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Inhibition of Neurite Outgrowth on Astroglial Scars *in vitro*

John S. Rudge and Jerry Silver

Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio 44106

Traumatic injury to the adult mammalian CNS results in the formation of an astroglial–mesenchymal scar that seals the wound site but blocks axonal regeneration in the process. The mechanism that leads to this inhibition of axon outgrowth has been proposed to be either a physical barrier blocking the advancement of the growth cone or chemical factors actively inhibiting axon outgrowth. At present, it is unknown whether one or both of these mechanisms are responsible for the inhibitory nature of the glial scar *in vivo*.

Using a model of CNS trauma that allows for removal of an adult rat glial scar intact on a nitrocellulose support and placement *in vitro* with the upper surface exposed, we addressed the question of whether the inhibitory effects could be accounted for by chemical components at the scar surface. A purified population of rat hippocampal neurons was seeded onto the scar explants as well as onto explants taken from neonatal rat cerebral cortex, and the extent of neurite outgrowth was compared. We found that the glial scar, at best, stimulates only minimal neurite outgrowth over its surface when compared to the immature environment explanted in the same manner. This growth-inhibitory state cannot merely be explained by neuronotoxic factors or fibroblasts preventing astrocyte-mediated neurite outgrowth. The inhibition is more probably due to the expression of molecules on the surface of the adult scar that either directly inhibit growth cones or inhibit them indirectly by occluding neurite-promoting factors in the extracellular matrix or on the astrocyte surface.

The intimate association that occurs between astrocytes and neuronal growth cones within the CNS during development has led to the suggestion that astrocytes are an important substrate for axon outgrowth *in vivo* (Grafe and Schoenfeld, 1982; Silver et al., 1982). This also appears to be the case after trauma to the immature mammalian CNS where astrocytes in the region of the wound support axonal elongation through or around the lesion (Barrett et al., 1984; Smith et al., 1986; Carlstedt et al., 1987). However, after a penetrating injury to the adult CNS, an astroglial–mesenchymal scar is formed, and it is this structure that has traditionally been thought of as a major obstacle to axonal regeneration (Ramon y Cajal, 1928; Brown and McCouch, 1947; Clemente, 1955; Reier et al., 1983). At present, it is unknown whether astrocytes in the adult CNS that respond to trauma (reactive astrocytes) are intrinsically incapable of supporting axon outgrowth or whether other factors serve to block the regeneration of CNS axons.

Attempts to analyze the efficacy of astrocytes as a substrate for axonal outgrowth have utilized glial cells from neonatal rat cerebral cortex cocultured with purified embryonic neurons (Noble et al., 1984; Fallon, 1985; Tomaselli et al., 1988; Smith et al., 1990). These studies have been instrumental in elucidating the molecular basis for neurite outgrowth on astrocytes *in vitro* where molecules such as the neural-cell adhesion molecule (NCAM) (Smith et al., 1990), laminin (Liesi et al., 1983), and *N*-cadherin (Tomaselli et al., 1988) have been implicated. They have also revealed that astrocytes allowed to mature for a prolonged period in culture are still capable of supporting 70% of the amount of neurite outgrowth that younger astrocyte cultures sustain (Smith et al., 1990). If mature astrocytes retain their capacity to support neurite growth *in vitro,* what other factors in the traumatized environment *in vivo* could contribute to block the capacity of the astrocyte to support axon outgrowth after trauma?

Strong candidates for inhibitory factors of axon outgrowth are 2 proteins in oligodendrocyte myelin that have been isolated and found to prevent neurite outgrowth *in vitro* (Schwab and Caroni, 1988). If the same is true *in vivo,* then white-matter debris at the wound site may actively inhibit axon outgrowth via these 2 repulsive molecules. However, axon outgrowth remains minimal even in wounds where myelin is not a major component of the scar. Thus, limited regeneration occurs in nonmyelinated retina (Goldberg and Frank, 1980; McConnell and Berry, 1982) following placement of dorsal roots into spinal-cord gray matter (Carlstedt, 1985; Kliot et al., 1988), after the placement of facial nerve into cerebral cortical gray matter (Windle et al., 1952), and after the placement of sciatic-nerve bridges into the optic tectum (Aguayo et al., 1982). All these examples of regenerative failure involve non-myelin-containing glial or glial–mesenchymal scar-forming regions and suggest that other factors in addition to or in collaboration with myelin nonpermissiveness are involved in the inhibition of axon outgrowth in gray matter.

