\beta\) integrin subunit other than \(\beta_1\). To obtain evidence for the presence of the \(\alpha_5\beta_1\) heterodimer in chick peripheral nerve, we determined by immunocytochemistry that all cells expressing the \(\alpha_5\) subunit also expressed the \(\beta_1\) subunit (data not shown); co-precipitation from chick nerve extracts with the \(\alpha_5\)-47 polyclonal antibody of an 110×10^3 \(M_r\) protein recognized specifically by anti-\(\beta_1\) antibodies provided direct biochemical evidence for the association of the two integrin subunits in peripheral nerve (data not shown). In cross sections

Immunocytochemistry

Regenerated and control nerves were removed from the animal and either frozen immediately in liquid nitrogen or fixed in 3% paraformaldehyde for 2 hours followed by overnight incubation in 15% sucrose. After embedding in Tissue-Tek OCT (Miles, Inc.), the nerve segments were sectioned at 10-14 \(\mu\)m and the slides stored at −20°C. Both the contralateral control nerve and regenerating nerves were embedded in the same block of OCT; thus each slide contained sections of both nerves. To examine developing nerve, medial nerves were removed from 1-day-old chicks and treated similarly. The sections taken from nerves that had not been previously fixed were then fixed in either 3% paraformaldehyde for 20 minutes or −20°C methanol for 10 minutes. Sections were blocked for 30 minutes in either 5% milk powder or 5% normal goat serum in Tris-buffered saline plus 0.2% Triton X-100 for 30 minutes followed by overnight incubation at 4°C in primary antibody (10 \(\mu\)g/ml). Secondary antibodies included goat anti-rabbit fluorescein (Cappel, 1:100), goat antimouse rhodamine (Accurate, 1:100), goat anti-rabbit biotin (Vectorstain, 1:200), strepavidin fluorescein (Amersham, 1:100). Sections were then mounted in gelvatol with 2% n-propyl gallate (Sigma) added as an antioxidant and observed on a Zeiss Axio photomicroscope. All photographs were taken with either hypersensitized Kodak Technical pan 2415 (Lumicon; Liverpool, CA) or Kodak TMAX 100. To verify the specificity of the antibody labeling, controls included sections incubated in secondary antibody alone and for the anti-\(\alpha_5\) cytoplasmic IgG, the antibody was incubated for 1 hour with 10 \(\mu\)g/ml of a 20mer peptide from the C terminus of the human \(\alpha_5\) sequence before application to sections. Additional experiments were performed to insure that there was no cross-reactivity to the cytoplasmic domains of other integrin \(\alpha\) subunits, focusing on three with highest sequence conservation in the cytoplasmic domain (\(\alpha_6\), \(\alpha_4\), \(\alpha_5\)). Preincubation with peptides from the C termini of both the chick \(\alpha_5\) integrin subunit (Bossy et al., 1991) and the human \(\alpha_5\) subunit, did not interfere with the \(\alpha_5\)-47 labeling of nerve. Polyclonal antibodies against the C termini of the \(\alpha_5\) and \(\alpha_6\) integrin subunits generated very different staining patterns from that of the anti-\(\alpha_5\) IgG in chick peripheral nerve (not shown).
of mature (4 week) peripheral nerve, both α5 (Fig. 3A) and FN (Fig. 3B) were prominently expressed within the perineurium and endoneurium. Overlapping expression of the two antigens was evident within both compartments. Examination with higher magnification showed strong expression of the α5 subunit on myelinated axons and myelinating Schwann cells (Fig. 3C). Non-myelinated axons and non-myelinating Schwann cells expressed lower levels of the α5 subunit. Non-neuronal cells, including capillary endothelial cells and fibroblasts also expressed the α5 subunit. Fibronectin was localized in rings surrounding the Schwann cell endoneurial tubes (Fig. 3B,D) corresponding to the site of basal lamina deposition (Peters et al., 1976). To confirm the localization of FN along Schwann cell endoneurial tubes, longitudinal sections of peripheral nerve were double labeled with anti-FN and a known Schwann cell marker, S-100 (Fig. 3E,F). The anti-S100 IgG labels Schwann cell cytoplasm (Fig. 3E). FN was distributed just outside and running along part of the length of the same endoneurial tube (Fig. 3F). The expression on axons of the integrin α5 subunit was also clearly demonstrated in longitudinal sections of peripheral nerve (Fig. 3G,H); being particularly evident under Nomarski optics (Fig. 3H) and revealing a punctate distribution at higher magnification with epi-fluorescence optics (Fig. 3G). In developing peripheral nerve, the α5 subunit (Fig. 3I) was prominently expressed on axons, identified with the neuronal-specific anti-neurofilament antibody (Fig. 3J), and non-neuronal cells.

