Fibrinogen inhibits neurite outgrowth via β3 integrin-mediated phosphorylation of the EGF receptor

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Changes in the molecular and cellular composition of the CNS after injury or disease result in the formation of an inhibitory environment that inhibits the regeneration of adult mammalian CNS neurons. Although a dramatic change in the CNS environment after traumatic injury or disease is hemorrhage because of vascular rupture or leakage of the blood–brain barrier, the potential role for blood proteins in repair processes remains unknown. Here we show that the blood protein fibrinogen is an inhibitor of neurite outgrowth that is massively deposited in the spinal cord after injury. We show that fibrinogen acts as a ligand for β3 integrin and induces the transactivation of EGF receptor (EGFR) in neurons. Fibrinogen-mediated inhibition of neurite outgrowth is reversed by blocking either β3 integrin or phoshorylation of EGFR. Inhibition of Src family kinases that mediate the cross-talk between integrin and growth factor receptors rescue the fibrinogen-induced phosphorylation of EGFR. These results identify fibrinogen as the first blood-derived inhibitor of neurite outgrowth and suggest fibrinogen-induced EGFR transactivation on neuronal cells as a molecular link between vascular and neuronal damage in the CNS after injury.

blood–brain barrier | regeneration | spinal cord injury | transactivation | scar

The identification of common molecular mechanisms that regulate vascular and neural development has expanded the role of the CNS vasculature from nutrition to regulation of axonal guidance, synaptic activity, metabolic trafficking, and adult neurogenesis (1). Although there is a causal interaction between the nervous system and the vasculature, a physical and metabolic barrier between the brain and the systemic circulation, namely the blood–brain barrier (BBB), prohibits the entry of blood proteins from the vasculature into the nervous tissue (2). Leakage of blood components in the CNS parenchyma is a common denominator of several CNS diseases characterized by edema formation and neuronal damage, such as stroke, HIV encephalitis, Alzheimer's disease (AD), multiple sclerosis (MS), glioblastoma, and bacterial meningitis (2). After traumatic injury, such as spinal cord injury (SCI), the primary mechanical injury results in pronounced hemorrhage into the spinal cord and disruption of the blood vessel walls (3). Evidence in AD that correlates microhemorrhages with amyloid plaque formation (4) and in MS that identifies leakage of blood components in the brain as one of the earliest histopathologic abnormalities (5) has postulated a role for blood components in the onset and progression of neurodegeneration. However, the cellular and molecular mechanisms of action of blood proteins within the CNS microenvironment and their contribution to disease pathogenesis remain poorly characterized. Given that inhibition of regeneration in the CNS results in part from the presence of inhibitory factors in the neuronal environment (6), investigating the role of blood proteins as modulators of neuronal functions could be crucial for the identification of inhibitors of axonal regeneration.

Fibrinogen is a 340-kDa protein secreted by hepatocytes in the liver and present in the blood at 3 mg/ml (7). Fibrinogen is cleaved by thrombin and, on conversion to fibrin, plays a major role in blood clotting and circulation via interaction with platelets. However, the biological functions of fibrinogen extend beyond blood coagulation. Fibrinogen is a classic acute-phase reactant that extravasates into tissues, including the brain via ruptured vasculature (for reviews, see refs. 7 and 8). Studies in animal models have identified fibrinogen as a major player in infection (9, 10), inflammation (11–13), and inhibition of tissue repair processes in muscle regeneration (14) and wound healing (15). The ability of fibrinogen to mediate a wide range of biological effects is because of its unique structure that contains multiple nonoverlapping binding motifs for different receptors, such as integrins, intracellular adhesion molecule-1, and vascular endothelial cadherin (7, 16). Depending on the cellular distribution of its receptors, fibrinogen acts as a ligand to induce diverse signal transduction pathways, such as activation of Rho GTPases and NF-κB and mediate cellular functions ranging from cytokine gene expression to cell adhesion, migration, and survival (17). Our studies in the nervous system identified a central role for fibrinogen as a regulator of peripheral nerve remyelination (18). We have demonstrated that fibrinogen exacerbates sciatic nerve degeneration (19) and, by activating ERK1/2 phosphorylation, arrests Schwann cell differentiation to a nonmyelinating state (18). Moreover, we showed that fibrinogen via the CD11b/CD18 integrin receptor activates microglia (20) and mediates inflammatory demyelination in animal models of MS (20, 21). Given the potential of fibrinogen for signal transduction via a wide range of cellular receptors and its presence in the CNS microenvironment only after injury or disease, we hypothesized that fibrinogen could be a component in the blood that regulates functions of neurons during degenerative and repair processes in the CNS.

In this study, we identify fibrinogen as a blood component that is deposited in the spinal cord after injury and inhibits neurite outgrowth by triggering an inhibitory signal transduction pathway in neurons. We show that fibrinogen inhibits neurite out-

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Abbreviations: AD, Alzheimer's disease; BBB, blood–brain barrier; CGN, cerebellar granule neuron; DH, dorsal hemisection; ECM, extracellular matrix; EGFR, EGF receptor; GF, growth factor; MS, multiple sclerosis; SCI, spinal cord injury; SCG, superior cervical ganglia neuron; SFK, Src family kinases.