After a penetrating injury to the CNS, the overriding imperative is to seal the wound to prevent infection, which results in a haphazard assembly of fibroblasts and reactive astrocytes at the wound site (Berry et al., 1983; Reier and Houle, 1988). The resultant "wall" of cells and extracellular matrix provides no recognizable avenue for ordered axonal regeneration. The apparent cellular disarray and lack of known gray-matter inhibitory molecules has led to the hypothesis that the scar acts as a physical barrier to axonal regeneration (Ramon y Cajal, 1928; Clemente, 1955) or is lacking in growth-promoting trophic or

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Correspondence should be addressed to Dr. John S. Rudge, Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707.

tropic molecules (Reier and Houle, 1988; Snider and Johnson, 1989).

We have developed a model of CNS wound healing that causes a penetrating injury in gray matter by implanting a nitrocellulose sheet into the immature and adult cerebral cortex. This allows for removal of the complex cellular environment that comprises the wound response and its placement as an explant into culture (Rudge et al., 1989). By removing the gray-matter scar environment *in toto* from an adult, we wanted to determine if the surface of a complete glial–mesenchymal scar was itself nonpermissive or inhibitory to axon outgrowth.

We found that neurite outgrowth on the scar explants is minimal compared to outgrowth on the neonatal explants. This is not due to neuronotoxic factors secreted by the scar, nor to fibroblasts occluding astrocyte-mediated neurite outgrowth. Therefore, though neurons have the capacity to extend neurites, they are unable to do so on scar tissue. A critical factor in the inhibition may be the presence of abundant ectopic basal laminae in the scar explants. Thus, the axon refractory properties of the glial scar appear to be due not only to its physical properties, but also to molecules on the surface either actively inhibiting growth cones or indirectly inhibiting them by occlusion of neurite-promoting factors.

Materials and Methods

Surgery. The surgical paradigm used for implantation of filters into young and adult brains was the same as described previously (Rudge et al., 1989). Briefly, nitrocellulose filters (8-μm pore size) were implanted into the cerebral cortex of 1-d-old ice-anesthetized rat pups or 20-d-old rats. A modification of this procedure is the introduction of the nitrocellulose filter into the cerebral cortex lateral to the midline, resulting in an increased astrocyte : fibroblast ratio compared to implantation at the midline. In the case of the rat pups, the filters were allowed to remain *in situ* for 6 d, during which time they became inhabited by those cells that responded to a traumatic injury in the neonate. The filters were removed on postoperative day 6 and placed into serum-free culture (1–6-d critical-period implants, 6 d *in vivo*). Filters placed into 20-d-old rats were removed after 10 d and were found to possess a complement of cells comprising the glial scar (20–30-d scar implants, 10 d *in vivo*). A permutation of this procedure involved introducing the filters into the pups at 1 d of age and removing them at 30 d of age. This resulted in cells occupying the filter that had at one time possessed the qualities of the immature environment but had aged past the critical period *in vivo* (1–30-d postcritical-period implants, 30 d *in vivo*).

Culture of explants. After removal from the animal, explants were placed into calcium- and magnesium-free Hanks balanced salt solution (CMF) containing 0.02% EDTA and incubated for 30 min at room temperature. After this time, the explants were vigorously shaken to remove any tissue that was not intimately associated with the filter, washed in CMF, and placed into Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F12 nutrient mixture (DME-F12) (GIBCO 430-2400EB), and the medium was changed on days 1 and 3 of the culture period.

Quantitation of cells populating explants. The characterization of the number of astrocytes populating the different explants was achieved by utilizing the same procedure described previously (Rudge et al., 1989), which involved immunocytochemically staining cells on the explant with cell-type-specific antibodies—anti-glial fibrillary acidic protein (GFAP) for astrocytes (a gift from Dr. Larry Eng), MRCOX42 for macrophages (Serotec MCA 275), and anti-galactocerebroside for oligodendrocytes (a gift from Dr. Barbara Ranscht)—and visualizing them using a secondary antibody linked to biotin (Vector BA1000 and BA2000) followed by streptavidin fluorescein (Amersham RPN1232). The explants were then counterstained with propidium iodide to determine total cell number, and the labeled cells were expressed as a percentage of the total cell number. We supplemented this quantification at the light level by examining *en face* sections of the explants under higher magnification with the electron microscope to determine whether other cells or structures were present within the explants (see Histology, below).