Changes in expression of FN and the α5 subunit during peripheral nerve maturation

Several molecules that promote neurite outgrowth and their receptors have been shown to be down regulated as development proceeds (Martini and Schachner, 1988; Dodd et al., 1988). Reduced expression of these molecules often correlates with the arrival of axons at their targets (Cohen et al., 1986; 1989; Hall et al., 1987; de Curtis et al., 1991).

To determine whether expression of FN and the α5 subunit might also be developmentally regulated within the peripheral nerve, we compared their expression levels in hatchlings (day 1) to 4-week-old chicks by immunocytochemistry (Fig. 4). We found the distribution of FN to be more sparse and dispersed in the older nerve (Fig. 4D) compared to its more prominent expression in the younger nerve (Fig. 4C) where it was broadly expressed along axon bun-

![Image](image-url)
Expression of fibronectin in chick peripheral nerve
The pattern of the α5 subunit expression changed considerably when comparing longitudinal sections from the two ages (Fig. 4A,B). In the day 1 nerve (Fig. 4A), all axons were brightly labeled with the anti-α5 antibody while in the 4 week nerve (Fig. 4B) brightly labeled myelinated axons were separated by bundles of more faintly labeled non-myelinated axons. While at both ages, α5 was expressed on non-neuronal cells, the proportion of such cells expressing α5 appeared to be greater in the younger nerve (see Fig. 3I,J). Between these two timepoints, the nerve undergoes a period of extensive cellular remodelling and differentiation, including myelination (Saxod and Bouvet, 1982). The visible decrease in α5 subunit expression within the more mature nerve corresponds primarily to a decreased expression on non-myelinated axons and partially to a diminished expression or presence of non-neuronal cells.

Expression of FN and α5 strongly increases during nerve regeneration

Nerve injury triggers a state of active growth both in terms of neurite extension and Schwann cell differentiation (for review see Fawcett and Keynes, 1990). To test whether there were changes in expression of FN or α5β1 in response to such an injury, we transected the medial-ulnar nerve in 3 week chickens. To examine regulation of the integrin α5 subunit, the relative amounts of α5 expression in both control and experimental nerves were compared on immunoblots by densitometry. We measured a sixfold increase (Fig. 5) in the expression of the α5 subunit in regenerating nerve relative to contralateral control nerve, peaking 1 week following nerve transection. This increase occurred both in the segment immediately proximal and distal to the transection site.

To determine the cell types in which these changes occurred, longitudinal sections of regenerating and control nerve at 1 week following nerve transection were labeled with the anti-α5 and FN antibodies as well as neuronal and Schwann cell markers (Fig. 6). In agreement with our densitometry results, we found that nerve injury strongly induced expression of the integrin α5 subunit. Compared to the contralateral control nerve (Fig. 6A), the α5 subunit was more highly expressed in the proximal (P2 and P1) and distal (D2 and D1) regions of the regenerating nerve as well as in the site of injury (Fig. 6H). In this nerve, the distal front of the regenerating axons had not yet reached the D1 region (see Fig. 8). Thus the expression of the α5 subunit observed distal to the transection site (D1 and D2 regions) must reflect expression on non-neuronal cells. In the more

