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Fibrin is a regulator of Schwann cell migration after sciatic nerve injury in mice

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Abstract

Fibrin, derived from the blood protein fibrinogen, is deposited in the sciatic nerve after injury and retards functional regeneration. Since Schwann cell migration is critical for remyelination of injured nerves, we investigated the effects of fibrin in this process. In vivo experiments showed that fibrin co-localizes with fibronectin deposition in the injured sciatic nerve. In vitro migration assays demonstrated that fibrin alone is not a permissive substrate for Schwann cell migration. Furthermore, migration assays of Schwann cells on mixed fibrin/fibronectin substrates showed that fibrin has a dose dependent inhibitory effect on Schwann cell migration on fibronectin. Our results show that fibrin, deposited in the sciatic nerve after injury, changes the composition of the extracellular matrix and inhibits Schwann cell migration. This negative effect of fibrin should be considered in the therapeutic application of biomaterials based on fibrin matrices.
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Nerve repair is a combined process of axonal elongation and remyelination by Schwann cells. After sciatic nerve injury, fibrin is deposited at the nerve and its deposition exacerbates nerve damage [2]. Fibrin clearance correlates with regeneration, while fibrin deposition delays nerve regeneration by arresting Schwann cells at a proliferating, non-myelinating state [3]. After nerve injury Schwann cells proliferate and migrate towards the newly formed axons. Schwann cell migration depends upon the extracellular matrix (ECM) and has been extensively studied in a variety of ECM substrates, such as laminin and fibronectin [6]. However, the effects of fibrin, as a component of the ECM after injury, on Schwann cell migration has not been investigated.

We first examined the contribution of fibrin and fibronectin to the composition of the ECM of the sciatic nerve after injury. Previous studies demonstrated that fibrin is deposited in the sciatic nerve after injury [2] and regulates fibronectin production [3]. While in the later study we observed that fibronectin and fibrin were both deposited in the nerve, their localization was unknown. To determine the localization of fibrin and fibronectin, we crushed the sciatic nerve of wild-type mice [2], isolated the sciatic nerves, prepared methanol-fixed 10 μm cross cryostat sections and incubated with a goat anti-mouse fibrin(ogen) antibody (Chemicon, 1/500), visualized by anti-goat Texas red antibody (Vector, 1/300). For the double staining with fibronectin, after visualization of fibrin, the sections were incubated with rabbit anti-mouse fibronectin (Dako, 1/500) and then with anti-rabbit fluorescein isothiocyanate (Vector, 1/300). We observed that uninjured wild type mice did not show any staining for fibrin (Fig. 1A), while they showed staining for fibronectin (Fig. 1B, green). Four days after crush injury, fibrin (Fig. 1D, red) and fibronectin (Fig. 1E, green) are both detected at the nerve distal to the crush site. Overlay of the fibrin and the fibronectin immunostainings demonstrate that fibrin and fironectin co-localize periaxonally after injury (Fig. 1F, yellow). To further characterize the localization of fibrin deposition we examined longitudinal sections of crushed sciatic nerve stained with anti-