Incorporation of dil into and coculture of rat hippocampal neurons with explants. The advantage of using hippocampal neurons as a test of neuronal population was that the majority of cells in the hippocampus on embryonic day 18 (E18) are pyramidal neurons (Banker and Cowan, 1979; Cowan et al., 1984), and the proportion of non-neuronal cells to neurons is less than 10% (Müller and Seifert, 1982; Barbin et al., 1984). Hippocampi were dissected free from the brains of E18 Sprague–Dawley rat pups and placed into 0.1% trypsin for 30 min at 37°C. DNAase was incorporated at 0.2 mg/ml for the final 10 min. Ten percent fetal calf serum (FCS) in DME-F12 was added to stop the reaction, the cells were centrifuged, and the supernatant was removed. The cells were then resuspended in DME-F12 without FCS, and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (diI) (Molecular Probes Inc., D282) in dimethyl sulfoxide was added to a final concentration of 3 μg/ml. The tube was shaken and left at room temperature for 10 min, placed on ice for 10 min, then centrifuged through twice the volume of horse serum for 5 min at 500 × *g*. The cells were resuspended and plated onto a polylysine-coated 100-mm tissue-culture plastic dish (0.1 mg/ml in distilled water for 2 hr) in DME-F12 plus 10% FCS and allowed to adhere for 3 hr at 37°C. The debris and aggregated diI particles were washed off and the dish placed on ice for 20 min. Neurons were removed from the surface of the dish by aspiration with a Pasteur pipette, counted on a hemocytometer, and seeded onto the surface of the explants (which had been in culture for only 3 d) at 5000 neurons per 16-mm well (25 neurons per mm²). Neurons and explants were cocultured in DME-F12 plus N2 supplement (Bottenstein et al., 1979) and pyruvate (2 mM) for 48 hr, fixed with 4% paraformaldehyde for 30 min, mounted in vinol (Air Products and Chemicals, Inc., 523; 10% vinol in 2 parts PBS to 1 part glycerol), and photographed immediately. The coculture period of 48 hr 3 d after placement of the explants *in vitro* was chosen to allow the cells in the explant to stabilize *in vitro* but also to maintain the cells as close to the *in vivo* state as possible.

Immunocytochemistry. Laminin expression was determined by incubating explants with an anti-laminin antiserum (GIBCO-BRL 680 3019AA) and visualized using the biotin–avidin procedure.

Neuronal-survival assay. To determine whether neurons seeded onto the surface of the explants were subject to trophic or toxic effects, the number of hippocampal neuronal cell bodies occupying 1-mm² grids on the surface of the explants was determined after fixation of the neurites as described above. Thirty separate grids were counted for each of the 1–6-d, 1–30-d, and scar explants. Comparison of neuronal survival on the explants was carried out using a Student's *t* test.

Quantification of neurite outgrowth. The length of outgrowth of diI-stained rat hippocampal neurons ($n = 60$ for each type of explant) on the explants was determined by photographing fields of neurite outgrowth on explants in different planes of focus, then building up the total neurite outgrowth by adding the different planes together into a single tracing. The length of all neurites greater than 2 somal diameters were measured by projecting the 35-mm negatives at a fixed magnification onto the screen of a Vanguard M-35C Motion Analyzer and digitizing lengths of individual neurites with a Numonics digitizer. In order to compare outgrowth on the 3 different types of explant, the lengths were then placed into bins of increasing 100-μm increments and graphically represented as a percentage of total number of neurons with neurites of a specific length. This results in a direct comparison of all the neurites extending from neurons plated onto the explants and directly addresses the capacity of all the neurons in the population to extend neurites over different substrates.

Comparison of neurite lengths on the 3 different types of explants was carried out using a Mann–Whitney *U* test.

Neurite outgrowth on a mixed culture of astrocytes and fibroblasts. Astrocytes were prepared from 1-d-old rat cerebral cortex by the method of Smith et al. (1990), while fibroblasts were prepared from the meninges of the same rats. Both cell types were grown in DME-F12 with 10% FCS for 30 d, then plated onto fibronectin-coated (10 μg/ml PBS overnight) coverslips in the following percentages: 100, 85, 60, 40, 15, and 0%. After 24 hr, diI-prelabeled rat hippocampal neurons were seeded onto their surface, allowed to extend neurites for 18 hr in serum-free medium, and fixed with 4% paraformaldehyde. Cocultures of rat hippocampal neurons were mounted in vinol, photographed, and digitized (see Quantification of Neurite Outgrowth, above). Data is presented as the mean neuritic length of hippocampal neurons ($n = 60$ for each ratio) over a monolayer containing increasing numbers of fibroblasts.