Fig. 4. Immunohistochemical comparison of expression levels of α5 and FN in nerves from a 1-day-old (A,C) versus 4-week-old (B,D) chick. (A,B) Anti-α5 labeling; (C,D) anti-FN labeling. All exposure times and development times (for the same given antigen) were of equal duration in order to compare fairly the levels of fluorescence intensity between the two ages. Note that for both antigens, their levels decrease with nerve maturation. Bar, 35 μm.
distal D2 region, the distribution and morphology of the α5 labeled cells closely resembled proliferating Schwann cells that align to form the Bands of Bungner during Wallerian degeneration (Allt, 1976; for review see Fawcett and Keynes, 1990). These cells could not be positively identified with anti-S100 antibodies since immature proliferating Schwann cells express little of this antigen (Neuberger and Cornbrooks, 1989; Jessen et al., 1989). Proliferating fibroblasts and endothelial cells may also contribute to the staining pattern. At the site of injury, the integrin α5 subunit colocalized with axons and sprouting growth cones as indicated by the overlap of neurofilament (Fig. 6l) and α5 expression (Fig. 6H). Since there were also cells in this region that expressed α5 but not the neuronal markers (neurofilaments or β-tubulin), the non-neuronal cells with which the axonal sprouts and growth cones were associated (most likely Schwann cells, see Hall, 1986; Fawcett and Keynes, 1990) must also express the α5 subunit. Thus, it appears that the number of cells expressing α5 increased in the regenerating nerve, both proximal and distal to the transection site.

The distribution of fibronectin was also examined in the regenerating nerve (Fig. 7). Compared to the contralateral control nerve (Fig. 7A), one week after nerve transection a dramatic increase in the level of fibronectin expression was observed in the regenerating nerve (Fig. 7B-F). Interestingly, while the P2 and D2 regions showed a slight increase compared to the control section, the area surrounding and including the transection site (P1, P1-D1, D1; Fig. 7C-D) showed a very strong increase compared to control nerve. In fact, the region with the highest expression was very discrete: it began in the area surrounding the nerve transection site in the vicinity of the distal front of the regenerating growth cones (distal edge of P1) and increased dramatically in the region connecting P1 to D1 (P-D), terminating in the proximal portion of the D1 region. This very circumscribed pattern of FN expression with respect to the disposition of the regenerating growth cones is more clearly illustrated in Fig. 8, where several mm of the regenerating nerve are reproduced. The region of highest FN intensity (Fig. 8B) coincided with the ‘bridge’ region connecting the proximal and distal stumps (Longo et al., 1984; Martini et al., 1990). The growth cones of the regenerating neurites (labelled in Fig. 8A with anti-β-tubulin antibody) had penetrated into the region of elevated FN expression, which extended distally in advance of the growth cones.

To determine whether there was a general increase in expression of ECM molecules in the bridge region, we examined the distribution of laminin, a representative constituent of basal lamina, in regenerating nerve (Fig. 9). Based on antibody perturbation studies, laminin isoforms have been implicated functionally in peripheral nerve regeneration (Sandrock and Mathews, 1987a,b). Interestingly, while laminin immunoreactivity was very prominently distributed proximally and distal to the cut site, mostly on the surface of Schwann cells (Fig. 9B,D) or in the endoneurial basal lamina (Fig. 9A; see also Sanes et al., 1990), there was little detectable laminin expression in the bridge region (Fig. 9C).

Two weeks following nerve transection, results illustrated in Figs 10 and 11 show that the expression of FN was still elevated in the regenerating nerve (compare Fig. 10A to panels B,C,E and G). The region of most intense expression still corresponded to the original transection site. Compared to one week after transection, the elevated FN expression extended further distally down the nerve into segment D2 (Fig. 10G). In addition, the regenerating axons had now traversed the bridge region and extended distally for several mm into the D2 region (Figs 10, 11). Both in the vicinity of the transection (Figs 10D,F, 11A,C,E) and further distally (D2 region, Figs 10H, 11B,D,F), α5 labeled regenerating axons were observed in regions with elevated FN expression. In the D2 segment, axons grew with straight trajectories, indicating that they were growing through endoneurial tubes (Ide et al., 1983; Longo et al., 1984; Fawcett and Keynes, 1990). However, in the bridge region, neurites were observed in several orientations (Figs 10F, 11C,E), an indication that they had not yet reached the parallel array of endoneurial tubes. Since regenerating neurites expressed α5 (Figs 6, 11), growth cones appear able to interact with FN in both the bridge and more distal regions.