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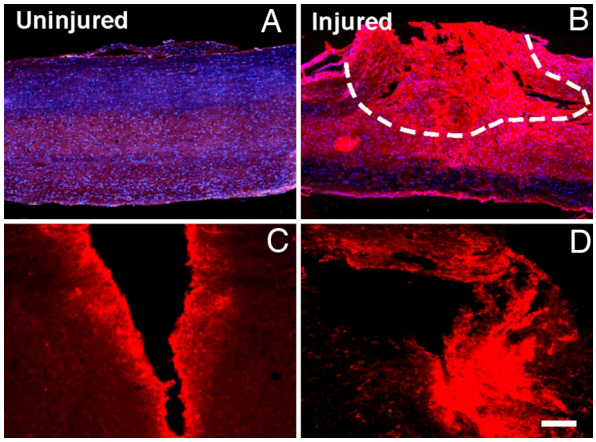

Fig. 1. Fibrinogen is deposited in the spinal cord after traumatic injury. (*A*) Immunolabeling longitudinal sections of uninjured control mouse spinal cord for fibrinogen (red) revealed that fibrinogen is undetectable in the intact spinal cord. (*B*) Fibrinogen deposition (red) at the lesion site of mouse spinal cord longitudinal sections 2 days after DH. (*C* and *D*) The boundary of the lesion site is indicated by dotted lines. Sagittal sections of rat dorsal column lesion (*C*) and contusion model (*D*) show deposition of fibrinogen (red). Nuclei are stained with DAPI (blue). Representative images of three independent experiments of three animals per condition are shown. (Scale bar: 105 μm.)

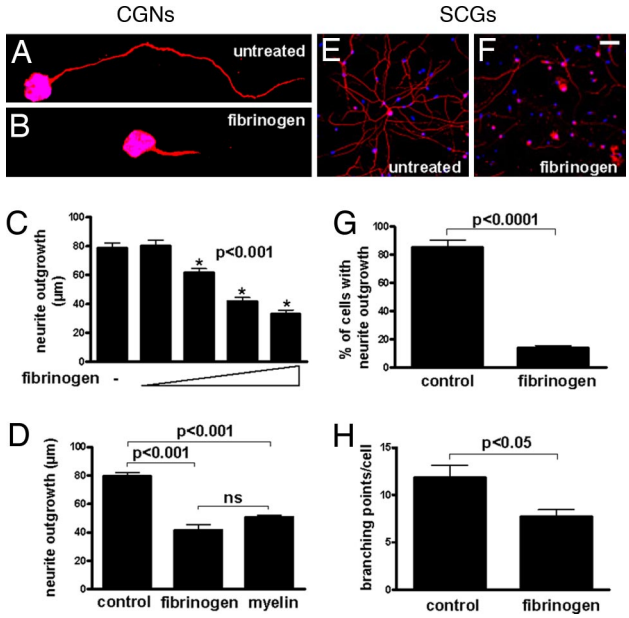

Fig. 2. Fibrinogen inhibits neurite outgrowth. Representative micrographs of mouse control CGNs (*A*) and SCGs (*E*) cultured for 24 h on poly-D-lysine, or CGNs (*B*) and SCGs (*F*) cultured in the presence of 1.5 mg of fibrinogen. (*C*) Quantification of neurite length of mouse control CGNs untreated or treated with different concentrations of fibrinogen (0.1, 0.3, 0.9, 1.5 mg/ml) indicated a concentration-dependent inhibition of neurite outgrowth. (*D*) Inhibition of neurite length of mouse CGNs either treated with 1.5 mg/ml fibrinogen or plated on the neurite outgrowth inhibitor myelin (1 μg of total protein per well). (*G*) Reduced number of neurite-bearing mouse SCGs on treatment with 1.5 mg/ml fibrinogen. (*H*) Quantification of branching points per cell in the neurite-bearing SCGs. CGNs and SCGs were stained with a β-tubulin antibody (red) and DAPI for nuclei (blue). Results are expressed as means $\pm$ SEM of a minimum of five different experiments. A minimum of 400 to 500 neurons per condition were analyzed. Statistical comparisons between means were made with one-way ANOVA (*, $P < 0.001$) (*C* and *D*) and Student's *t* test (*G* and *H*). (Scale bar: 75 μm.)

growth in two different neuronal cell types, cerebellar granule neurons (CGNs) and superior cervical ganglia neurons (SCGs). Fibrinogen is equally potent to known inhibitors, such as myelin (22), to inhibit neurite outgrowth. Fibrinogen binding to its neuronal receptor $\alpha v\beta 3$ integrin induces transactivation of EGF receptor (EGFR), resulting in inhibition of neurite outgrowth. Moreover, fibrinogen is deposited in the CNS in three different models of SCI, and its deposition spatially correlates with axonal damage and phosphorylated EGFR. Identification of fibrinogen as a blood-derived inducer of EGFR transactivation reveals an inhibitory mechanism of neurite outgrowth and provides a molecular link between vascular and neuronal damage in the CNS after injury.