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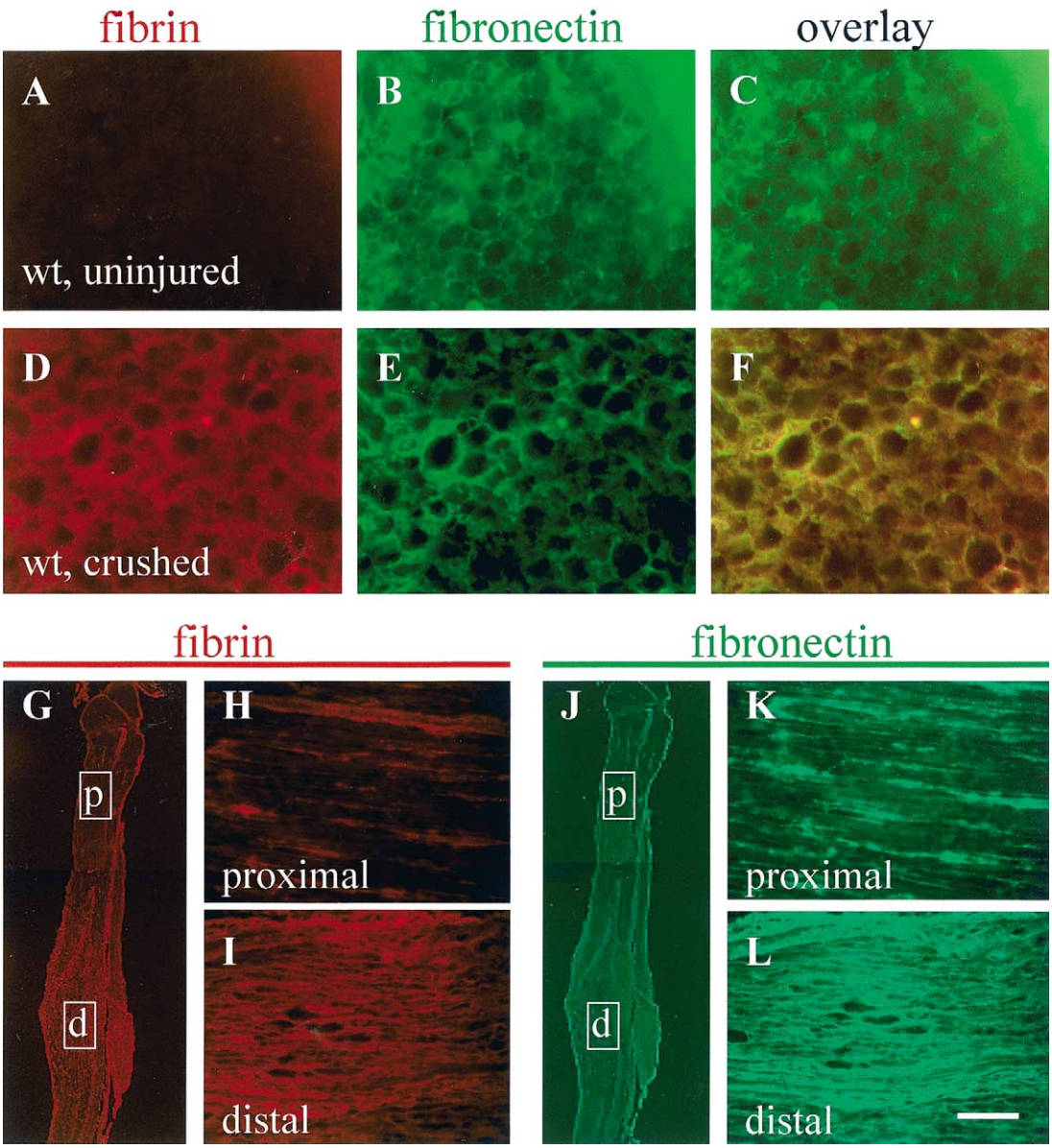

Fig. 1. Cross-sections of uninjured sciatic nerve show no staining for fibrin (A), while there is staining for fibronectin (B, green). Overlay of (A) and (B) shows periaxonal fibronectin immunoreactivity (C, green). Four days after injury fibrin (D, red) and fibronectin (E, green) are both detected at cross-sections of sciatic nerve distal to the crush site. Overlay of (D) and (E), shows a colocalization of fibrin and fibronectin periaxonally after injury (F, yellow). Immunostaining on longitudinal sections showed that fibrin (G) and fibronectin (J) were mainly present distal to the crush site (d), while minimal staining was observed proximally (p). High power of the distal and proximal sites of sciatic nerves stained for fibrin (H,I) and fibronectin (K,L). Bar: (A–F): 30 μm; (G,J): 825 μm; (H,I,K,L): 75 μm.

fibrinogen (Fig. 1G) and anti-fibronectin (Fig. 1J). Fibrin localized at the distal site (Fig. 1I) and minimal staining was observed proximally (Fig. 1H). Fibronectin was also upregulated distally (Fig. 1L) and was also present at the nerve proximal to the crush site (Fig. 1K).