**Discussion**

We report here on the spatiotemporal expression pattern of fibronectin and one of its receptors, the α5β1 integrin heterodimer, during development and regeneration of peripheral nerve. We and others have not found any instances...
Fig. 6. αS expression in regenerating nerve one week following nerve transaction. (A-C) Control nerve; (D-H) regenerating nerve labeled with anti-αS antibody. To compare levels of αS expression, all photographic exposures and development times were of equal length (see text). (A, B) Same section of contralateral control nerve double labeled with αS (A) and neurofilament antibodies (B). Note how all axons express αS (arrow). (C) Adjacent section of control nerve which was incubated with the C-terminal αS peptide and the αS ab. The staining is considerably reduced indicating that this peptide can specifically compete with the endogenous ligand for recognition by the αS polyclonal antibody. (D) P2 region of regenerating nerve, (E) P1 region, (F) D1 region, (G) D2 region, where Schwann cells are dividing and starting to align to form the Bands of Bungner. (H, I) Double label with anti-αS (I) and anti-neurofilament antibodies (J) at transaction site to indicate that axonal sprouts (as labeled by neurofilament abs) also express αS. Note how the antigens overlap. Bar; 25 μm.
Expression of fibronectin in chick peripheral nerve

where the \( \alpha_5 \) subunit is expressed in the absence of \( \beta_1 \); in immunoprecipitations \( \alpha_5 \) is invariably associated with \( \beta_1 \) and has not been detected in association with any other \( \beta \) subunit. Thus we believe in peripheral nerve the distribution of the \( \alpha_5 \) subunit reflects that of the \( \alpha_5\beta_1 \) receptor. We have found that (1) both FN and \( \alpha_5\beta_1 \) are prominently expressed in developing nerve with strong \( \alpha_5 \) subunit expression on neurons and non-neuronal cells, (2) the prevalence of both \( \alpha_5 \) and fibronectin decreases with maturation of the peripheral nerve, (3) in response to injury to the mature nerve, both FN and \( \alpha_5 \) are strongly induced, (4) the elevated FN expression at the site of injury suggests that it is functionally important for reestablishing connections between the severed nerve segments and (5) the distribution of the fibronectin receptor, \( \alpha_5\beta_1 \), suggests that it is utilized by both axons and Schwann cells during nerve regeneration.

As others have noted (Palm and Furcht, 1983; Longo et al., 1984; Sanes, 1989), in mature nerve, we observed fibronectin distributed around the endoneurial tubes, the region known to correspond to the endoneurial basal lamina (Peters et al., 1976; Bunge et al., 1989a) and in the perineurium. Thus \( \alpha_5\beta_1 \) receptors on the outer Schwann cell membrane seem likely to interact with fibronectin in the basal lamina. Since the \( \alpha_5\beta_1 \) receptor has been demonstrated to be required for optimal FN matrix assembly (McDonald et al., 1987; Roman et al., 1989), its expression on Schwann cells is consistent with such a role in the nerve.