Results

Fibrinogen Is Deposited in the Spinal Cord After Injury and Correlates with Axonal Damage. Several studies have shown the presence of fibrinogen in CNS neurodegenerative diseases, such as MS (23), AD (24), and cerebral ischemia (25). The inability of CNS axons to regenerate is exemplified in SCI (26). However, whether fibrinogen is present in the CNS after traumatic injury is unknown. Therefore, we examined the spatial and temporal regulation of fibrinogen in the CNS in three different models of SCI. In the presence of an intact BBB in the uninjured mouse spinal cord, there was no fibrinogen deposition (Fig. 1*A*). Strikingly, 2 days after dorsal hemisection (DH) in the mouse, there was a massive deposition of fibrinogen (Fig. 1*B*). Composite images of the entire spinal cords are shown in supporting information (SI) Fig. 6. In addition, fibrinogen deposition occurred in the rat after either dorsal column lesion (Fig. 1*C*) or spinal cord contusion (Fig. 1*D*). Fibrinogen deposition occurred as early as 1 day after injury, peaked at 7 days, and decreased in the following weeks (SI Fig. 7). We further showed that fibrinogen deposition correlated with axonal damage in both the murine DH model (SI Fig. 8) and the rat dorsal column lesion (SI Fig. 9). Overall, these results suggest that fibrinogen deposition is a general feature after SCI that occurs in all three animal models examined, demonstrating that fibrinogen is present in the spinal cord after injury.

Fibrinogen Inhibits Neurite Outgrowth. We further sought to determine whether fibrinogen affects neuronal functions after injury as they relate to axonal regeneration. In an established assay using postnatal CGNs previously used to identify inhibitors of axonal regeneration (27), fibrinogen induced a dramatic decrease of neurite outgrowth (Fig. 2*B*), when compared with untreated CGNs (Fig. 2*A*). Fibrinogen-mediated neurite outgrowth inhibition was concentration-dependent, as shown on treatment with increasing concentrations of fibrinogen ranging from 0.1 to 1.5 mg/ml (Fig. 2*C*). Interestingly, concentrations as low as 0.3 mg/ml, which is 10-fold below the physiological 3 mg/ml concentration of fibrinogen, inhibited neurite outgrowth (Fig. 2*C*; $P < 0.001$). Fibrinogen showed similar inhibition of neurite outgrowth when compared with myelin (Fig. 2*D*). Fibrinogen did not induce apoptosis in CGNs (SI Fig. 10), suggesting that the effects of fibrinogen were specific on inhibition of neurite outgrowth. Similar to CGNs, SCGs showed a dramatic reduction in neurite outgrowth in the presence of fibrinogen (Fig. 2*F*), compared with untreated SCGs (Fig. 2*E*). Quantification showed a 6-fold decrease of neurite-bearing SCGs on fibrinogen treatment (Fig. 2*G*; 85.5 $\pm$ 5.0% in control vs. 14.0 $\pm$ 1.7% of SCGs in the fibrinogen treated group; $P < 0.0001$). Furthermore, the small percentage of fibrinogen-treated SCGs bearing neurites exhibited a decrease in branching points (Fig. 2*H*).

Fibrinogen Inhibits Neurite Outgrowth via β3 Integrin. Fibrinogen regulates cellular functions as a ligand for different integrins (17). For example, binding of fibrinogen to the platelet αIIbβ3 integrin mediates platelet aggregation, whereas binding of fi-

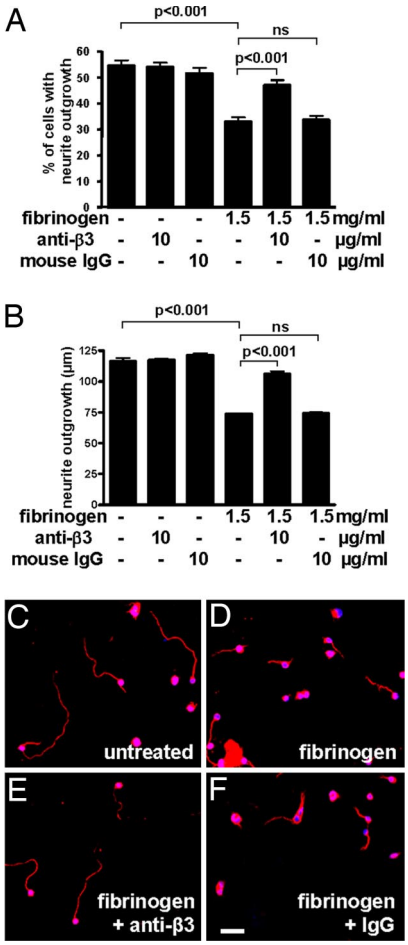

Fig. 3. Fibrinogen mediates inhibition of neurite outgrowth via β3 integrin. (*A* and *B*) Blocking of β3 integrin using a mouse monoclonal antibody that specifically inhibits rat β3 integrin significantly reduces the inhibitory effects of fibrinogen on both the percentage of rat CGNs that bear neurites (*A*; $P < 0.001$) and the neurite length of the CGNs (*B*; $P < 0.001$). Treatment with mouse IgG does not affect the inhibitory effects of fibrinogen in rat CGNs (*A* and *B*). (*C–E*) Representative images of rat CGNs stained with a β-tubulin antibody (red) and DAPI (blue) show that treatment with 1.5 mg/ml fibrinogen inhibits neurite outgrowth (*D*), when compared with untreated rat CGNs (*C*), whereas its effects are reduced in the presence of 10 µg/ml of a mouse anti-β3 blocking antibody (*E*). (*F*) Rat CGNs treated with both fibrinogen and 10 µg/ml of mouse IgG are shown for control. Results are expressed as means ± SEM of a minimum of three different experiments. A minimum of 400 to 500 neurons per condition were analyzed. Statistical comparisons between means were made with one-way ANOVA. (Scale bar: 100 µm.)