Since fibrin and fibronectin co-localize in vivo, we examined whether Schwann cells migrated on mixed fibronectin/fibrin matrices. To examine the effect of fibrin on Schwann cell migration, Schwann cell primary cultures were established [9] and their migration capacity on different ECM substrates was determined [12]. Briefly, Schwann cells were isolated from neonatal P0–P2 mice and cultured in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium [9]. Cell migration was assessed by examination of migration from agarose drops [12]. Schwann cells were resuspended at 3×10^6 cells/ml in Sato medium [9] containing 10% FBS and 0.3% low melting point agarose (Sigma). Drops (1.5 μl) of the cell suspension were applied to the center of wells within a 24-well tissue culture dish (Costar), which was then placed at 4 °C for 15 min to allow the agarose to solidify. Following this the cooled drop was covered with 50 μl of Sato medium containing 10 μg/ml of the chosen ECM molecule and incubated for 2 h at 37 °C. Since in this assay Schwann cells

do not migrate in the absence of ECM molecules [8], we used SATO medium without adding ECM proteins as a negative control. Murine fibronectin (Life Technologies) was used at 10 μg/ml. For the preparation of the clotted murine fibrinogen (Sigma), we added 50 μl of Sato medium containing 10 μg/ml fibrinogen (Sigma), 0.01 U/ml thrombin (Sigma) and 0.01 U/ml coagulation factor XIII from human plasma (Calbiochem, San Diego, CA) and overlaid the cell agarose drop. Serum-free Sato medium was added to each well. Cell migration was monitored daily using a Nikon phase contrast tissue culture microscope. Within single experiments each condition was tested in duplicate.

In the presence of fibronectin alone, Schwann cells migrated outside of the agarose drop (Fig. 2D). By contrast, Schwann cells showed no migration on fibrin (Fig. 2A). To obtain higher numbers of cells for quantifying the migration assay, Schwann cells were isolated from rat sciatic nerve [4]. Compared to extensive migration on fibronectin alone (Fig. 2D) quantified as 100% (Fig. 2I), migration was decreased to 73.68 ± 25.1% ($n = 3$) on 10:1 fibronectin:fibrin matrices (Fig. 2C,I). Alteration of the fibronectin:fibrin ratio to 1:10 further decreased migration to 35.52 ± 3.95% ($n = 2$, $P < 0.001$) (Fig. 2B,I). Observation of cells growing on fibrin (Fig. 2E), mixed fibrin:fibronectin matrices (Fig. 2F,G) and fibronectin (Fig. 2H) show the characteristic bipolar spindle morphology of Schwann cells. These results are in accordance with recent data on fibrin/fibronectin biomaterials that show that fibrinogen inhibits Schwann cell migration on fibronectin [1].

Overall our in vitro data (Fig. 2), combined with our in vivo observations (Fig. 1), suggest that fibrin is not a permissive substrate for Schwann cell migration, and that its presence delays migration on permissive substrates such as fibronectin. A mechanism to explain the inhibitory effect of fibrin on Schwann cell migration on fibronectin could be the