Fig. 7. Fibronectin expression in regenerating nerve one week post-transection. All photographic exposure times were equivalent and all negatives were processed equally. (A) Contralateral control nerve stained with anti-fibronectin antibody. (B-F) Regenerating nerve stained with anti-fibronectin antibody; (B) P2 region; (C) P1 region; (D) the ‘bridge’ region connecting P1 and D1 regions of nerve; (E) D1 region; (F) D2 region. Bar, 45 \( \mu \)m.
Fig. 8. Adjacent sections of regenerating nerve one week following nerve transection, labelled with (A) anti-β-tubulin, a neuronal marker, to illustrate position of regenerating growth cones and (B) anti-FN to demonstrate discrete pattern of FN expression in the regenerating nerve. Note how FN expression is highest in the 'bridge' region connecting the proximal (P1) and distal (D1) stumps. Arrow marks the site of transection. Note how the most distally extended growth cones (star) have penetrated into the region of intense FN expression. Bar (A) 300 μm; (B) 450 μm.
Expression of fibronectin in chick peripheral nerve

Non-myelinated axons and non-myelinating Schwann cells, which also expressed the \( \alpha_5 \) subunit but at lower levels, have been observed in direct contact with the endoneurial basal lamina (Kuecherer-Ehret et al., 1990) and thus are also likely to interact with FN. In the mature nerve, FN would not be accessible to the \( \alpha_5 \beta_1 \) receptors on myelinated axons. It is thus possible that, in addition to FN, \( \alpha_5 \beta_1 \) may also interact with an unidentified ligand on the interior surface of myelinating Schwann cells. Many other integrins have been shown to have more than one ligand and these include integral membrane proteins (for example, see Elices et al., 1990).

In contrast to their more restricted expression in the older nerve, fibronectin and \( \alpha_5 \beta_1 \) were much more prominently expressed in a relatively immature nerve. In the hatchling nerve, FN was rather continuously expressed along axon bundles whereas in the older nerve it was sparse and more dispersed within the endoneurium. In the younger nerve, the \( \alpha_5 \) subunit seemed ubiquitously expressed throughout the nerve with strong expression on all axons. In the older nerve, strong axonal \( \alpha_5 \) expression was restricted to myelinated axons in contrast to the considerably weaker expression on non-myelinated axons. Between these two time points (day 1 versus 4 weeks), the nerve undergoes a period of extensive myelination; at hatching, fewer than 4% of axons are myelinated, while by 6 weeks, 40% of the axons are myelinated. (In the adult, the maximum number of myelinated fibers reaches 60%; Saxod and Verna, 1979.)

In the nerves of hatchlings (day 1), none of the broader (myelinated) axons observed in the older nerves were evident; instead most of the axons were thin and appeared in broad bundles, characteristic of an immature nerve where several (non-myelinated) axons are often associated with an individual Schwann cell (cf. Webster and Favilla, 1984). Thus our data suggest that the diminished expression of \( \alpha_5 \) observed on the neuronal cells with nerve maturation is primarily due to a decreased expression on axons that remain non-myelinated. The prominent expression of \( \alpha_5 \beta_1 \) at this early time point also coincides with a period of pronounced Schwann cell proliferation which later ceases (Webster and Favilla, 1984; Asbury, 1967). As Schwann cells mature they alter their expression of several proteins including cell surface adhesion molecules (Daston and Ratner, 1991; Neuberger and Cornbrooks, 1989; Jessen et al., 1989).

**Fibronectin and the fibronectin receptor \( \alpha_5 \beta_1 \) are induced in response to nerve injury**

Our results show that FN is strongly induced in peripheral nerve in response to injury. Both one and two weeks following transection, fibronectin expression was most strongly increased in the vicinity of the site of injury. Laminin, another representative ECM constituent, is virtually absent from this region, although the expression of tenascin increases distally following nerve injury (Martini...
non-neuronal cells and incorporated by Schwann cells into endothelial cells in the bridge region (Longo et al., 1984; Woolley et al., 1990; Cornbrooks et al., 1983). This bridge region is not simply an acellular matrix characteristic of a fibrin clot; labelling with nuclear markers (e.g., DAPI, data not shown) demonstrates a rather uniform cellular array throughout this region connecting the two severed stumps and most likely corresponds to the fibroblasts reforming the outer nerve sheath (peri or epineurium; Bunge et al., 1989b). Since, in adult nerve, FN is localized along the external surface of Schwann cell endoneurial tubes, it is conceivable that FN might be synthesized by Schwann cells in vivo or alternatively synthesized by other neighboring non-neuronal cells and incorporated by Schwann cells into their overlying matrix (McDonald et al., 1987; Roman et al., 1989). In vitro, Schwann cell synthesis of FN has been observed in the presence of cAMP analogs and ascorbate (Baron Van Evercooren et al., 1986).