brinogen to the αMβ2 (CD11b/CD18) integrin on monocytes mediates inflammatory responses (10, 20). Therefore, we hypothesized that the fibrinogen αvβ3 integrin receptor expressed on neurons (28–30) could mediate the inhibition of neurite outgrowth. Inhibition of β3 integrin was performed in rat CGNs; because of species-specificity, the neutralizing antibody recognizes only the rat, but not the murine β3 integrin (31). Comparative species analysis of neurite inhibition showed that fibrinogen was a potent inhibitor of both rat and murine CGNs (SI Fig. 11). Neutralization of β3 integrin resulted in a statistically significant 30% increase in both the number of rat CGNs extending neurites (Fig. 3*A*; $P < 0.001$), as well as in neurite length (Fig. 3*B*; $P < 0.001$) on fibrinogen treatment. In contrast, IgG treatment did not affect the number of neurite-bearing CGNs or neurite length (Fig. 1 *A* and *B*). Representative images are shown in Fig. 3 *C–F*. These results suggest that β3 integrin is involved in the fibrinogen-mediated inhibition of neurite outgrowth.

Fibrinogen Induces Phosphorylation of EGFR on Neurons via β3 Integrin. An established signal transduction pathway initiated by integrins on their activation by an extracellular matrix (ECM) ligand is transactivation of growth factor (GF) receptors (32, 33). β3 integrin in particular physically interacts and induces phosphorylation of EGFR in fibroblasts (34). Phosphorylation of EGFR mediates the inhibitory effects of several inhibitors of axonal regeneration, such as myelin, Nogo, and chondroitin sulfate proteoglycans (35). Therefore, we hypothesized that fibrinogen as a ligand for β3 integrin could be involved in the activation of EGFR in neurons.

Pharmacological inhibition of EGFR phosphorylation using the irreversible inhibitor PD168393 increased both the number of mouse CGNs extending neurites (Fig. 4*A*; $P < 0.001$), as well as their neurite length (Fig. 4*B*; $P < 0.001$) on fibrinogen treatment. In contrast, PD168393 did not affect the number of neurite-bearing CGNs or their neurite length on a control PDL substrate (Fig. 4 *A* and *B*). To examine whether fibrinogen induces EGFR phosphorylation on neurons, we incubated CGNs with fibrinogen or EGF for positive control. Fibrinogen alone in the absence of EGF was sufficient to induce a 5.1-fold increase in phosphorylation of EGFR in neurons (Fig. 4*C*). Fibrinogen also induced an ≈1.5-fold increase in the total levels of EGFR (Fig. 4*C*). Double immunofluorescence after SCI showed axons positive for phosphorylated EGFR with abundant fibrinogen deposition at the lesion site (Fig. 4*D*). High-magnification images and individual channels are shown in SI Fig. 12. A blocking antibody against β3 integrin reduced the fibrinogen-mediated phosphorylation of EGFR (Fig. 4*E*), suggesting that fibrinogen interactions with β3 integrin was mediating EGFR phosphorylation. A common mechanism that regulates cross-talk between integrins and GFs receptors is activation of Src family kinases (SFK) (33). Inhibition of SFK using the inhibitor PP2 (36) reduced the fibrinogen-mediated EGFR phosphorylation (Fig. 4*F*). Moreover, endogenous coimmunoprecipitation in primary neurons showed interaction between phosphorylated EGFR with β3 integrin only on exposure to fibrinogen (Fig. 4*G*). Overall, these results suggest that fibrinogen induces the cross-talk between β3 integrin and EGFR on neurons that results in the transactivation of EGFR to mediate inhibition of neurite outgrowth.

Discussion

Studies of the inhibition of axonal regeneration have mainly focused on proteins of the nervous system, such as myelin-derived neurite outgrowth inhibitors expressed by oligodendrocytes, guidance molecules expressed by neurons, and proteoglycans that are secreted by the glial scar (26). Our study identified fibrinogen as a major inhibitor of neurite outgrowth that is not synthesized within the CNS, but leaks from the bloodstream into the CNS parenchyma after vascular damage or BBB disruption. Our study suggests the following model for the role of fibrinogen in axonal regeneration (Fig. 5). (*i*) Traumatic injury or other neurodegenerative conditions associated with compromised BBB allow the leakage of fibrinogen in the CNS. (*ii*) Fibrinogen interaction with β3 integrin on neurons induces clustering of β3 integrin with EGF receptor, leading to EGF receptor autophosphorylation in the absence of EGF. (*iii*) Cross-talk of β3 integrin and EGFR is mediated by SFK. (*iv*) Fibrinogen-mediated phosphorylation of the EGF receptor in neurons leads to inhibition of neurite outgrowth. Because fibrinogen is essential for the interaction between β3 and EGFR, the mechanism of EGFR transactivation by integrins could be triggered in the CNS only in pathological states associated with hemorrhage, vascular