Table 1. Percentage of cell types occupying explants after 3 d *in vitro*

	1–6-d	1–30-d	Cortical scar
Astrocytes	71	81	65
Macrophages	21	11	16
Fibroblasts	4	4	15
Endothelial cells	2	3	4

10-μm cryostat frozen sections were cut and stained with cell-specific antibodies: glial fibrillary acidic protein, astrocytes; MRCOX42, macrophages; anti-fibronectin, fibroblasts; anti-Factor VIII, endothelial cells. Cells were visualized with biotin-linked secondary antibody and streptavidin fluorescein and counterstained with propidium iodide. Values represent the means of 3 implants, 20 sections from each implant. SDs ranged from 7 to 15%.

Histology. Animals were prepared for histological analysis as described previously (Rudge et al., 1989). Briefly, explants that had remained in culture for 3 d were fixed in 0.5% glutaraldehyde with 2% paraformaldehyde, postfixed with OsO_4, dehydrated, and embedded in Spurr's resin using standard procedures. Serial 1-μm sections were taken through the implant either coronally or tangentially and stained with Toluidine blue. Ultrathin sections were cut on a Reichert microtome, stained with uranyl acetate and lead citrate, and viewed with a JEOL 100CX electron microscope at 80 kV.

Results

Quantitation of cells occupying explants

The major cell type occupying all 3 types of explant was the astrocyte (Table 1) with macrophages, fibroblasts, and endothelial cells comprising the remainder. We modified the original method of implanting nitrocellulose filters for scarring (Rudge et al., 1989) by inserting the filter lateral to the midline in the cerebral cortex of adult rats in order to maximize the number of astrocytes in the scars and to exclude oligodendrocytes. The procedure allows for only limited migration of fibroblasts onto the filter because it is placed away from the midsaggital fissure, resulting in a high astrocyte : fibroblast ratio within the wound tissue that forms around the implant (Table 1, cortical scar, 10 d *in vivo*). Oligodendrocytes were not detectable in or on such scars, while the 1–6- and 1–30-d explants possessed less than 0.05% oligodendrocytes.

Outgrowth of rat hippocampal neurons on the 3 types of explant

The outgrowth of rat hippocampal neurons on the 1–6-d explants (Fig. 1*A*) was characterized by long axonlike processes that exhibited branch points along their length, as well as collateral neurites extending from the cell body. The neurites did not appear to follow any prescribed course, such as along blood vessels, but rather extended uniformly over the entire surface of the explant. The substrate over which these neurites extended was comprised primarily of astrocytes, as shown in Figure 1*B* and Table 1.

Hippocampal neurons on the surface of the 1–30-d explants (Fig. 1*C*) generated neurites with similar morphology to those on the 1–6-d explants but with a reduced length. The substrate over which these neurites grew was predominantly hypertrophied astrocytes (Fig. 1*D*) with a markedly different morphology to those seen on the 1–6-d explants (Fig. 1*B*).

Outgrowth on the surface of the scar explants, however, was dramatically different than the 1–6- and 1–30-d explants in that the majority of neurons possessed only very short processes extending out over the substrate (Fig. 1*E*). The scar-forming cells that had responded to traumatic injury in the adult and comprised the explanted substrate were largely hypertrophied astrocytes (Fig. 1*F*), with fewer numbers of macrophages and fibroblasts (Table 1). The differences in neurite outgrowth are more clearly shown in camera lucida drawings of representative rat hippocampal neurons extending neurites over the surface of 1–6-d explants (Fig. 2*A–E*) and scar explants (Fig. 2*F–J*). The neurites on scar are much shorter with multiple processes, none of which predominate in length.

A quantitative comparison of this data is shown in Figure 3*A*, which depicts the differences in hippocampal neurite outgrowth on 1–6-d, 1–30-d, and scar explants. The most extensive neurite outgrowth was seen on the 1–6-d explants. The mean length of processes was 245 μm, with the longest neurite achieving a length of 639 μm in 48 hr. On the 1–30-d explants, neurite outgrowth was reduced to a mean length of 139 μm (longest was 477 μm) and was further reduced to 109 μm on the scar explants (44% of the mean outgrowth on the 1–6-d explants; longest was 341 μm). To ensure that the change in neurite length was not simply a redistribution of the extent of neurite outgrowth to collaterals, we determined total neuritic length. We observed the same reduction in the capacity for hippocampal neurites to grow as was observed with the length of the longest neurite, with total mean lengths of 478 μm on 1–6-d, 364 μm on 1–30-d, and 218 μm on scar explants (see Fig. 5*B*).