The fibronectin receptor \(\alpha_5\beta_1\) was also strongly induced in regenerating peripheral nerve on both axons and Schwann cells. Notable increases in \(\alpha_5\beta_1\) expression were observed both proximal and distal to the site of injury. Proximal to the transection, a major proportion of the increase in \(\alpha_5\beta_1\) appeared to be neuronal. This reflected both elevated \(\alpha_5\beta_1\) expression on individual axons (compare the intensity of Fig. 6H to 6A), and an increased density of neurites due to axonal sprouting, which is triggered by nerve injury (Cajal, 1928; Diamond et al., 1987). The growing tips of regenerating axons strongly expressed \(\alpha_5\beta_1\) (Fig. 6H,I). Further, \(\alpha_5\) subunit was also induced in non-neuronal cells both proximal and distal to the transection site (Fig. 6F,G). This was particularly striking in the D2 region (about 3-6 mm distal to the transection site) where at one week following transection, Schwann cells were proliferating and aligning to form the bands of Bungner (Fawcett and Keynes, 1990; Allt, 1976). Thus the induction of \(\alpha_5\) subunit in this segment must be on Schwann cells since they constitute the vast majority of cells at this time. \(\alpha_5\beta_1\), then, appears to be reexpressed on dedifferentiating Schwann cells as well as on regenerating axons. Overall, the expression patterns of the \(\alpha_5\) subunit in the developing and regenerating nerve were similar; in both, expression was strong on several cell types.

Previous studies have demonstrated that fibronectin levels are greatly elevated during wound healing in adult rat skin (ffrench-Constant et al., 1989; Grinnell et al., 1981), resembling its embryonic expression pattern. The authors suggest that the reexpression of FN, whose localization during development coincides with a period of active cell migration, would facilitate the enhanced migration that occurs as part of the wound response. For successful nerve regeneration, migration of Schwann cells into the wound region is critical; in their absence axons fail to regenerate (Hall, 1986; Fawcett and Keynes, 1990). Since FN has been shown to act not only as a substratum for Schwann cell migration, but also as a chemoattractant and mitogen for Schwann cells (Baron Van Evercooren et al., 1982), it seems possible that the elevated FN expression in the transection region may be an integral component of successful nerve regeneration by inducing the proliferation and migration of Schwann cells to the site of injury.