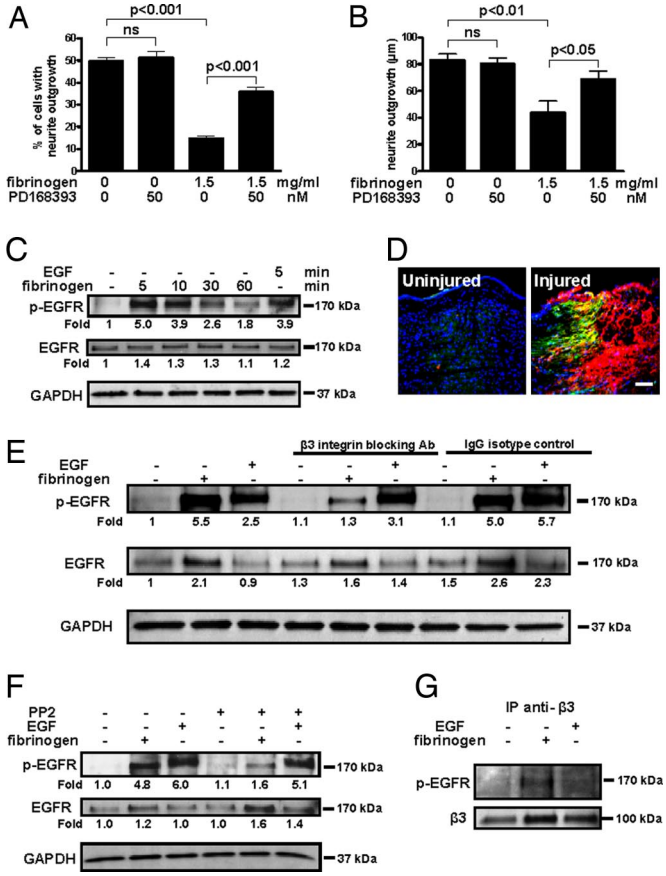

Fig. 4. Fibrinogen induces transactivation of EGFR via β3 integrin to inhibit neurite outgrowth. (*A* and *B*) Treatment with the EGFR phosphorylation inhibitor PD168393 (50 nM) on 1.5 mg/ml fibrinogen treatment results in a statistically significant increase in the percentage of mouse CGNs bearing neurites (*A*; $P < 0.001$) and the length of neurite outgrowth (*B*; $P < 0.05$). Results are expressed as means ± SEM of a minimum of four different experiments. A minimum of 400 to 500 neurons per condition were analyzed. Statistical comparisons between means were made with one-way ANOVA. Error bars indicate SEM. (*C*) Fibrinogen induces phosphorylation of EGFR. Serum-starved mouse CGNs were treated with 1.5 mg/ml fibrinogen for the indicated time points, and the levels of pTyr1173 EGFR (*p*-EGFR) and total EGFR were analyzed by Western blotting. GAPDH was used as loading control. (*D*) Fibrinogen (red) and *p*-EGFR (green) immunostaining of longitudinal sections of mouse spinal cord 2 days after DH revealed axonal colocalization of fibrinogen and *p*-EGFR (yellow) at the lesion area (*Right*). Fibrinogen and *p*-EGFR are undetectable in the uninjured mouse spinal cord (*Left*). Nuclei are stained with DAPI (blue). Representative images of three independent experiments of three animals per condition are shown. (Scale bar: 95 μm.) (*E*) Fibrinogen induces EGFR phosphorylation via β3 integrin. Rat CGNs untreated, pretreated with 10 μg/ml mouse anti-rat β3 blocking antibody or 10 μg/ml control mouse IgG for 1 h, and treated with 1.5 mg/ml fibrinogen, and the levels of pTyr1173 EGFR (*p*-EGFR) and total EGFR were analyzed by Western blotting. GAPDH was used as loading control. (*F*) Fibrinogen induces EGFR phosphorylation via SFK. Mouse CGNs were untreated or pretreated with 10 μM of the general Src inhibitor PP2 for 1 h and treated with 1.5 mg/ml fibrinogen, and the levels of pTyr1173 EGFR (*p*-EGFR) and total EGFR were analyzed by Western blotting. GAPDH was used as loading control. (*G*) Fibrinogen induced the endogenous coimmunoprecipitation of phosphorylated EGFR with β3 integrin in primary neurons. Serum-starved mouse CGNs were treated with 1.5 mg/ml of fibrinogen for 10 min, and cell extracts were immunoprecipitated (IP) with an antibody against β3 integrin (anti-β3). Immunoblotting was performed with antibodies against *p*-EGFR and β3 integrin. Immunoblots were performed at least five times, and representative blots are shown. Blots were quantitated by densitometry and normalized to GAPDH.

damage, or BBB disruption. Interestingly, in the peripheral nervous system, EGFR is not expressed by axons distal to the site of injury (37), which is the site of fibrinogen deposition (18, 19).

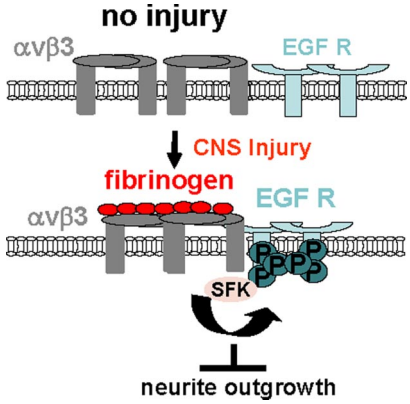

Fig. 5. Proposed model of fibrinogen-mediated inhibition of neurite outgrowth. Traumatic injury or other neurodegenerative conditions associated with compromised BBB or vascular damage allow the leakage of fibrinogen in the CNS. Fibrinogen binding to β3 integrin on neurons induces coclustering of β3 integrin with EGFR, leading to EGFR phosphorylation and subsequent inhibition of neurite outgrowth.