These experiments were repeated using another neuronal population, E7 chick retinal ganglion neurons, and visualizing with anti-NCAM and the ganglion-cell-specific marker TuJ1 (Moody et al., 1989). We found the same trend in reduction of outgrowth of TuJ1-positive cells as is shown in Figure 3, with the mean length of neurite outgrowth exhibiting a 76% reduction on scar explants compared to outgrowth on the 1–6-d explants (data not shown), suggesting that this phenomenon is not specific to cell type or species.

Survival of neurons on the surface of 1–6-d, 1–30-d, and scar explants

The capacity of the different explants to exert an influence on neurite outgrowth through a humoral trophic or toxic effect was examined, and the results are shown in Table 2. The number of rat hippocampal neurons that survive on the surface of the 3 different types of explant remains fairly constant. Although the number of neurons surviving on scar explants seems to be slightly greater than on the other 2 explants, this value is not statistically significant. Therefore, hippocampal neurons do not appear to redistribute their neuritic length through differential survival of subpopulations, but rather, the same population of neurons responds to the substrate in different ways.

Outgrowth of neurites on mixed astrocyte–fibroblast cultures

One possible explanation for the results shown in Figure 3 is that fibroblasts infiltrating in from the meninges after a penetrating injury modulate the capacity for growth cones to interact with the astrocyte surface. As fibroblasts are known to be a poor substrate for neurite outgrowth (Noble et al., 1984), their presence in scar explants could be a simple explanation for the lack of growth that we have observed. This issue was addressed by seeding rat hippocampal neurons (Fig. 4*A,B*) onto astrocytes and fibroblasts mixed in defined ratios in monolayer cultures. The hippocampal neurons showed similar morphologies when seeded onto monolayers as when seeded onto explants.