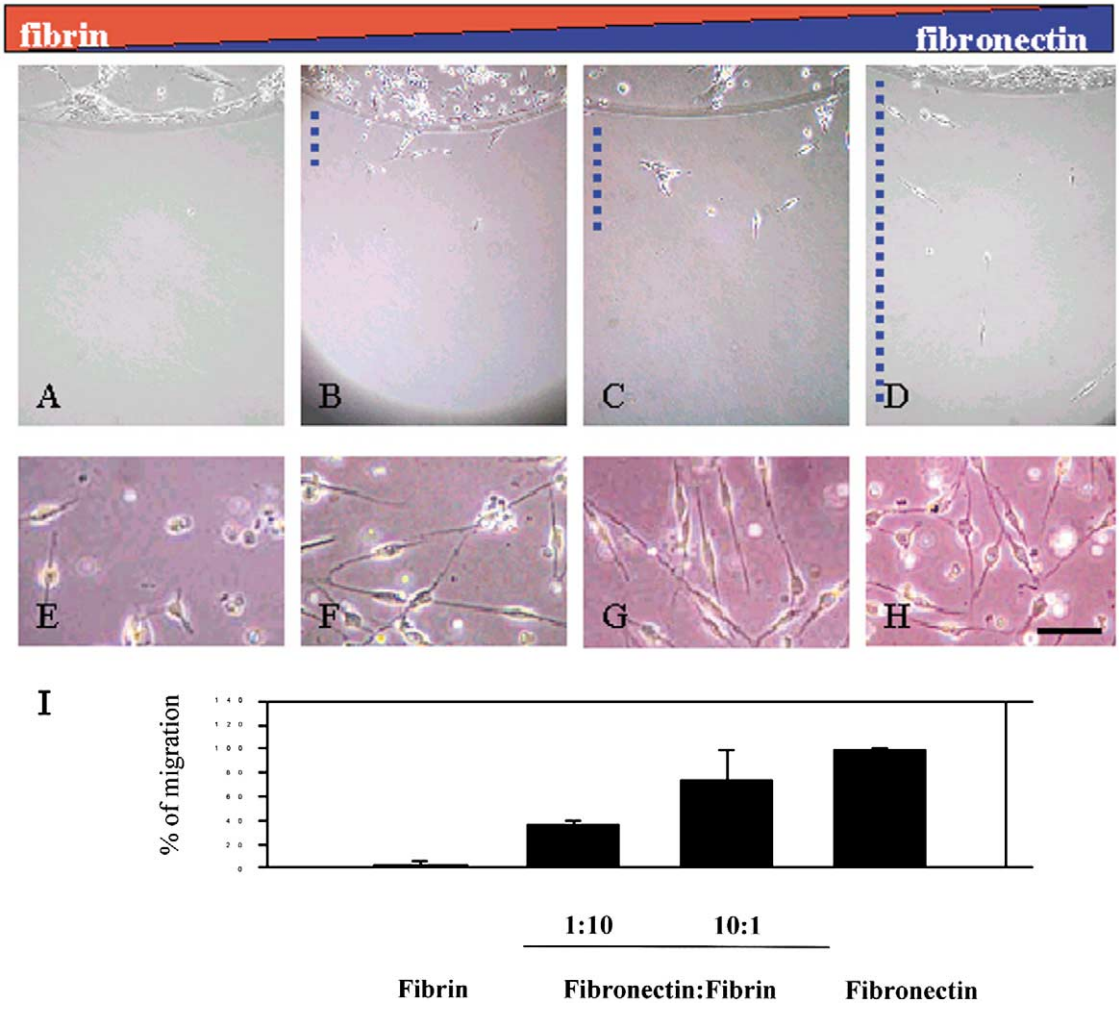

Fig. 2. Schwann cells do not migrate on fibrin (A), however addition of fibronectin in a fibrin:fibronectin ratio of 10:1 induces migration (B). The migration front (blue dotted line) increases with the addition of fibronectin in a fibrin:fibronectin 1:10 ratio (C), but does not reach the extent of migration observed only in the presence of fibronectin (D). Schwann cell morphology looks similar when cells grow on fibrin (E), fibrin:fibronectin 10:1 (F), fibrin:fibronectin 1:10 (G), or fibronectin (H). (I) Quantification of rat Schwann cell migration. Results are the mean value of separate experiments. Each experiment was performed in triplicate. Bar: 75 μm (A–D); 30 μm (E–H).

blockade of the fibronectin mediated integrin signaling. Schwann cell migration on fibronectin is mediated by an arg-gly-asp (RGD)-dependent integrin [9]. Fibronectin signals primarily through integrin receptors binding to RGD and adjacent sequences in the central binding domain [10]. During sciatic nerve regeneration, the levels of fibronectin and its integrin receptors are increased at similar levels observed during sciatic nerve development [8]. Fibrin, which is not present during development, but gains access in the nervous system after injury [2], contains two RGD peptides [11]. Therefore, fibrin might act as an in vivo antagonist of fibronectin and interfere with integrin signaling on Schwann cells. Fibrin deposition after crush injury might contribute to destabilizing the ligand-receptor interactions of the extracellular matrix with the Schwann cell integrins and through this mechanism interfere with the regeneration process.

This study demonstrates that fibronectin and fibrin co-localize in the sciatic nerve after injury. Since fibrin modulates Schwann cell migration on a fibronectin matrix, fibrin deposition and degradation could be a major regulatory mechanism for Schwann cell migration in vivo. Schwann cells respond with active migration after nerve injury [5]. Since migration is a necessary process for the differentiation of Schwann cells to myelin-producing cells, fibrin could affect Schwann cell differentiation by affecting its migration capacity. In addition, the success of therapeutic strategies for human demyelinating diseases based on Schwann cell transplantation relies upon the capacity of the Schwann cells to migrate in the injured nervous system [7]. Our results suggest that the presence of fibrin in a demyelinated lesion [13] might be inhibitory for therapeutic approaches based on glial cell migration. In addition, the design of biomaterials for nerve injury needs to take into account the active role of fibrinogen in glial cell biology and supplement these materials with fibronectin.

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