The spatial expression of EGFR is in accordance with the role of fibrinogen in peripheral nerve repair as an inhibitor of Schwann cell myelination, but not axonal elongation (18). Because fibrinogen functions in tissues depend on receptor-mediated signal transduction (10, 20, 38, 39), it is possible that the spatial and temporal distribution of the fibrinogen receptors in the nervous system would determine its role in the regenerative process.

The molecular interaction between integrin and GF receptors regulates GF receptor functions in response to changes in the extracellular environmental (32). Our study demonstrates that GF receptor transactivation by integrins occurs in neurons. Moreover, we show that EGF-independent, β3 integrin-mediated phosphorylation of EGFR produces the unique biological effect of inhibition of neurite outgrowth, which is a central impediment for CNS repair. In that respect, fibrinogen, which is present in the CNS only on injury or disease, could serve as the "signal," and β3 integrin might function as a "sensor" to changes in the CNS microenvironment, such as hemorrhage, to modulate neuronal responses by inducing activation of EGFR. The small, rapid 1.5-fold increase in the total levels of EGFR expression on fibrinogen induction might reflect pathways of integrin–GF cross-talk, such as modulation of EGFR recycling (40, 41), which may function in parallel with phosphorylation to potentiate the biological effects of EGFR activation (42). The fibrinogen-induced transactivation of EGFR by β3 integrin could therefore synchronize EGFR functions with changes in the tissue environment that are dictated by vascular rupture. Up-regulation of cAMP (22) or inhibition of EGFR phosphorylation rescues myelin-mediated inhibition of neurite outgrowth (35). Myelin/NgR1 signaling induces phosphorylation of the EGFR in a calcium-dependent manner to inhibit neurite outgrowth (35). On myelin stimulation, NgR1 does not form a complex with EGFR (35). By contrast, on fibrinogen stimulation, β3 integrin forms a complex with EGFR as shown by endogenous coimmunoprecipitation in primary neurons (data not shown). Future studies will determine the individual contributions and potential synergistic effects of fibrinogen and myelin signal transduction pathways in the regulation of neurite outgrowth.

Although other blood proteins such as thrombin exert apoptotic effects on neurons via protease-activated receptors (43), fibrinogen might be unique in its ability to transactivate EGFR and inhibit neurite outgrowth because of its unique structure that induces β3 integrin signaling. β3 integrin acts as a mediator of the maturation of excitatory synapses in hippocampal neurons

(30) and regulates formation of focal adhesion in astrocytes (44). Our study provides inhibition of neurite outgrowth as a biological function for β3 integrin signaling in the CNS. In addition to β3 integrin, fibrinogen binds to CD11b/CD18 integrin receptor and induces activation of microglia to phagocytes in the CNS (20). Therefore, it is possible that fibrinogen signal transduction via different cellular receptors might regulate both inflammatory and repair processes in the CNS after injury or disease associated with BBB disruption or vascular damage.

Although cerebrovascular dysregulation was originally considered a feature of CNS pathologies, such as ischemia and stroke, vascular abnormalities and hemorrhage have emerged as major players in a wide range of neurodegenerative diseases (45). In AD, amyloid plaques represent the sites of microhemorrhages (4), vascular disease correlates with cognitive impairment (46), and fibrinogen deposition is persistent in the AD brain (24). In MS, fibrinogen deposition is sustained and correlates with axonal damage and demyelination (47, 48), and pharmacological or genetic depletion of fibrinogen ameliorates disease pathogenesis in MS animal models (7). In SCI, traumatic injury severs both axons and the vasculature, leading to prominent intraparenchymal hemorrhage (49) and results in dramatic fibrinogen deposition (data not shown). Our study identifies a functional role for fibrinogen at the neurovascular interface as a molecular link between the blood and the CNS in neurodegenerative disease. The inhibitory functions of fibrinogen on neurite outgrowth could therefore be relevant in a variety of neurodegenerative diseases. Depletion of fibrinogen either by anticoagulants such as ancrod (18) or inhibition of β3 integrin could be tested alone or in combination with other therapeutic strategies for their efficacy in the promotion of axonal regeneration in the CNS. In that respect, identification of fibrinogen as a new inhibitor of neurite outgrowth may yield additional strategies to promote axonal regeneration in CNS after trauma or neurodegenerative disease.

Methods

SCI in Mice and Rats. The surgical procedures for mouse DH, rat dorsal column lesion, and contusion on adult female C57BL/6 mice or Fischer 344 rats (The Jackson Laboratory, Bar Harbor, ME) were performed as described (50, 51).

Histopathology. Analysis of the spinal cord tissue was performed as described (50). Primary antibodies used were sheep anti-fibrin(ogen) (1:100; U.S. Biological, Swampscott, MA), mouse anti-SMI-32 (1:2,000; Sternberger Monoclonals, Lutherville, MD), and rabbit anti-P-EGFR1173 (Abcam, Cambridge, MA). Sections were counterstained with DAPI (Invitrogen, Carlsbad, CA), and images were collected by using an Axioplan 2 Zeiss microscope (Carl Zeiss, Thornwood, NY) with an Axiocam HRc camera or were processed for confocal microscopy using Olympus (Tokyo, Japan) and Zeiss confocal microscopes.