Outgrowth of hippocampal neurons (Fig. 5) on the surface of

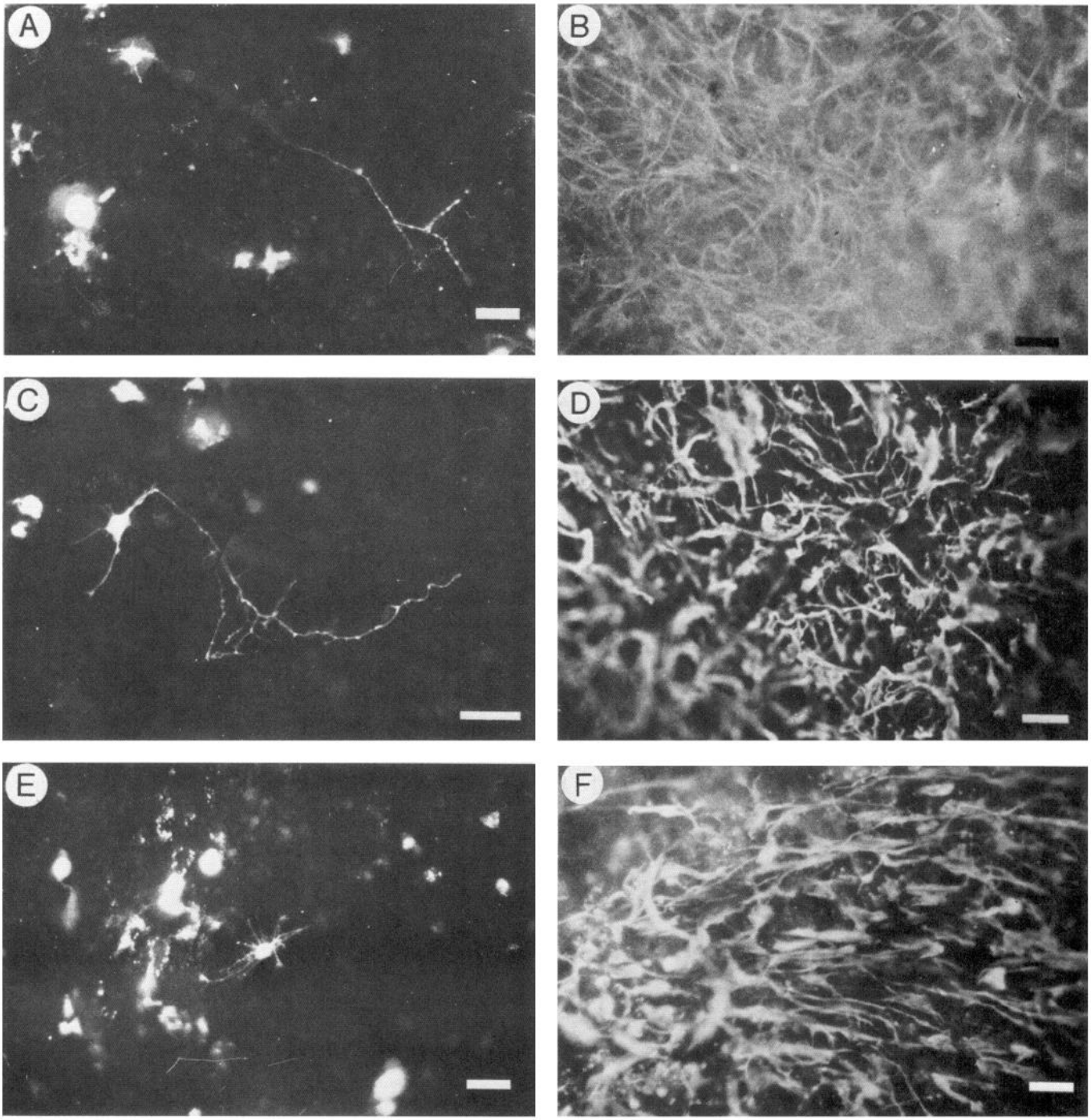

Figure 1. Representative neurite outgrowth from E18 rat hippocampal neurons on surface of 1–6-d (*A*), 1–30-d (*C*), and scar explants (*E*). Hippocampal neurons are stained with dil and cocultured with the explants for 48 hr. The explants are composed mainly of astrocytes, as shown by staining with anti-GFAP (see Materials and Methods) on the 1–6-d explant (*B*), the 1–30-d explant (*D*), and the scar explant (*F*; see Table 1). Note the hypertrophied, intensely GFAP-positive astrocytes on the surface of the 1–30-d (*D*) and scar (*F*) explants compared to the astrocytes with thin processes on the surface of the 1–6-d explants (*B*). Other stained areas in *A, C,* and *E* are due to macrophages on the surface of the explants ingesting dil particles introduced with the hippocampal seed. Scale bar, 20 μm.

a 100%-mature astrocyte culture (30 d *in vitro*) revealed that the mean total neuritic outgrowth (about 550 μm in 18 hr) was similar to neuritic outgrowth seen up to a ratio of 60% fibroblasts:40% astrocytes. Increasing the percentage of fibroblasts over 60% resulted in a drop in mean total neuritic length to about 150 μm in 18 hr on 100% fibroblasts. The same profile was observed for the longest hippocampal neurites (data not shown).

Laminin staining of explants

In the course of defining cell types and structures on the surface of the explants, labeling with an antibody to laminin revealed distinct differences in the staining pattern. In the case of the 1–6-d (Fig. 6*A*) and 1–30-d (Fig. 6*B*) explants, the majority of laminin staining was found to be around blood vessels ramifying in a polygonal pattern across the surface of the explants. Laminin

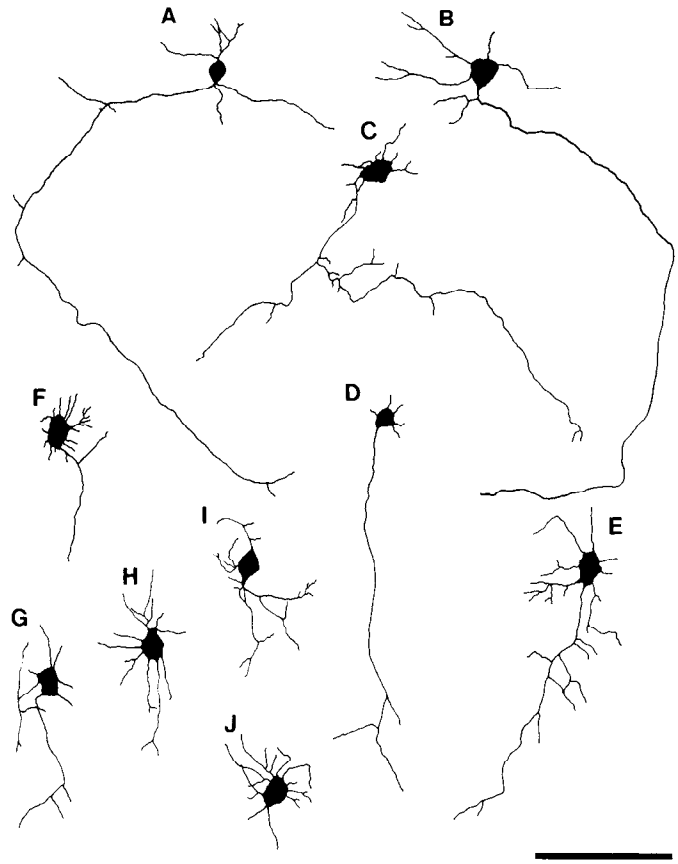

Figure 2. Camera lucida drawings of representative dil-labeled E18 rat hippocampal neurons that have extended neurites on surface of 1–6-d (*A–E*) or scar explants (*F–J*). Note the more extensive neurite outgrowth on the 1–6-d explants compared to the scar explants. Scale bar, 100 μm.

staining of the scar explants showed a very different profile, with sheets of basal lamina extending out across the surface of the explant (Fig. 6*C*), as well as being present around blood vessels.