Thus fibronectin might facilitate nerve regeneration via two mechanisms. (1) One mechanism could be indirect, by inducing an influx of Schwann cells into the wound region. Since Schwann cell surfaces contain several adhesion molecules that potently promote neurite outgrowth (Bixby et al., 1988; Tomaselli et al., 1986), these cells would provide an attractive substratum for axonal outgrowth. (2) A second mechanism could be direct by virtue of itself being a
Expression of fibronectin in chick peripheral nerve demonstrated an attractive glycoprotein for neuronal adhesion and neurite extension (for review, see Reichardt and Tomaselli, 1991). Its elevated expression in the vicinity of the regenerating growth cones and axons strongly implicates FN as a substratum for regenerating axons. At one week following transection, FN levels were highest in the vicinity of the regenerating growth cones and axons. Fig. 11. 2 weeks post-transection, regenerating axons express α5 and grow in regions of elevated FN expression. Double-labeled sections of regenerating nerve 2 weeks post-transection with anti-α5 (A,B) and anti-neurofilament abs (C,D). In a proximal portion of the D1 region (A,C) just distal to the site of transection, regenerating neurites labeled with neurofilament abs (C) all express the α5 subunit (A). The curvilinear growth is typical of the morphology of regenerating neurites in this region near the site of transection. Further distally (B,D) in the D2 region, axons have aligned themselves along the endoneurial tubes and hence grow relatively straight. Note how here too, all axons (D) express α5 (B). (E,F) Double labeling of an adjacent section with anti-neurofilament and anti-FN antibodies reacted with the same secondary antibody so that both antigens could be photographed on the same negative and yet distinguished by the relatively cylindrical neurofilament staining versus the punctate FN distribution. These photographs illustrate that FN is expressed in the same area as regenerating neurites both in the D1 region (E) and further distally in the D2 region (F), although FN expression is higher in the D1 region than in the D2 region. Bar, 20 µm.
ity of the regenerating growth cones and decreased further distally where the axons had not yet extended. By two weeks following nerve transection, the regenerating axons had grown into the distal stump and were now regrowing through the Schwann cell endoneurial tubes. In this distal region, axons are known to grow between the Schwann cell surface and its basal lamina in contact with both (Ide et al., 1983; Fawcett and Keynes, 1990). By this time, FN expression was elevated surrounding the reformed endoneurial tubes perhaps in response to axonal contact (Bunge et al., 1989a). Significantly, at both time points, we always observed prominent expression of α5β1 on regenerating neurites. The elevated expression of α5β1 on both Schwann cells and regenerating growth cones could facilitate their motility through a FN-rich region since previous work has positively correlated enhanced motility on FN with elevated expression of the α5β1 receptor (Schreiner et al., 1989).

Peripheral nerve transaction induces an inflammatory response (Brown et al., 1991). For sensory axons, this response is thought to be an essential element for their successful regeneration. Macrophages recruited into the wound area secrete interleukin 1 (II-1) which causes an elevation in NGF secretion by the non-neuronal cells in the nerve thereby trophically supporting the sensory neurons (Heumann et al., 1987; Lindholm et al., 1987; Brown et al., 1991). The increase in α5β1 in the nerve may similarly be regulated by cytokines such as II-1. Another cytokine centrally involved in wound healing, TGF-β1 (for review see Clark, 1990), has been found to induce Schwann cell proliferation (Ridley et al., 1989). Further, levels of TGFβ1 mRNA significantly increase during nerve regeneration (Scherer and Jakolew, 1991). Interestingly, TGFβ1 has been found to upregulate expression of both FN and α5β1 on fibroblasts in vitro (Ignotz and Massague, 1987). Thus, there are several growth and differentiation factors that may directly upregulate the α5β1 and/or FN expression in regenerating peripheral nerve. In addition to regulating integrin expression levels, cytokines may also directly activate the α5β1 receptor (for review, see Hynes, 1992).

Our results suggest that one feature of the wound response induced by nerve transection, elevated FN expression, may be an integral component in the promotion of Schwann cell migration and axonal extension through a complicated terrain undergoing extensive cellular remodeling. The prominent expression of its receptor, α5β1, on Schwann cells and on regenerating and developing neurites suggests that this receptor-ligand interaction is important for successful peripheral nerve development and regeneration.

We thank T. Broekelmann for assistance in production of the α5-47 antibody, J. Muschler and A. F. Horwitz for generously providing the A2F7 monoclonal antibody, A. Frankfurter for the gift of the TuJ1 monoclonal antibody, M. Kirschnner and T. Mitchison for the use of their microscopes, and M. Meyerson for expert typing assistance. We gratefully acknowledge D. Clary, J. D. Curtis and K. Jones for critically reading the manuscript and informative discussions. Grant support included NIH#1F32NS08451-01A1 to F. L., NS19090 to L. F. R., 2PO1HL29594 and 1RO1HL43418 to J. A. M.; L. F. R. is an investigator of the Howard Hughes Medical Institute.

References


Roman, J. and McDonald, J. A. (1992). Expression of fibronectin, the
integrin α5 and α-smooth muscle actin in heart and lung development. 


(Accepted 20 August 1992)