Neurite Outgrowth Assays. CGNs and SCGs were isolated as described (27, 52). We plated 7.5×10^4 mouse CGNs, 2.5×10^4 rat CGNs, and 2×10^4 mouse SCG neurons per well of poly-D-lysine-coated eight-well Nunc plates. Neurons were treated with human fibrinogen (Calbiochem, San Diego, CA) at concentrations ranging from 0.1 mg/ml to 1.5 mg/ml for 24 h. To inhibit EGFR phosphorylation, neurons were treated with 50 nM of PD168393 (Calbiochem) for 24 h. To inhibit β3 integrin, neurons were treated with a mouse monoclonal anti-rat β3 neutralizing antibody (10 μg/ml; BD Biosciences, San Diego, CA) or mouse IgG (10 μg/ml; BD Biosciences) as control. Myelin from mouse spinal cord was prepared as decribed (53). For myelin membranes, mouse CNS myelin at 1 μg total protein per well was dried overnight and used as substrate (54). Neurons extended processes for 24 h on different treatments and were then fixed in 4% paraformaldehyde and stained for β-tubulin. Quantification was performed as described (27, 50, 55). For SCGs, the number of neurite-bearing cells and branching points per cell, and for CGNs, the number of neurite-bearing cells and the neurite length, were measured from 400 to 500 neurons per condition. All experiments were repeated four times and were performed in triplicate.

Apoptosis Assay. Mouse CGNs were isolated and 5×10^4 neurons were plated per well of a poly-D-lysine-coated 96-well plate. CGNs were treated for 20 h using the indicated fibrinogen concentrations or 1 μM Staurosporine (Sigma–Aldrich, St Louis, MO) as positive control, and apoptosis was quantified by using the Cell Death Detection Elisa Kit (Roche Diagnostics, Indianapolis, IN). Apoptotic assays were performed three times in triplicate.

Immunoblots and Immunoprecipitation. CGNs were serum-starved for 2 h and treated with 1.5 mg/ml of fibrinogen for the indicated time points. Cells were extracted on ice for 30 min in lysis buffer [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease and phosphatase inhibitor]. For EGFR, immunoblot samples were incubated with agarose-bound Concanavalin-A (Vector Laboratories, Burlingame, CA) for 4 h at 4°C as described (56). For endogenous coimmunoprecipitation, cell lysates were incubated with rabbit anti-mouse integrin β3 antibody (1:100; Cell Signaling, Beverly, MA) bound to A-agarose beads for 4 h at 4°C. After washing three times, the beads were resuspended in sample buffer, boiled for 10 min, and centrifuged. Supernatants were electrophoresed on SDS/4–12% PAGE gels and probed with the following antibodies: phospho-EGFR (Tyr-1173), total-EGFR, and integrin β3 antibodies (1:1,000; Cell Signaling). For the inhibition of β3 integrin, neurons were pretreated with a mouse monoclonal anti-rat β3 neutralizing antibody (10 μg/ml; BD Biosciences) or mouse IgG (10 μg/ml; BD Biosciences) for 1 h before the addition of 1.5 mg/ml fibrinogen. For the inhibition of SFK, neurons were pretreated for 1 h with the inhibitor PP2 (10 μg/ml; Biomol, Plymouth Meeting, PA) before the addition of 1.5 mg/ml fibrinogen. Cell lysate was electrophoresed on SDS/4–12% PAGE gels and probed for GAPDH (Abcam) as loading control. Immunoblots were performed as described (18) at least five times, and representative blots are shown. Densitometry analysis using NIH Scion Imaging Software was performed on all blots, and the average values are shown.

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1. Carmeliet P, Tessier-Lavigne M (2005) *Nature* 436:193–200.
2. Abbott NJ, Ronnback L, Hansson E (2006) *Nat Rev Neurosci* 7:41–53.
3. Popovich PG, Horner PJ, Mullin BB, Stokes BT (1996) *Exp Neurol* 142:258–275.
4. Hardy J, Cullen K (2006) *Nat Med* 12:756–757.
5. Vos CM, Geurts JJ, Montagne L, van Haastert ES, Bo L, van der Valk P, Barkhof F, de Vries HE (2005) *Neurobiol Dis* 20:953–960.
6. Horner PJ, Gage FH (2000) *Nature* 407:963–970.
7. Adams RA, Passino M, Sachs BD, Nuriel T, Akassoglou K (2004) *Mol Interv* 4:163–176.