Ultrastructure of the scar astroglial

To determine which cells were associated with the basal lamina, as well as to assess what cell types or structures were in contact with the growth cone on the surface of the glial scar, we cut tangential sections of the cells at the surface of scar explants and processed them for electron microscopy. Figure 7*A* shows the surface of the scar explant inhabited by reactive astrocytes packed with glial filaments and lipid inclusions. In the extracellular spaces of some explants, sworls of basal lamina (Fig. 7*B*) and type 1 and 2 collagen were found. Mostly, the basal lamina was present as a single layer coating the surface of the astrocyte, as shown in Figure 7*C*.

Basal lamina expression on the surface of astrocytes of the 1–6-d and 1–30-d explants was confined to the interface between astrocytes and endothelial cells and the small numbers of fibroblasts (see Table 1) that migrated onto the surface of the filter on day 1. The basal lamina seen on the scar explants was not present on these explants (data not shown). When examined *in situ* as well as after 3 d *in vitro*, myelin debris and oligodendrocytes were at very low levels on all 3 types of explant, reflecting that the wound occurred in the gray matter of the cerebral cortex.

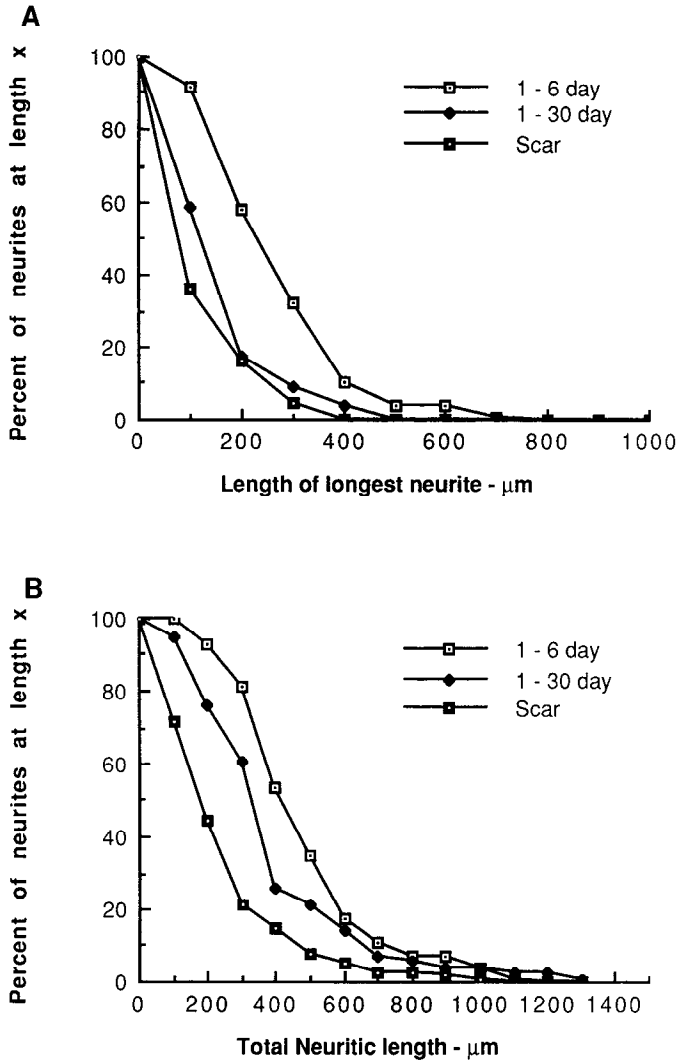

Figure 3. Length of E18 rat hippocampal neurons on 1–6-d, 1–30-d, and scar explants. E18 rat hippocampal neurons were prelabeled with dil and cultured for 48 hr on the surface of the explants in serum-free medium. Tracings of the extent of labeled neurite outgrowth were measured as described in Materials and Methods, and the percentage of neurites at or greater than a specific length (*vertical axis*) are expressed as a function of neurite length in microns (*horizontal axis*). *A*, Distribution of lengths of the longest neurite from matched populations of hippocampal neurons on the surface of 1–6-d, 1–30-d, and scar explants ($n = 60$ for each explant). A Mann–Whitney U test revealed significant differences between the distribution of neurite outgrowth: 1–6-d/1–30-d, $p < 0.001$; 1–6-d/scar, $p < 0.001$; 1–30-d/scar, $p < 0.05$. *B*, Distribution of total neuritic length from matched populations of hippocampal neurons on the surface of 1–6-d, 1–30-d, and scar explants ($n = 60$). Significant differences were also observed between the distribution of total neurite outgrowth: 1–6-d/1–30-d, $p < 0.01$; 1–6-d/scar, $p < 0.001$; 1–30-d/scar, $p < 0.001$.

Discussion

In this study, we show that explants containing cells responding to a wound of immature cortex have the capacity to support substantial outgrowth of neurites from rat hippocampal neurons *in vitro*. When matched populations of neurons are plated *in vitro* onto explants possessing the cellular and extracellular components of an adult gray-matter scar, very little neurite outgrowth is seen. This inhibition is not due to humoral neurotoxic factors nor to fibroblasts inhibiting astrocyte-mediated

Table 2. Survival of E18 rat hippocampal neurons on 1-6-d, 1-30-d, and scar explants

Explant	Cell bodies/mm²
1–6-day (6 d *in vivo*)	18.45 ± 4.37
1–30-d (30 d *in vivo*)	19.33 ± 5.44
Scar (10 d *in vivo*)	22.43 ± 7.95