8. Degen JL, Drew AF, Palumbo JS, Kombrinck KW, Bezerra JA, Danton MJ, Holmback K, Suh TT (2001) *Ann NY Acad Sci* 936:276–290.
9. Herwald H, Cramer H, Morgelin M, Russell W, Sollenberg U, Norrby-Teglund A, Flodgaard H, Lindbom L, Bjorck L (2004) *Cell* 116:367–379.
10. Flick MJ, Du X, Witte DP, Jirouskova M, Soloviev DA, Busuttil SJ, Plow EF, Degen JL (2004) *J Clin Invest* 113:1596–1606.
11. Tang L, Ugarova TP, Plow EF, Eaton JW (1996) *J Clin Invest* 97:1329–1334.
12. Tang L, Eaton JW (1993) *J Exp Med* 178:2147–2156.
13. Busso N, Peclat V, Van Ness K, Kolodziesczyk E, Degen J, Bugge T, So A (1998) *J Clin Invest* 102:41–50.
14. Lluis F, Roma J, Suelves M, Parra M, Aniorte G, Gallardo E, Illa I, Rodriguez L, Hughes SM, Carmeliet P, *et al*. (2001) *Blood* 97:1703–1711.
15. Bugge TH, Kombrinck KW, Flick MJ, Daugherty CC, Danton MJ, Degen JL (1996) *Cell* 87:709–719.
16. Mosesson MW (2005) *J Thromb Haemost* 3:1894–1904.
17. Akassoglou K, Strickland S (2002) *Biol Chem* 383:37–45.
18. Akassoglou K, Yu W-M, Akpinar P, Strickland S (2002) *Neuron* 33:861–875.
19. Akassoglou K, Kombrinck KW, Degen JL, Strickland S (2000) *J Cell Biol* 149:1157–1166.
20. Adams RA, Bauer J, Flick MJ, Sikorski SL, Nuriel T, Lassmann H, Degen JL, Akassoglou K (2007) *J Exp Med* 204:571–582.
21. Akassoglou K, Adams RA, Bauer J, Tseveleki V, Mercado P, Lassmann H, Probert L, Strickland S (2004) *Proc Natl Acad Sci USA* 101:6698–6703.
22. Filbin MT (2003) *Nat Rev Neurosci* 4:703–713.
23. Claudio L, Raine CS, Brosnan CF (1995) *Acta Neuropathol* 90:228–238.
24. Lipinski B, Sajdel-Sulkowska EM (2006) *Alzheimer Dis Assoc Disord* 20:323–326.
25. Adhami F, Liao G, Morozov YM, Schloemer A, Schmithorst VJ, Lorenz JN, Dunn RS, Vorhees CV, Wills-Karp M, Degen JL, *et al*. (2006) *Am J Pathol* 169:566–583.
26. Case LC, Tessier-Lavigne M (2005) *Curr Biol* 15:R749–R753.
27. Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002) *Nature* 417:941–944.
28. Ugarova TP, Yakubenko VP (2001) *Ann NY Acad Sci* 936:368–385.
29. Pinkstaff JK, Detterich J, Lynch G, Gall C (1999) *J Neurosci* 19:1541–1556.
30. Chavis P, Westbrook G (2001) *Nature* 411:317–321.
31. Thompson RD, Wakelin MW, Larbi KY, Dewar A, Asimakopoulos G, Horton MA, Nakada MT, Nourshargh S (2000) *J Immunol* 165:426–434.
32. Giancotti FG, Ruoslahti E (1999) *Science* 285:1028–1032.
33. Yamada KM, Even-Ram S (2002) *Nat Cell Biol* 4:E75–E76.
34. Moro L, Venturino M, Bozzo C, Silengo L, Altruda F, Beguinot L, Tarone G, Defilippi P (1998) *EMBO J* 17:6622–6632.
35. Koprivica V, Cho KS, Park JB, Yiu G, Atwal J, Gore B, Kim JA, Lin E, Tessier-Lavigne M, Chen DF, He Z (2005) *Science* 310:106–110.
36. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA (1996) *J Biol Chem* 271:695–701.
37. Toma JG, Pareek S, Barker P, Mathew TC, Murphy RA, Acheson A, Miller FD (1992) *J Neurosci* 12:2504–2515.
38. Petzelbauer P, Zacharowski PA, Miyazaki Y, Friedl P, Wickenhauser G, Castellino FJ, Groger M, Wolff K, Zacharowski K (2005) *Nat Med* 11:298–304.
39. Coller BS (1997) *J Clin Invest* 99:1467–1471.
40. Rubin C, Litvak V, Medvedovsky H, Zwang Y, Lev S, Yarden Y (2003) *Curr Biol* 13:297–307.
41. Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, Beguinot L, Geiger B, Yarden Y (1998) *Genes Dev* 12:3663–3674.
42. Miranti CK, Brugge JS (2002) *Nat Cell Biol* 4:E83–E90.
43. Wang H, Reiser G (2003) *Biol Chem* 384:193–202.
44. Leyton L, Schneider P, Labra CV, Ruegg C, Hetz CA, Quest AF, Bron C (2001) *Curr Biol* 11:1028–1038.
45. Iadecola C (2004) *Nat Rev Neurosci* 5:347–360.
46. Selnes OA, Vinters HV (2006) *Nat Clin Pract Neurol* 2:538–547.
47. Gveric D, Herrera B, Petzold A, Lawrence DA, Cuzner ML (2003) *Brain* 126:1–9.
48. Gay FW, Drye TJ, Dick GW, Esiri MM (1997) *Brain* 120:1461–1483.
49. Noble LJ, Wrathall JR (1989) *Brain Res* 482:57–66.
50. Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M (2003) *Neuron* 38:213–224.
51. Lu P, Yang H, Jones LL, Filbin MT, Tuszynski MH (2004) *J Neurosci* 24:6402–6409.
52. Lee KF, Li E, Huber LJ, Landis SC, Sharpe AH, Chao MV, Jaenisch R (1992) *Cell* 69:737–749.
53. Schwab ME, Caroni P (1988) *J Neurosci* 8:2381–2393.
54. Cai D, Shen Y, De Bellard M, Tang S, Filbin MT (1999) *Neuron* 22:89–101.
55. Niederost BP, Zimmermann DR, Schwab ME, Bandtlow CE (1999) *J Neurosci* 19:8979–8989.
56. Jo M, Thomas KS, Takimoto S, Gaultier A, Hsieh EH, Lester RD, Gonias SL (2007) *Oncogene* 26:2585–2594.