E18 rat hippocampal neurons prelabeled with dil were seeded onto the surface of the 3 types of explant which had been *in vitro* for 3 d, at 25 neurons/mm². After 48 hr, cocultures were fixed, and the number of surviving neuronal cell bodies per mm² were counted ($n = 30$ for each type of explant). Between 73 and 89% of the hippocampal neurons survived on the explants after 48 hr in culture. No significant differences in neuronal survival on any of the explants was detected using the Student's t test. Values are expressed as mean ± SD.

outgrowth, suggesting that the surface of the scar environment in the absence of oligodendrocytes is inhibitory or nonpermissive to neurite outgrowth.

A recent study using a culture model devoid of oligodendrocytes or myelin debris has implied that the 3-dimensional aspect of an aged astroglial environment packed into a tube may contribute an important factor to the inhibition of axon outgrowth in the adult brain (Fawcett et al., 1989). This may well account for a proportion of the axons that are unable to regenerate *in vivo,* but there are many different kinds of traumatic injury to the adult CNS, and in those instances where minimal penetration occurs, certain types of axons are capable of regenerating long distances through reactive (intensely GFAP-positive) astrocytes in the gray matter. Examples of this regenerative phenomenon occur after nondisruptive chemical lesions that axotomize the monoaminergic and cholinergic neurons of the brain stem, resulting in axon regeneration over several centimeters (Bjorklund et al., 1973; Nygren and Olson, 1977). Interestingly, after mechanical or electrolytic lesion to the same area, some of these axons exhibit terminal sprouting, but most fail to cross the lesion site (Katzman et al., 1971; Bjorklund et al., 1973). If the site of mechanical injury is bypassed, for example, by a bridge between the septum and the hippocampus after fimbria–fornix lesion (Kromer and Cornbrooks, 1985; Davis et al., 1987), then septal axons will grow into and reinnervate the intensely GFAP-positive gray matter of the hippocampus (Gage et al., 1988). This is also the case for fetal serotoninergic neurons that survive and differentiate after transplantation into the deafferented spinal cord (Nornes et al., 1983; Privat et al., 1988). This suggests that the cues for axon outgrowth, at least for these neuronal subtypes, are still present within regions of the adult CNS that are gliotic but unscarred.

In our *in vitro* model, astrocytes that comprise 1–30-d explants are intensely GFAP positive and hypertrophied in much the same way that "gliotic" astrocytes are *in vivo.* Why the high level of GFAP expression is maintained in astrocytes associated with the polymer is unknown, but may be due to the mere presence of a solid filter in the brain, a continuing immune response to the nitrocellulose, or the lack of a suppression signal from axons that are not present inside the filter. Whatever the cause, such intensely GFAP-positive cells appear to support neurite outgrowth over their surface about 54–56% as well as astrocytes on the 1–6-d explants. This supports the findings of Smith et al. (1990), who have shown that astrocytes matured *in vitro* show a reduced capacity to support neurite outgrowth when compared to immature astrocytes *in vitro,* an effect apparently mediated by changing amounts of laminin, NCAM,

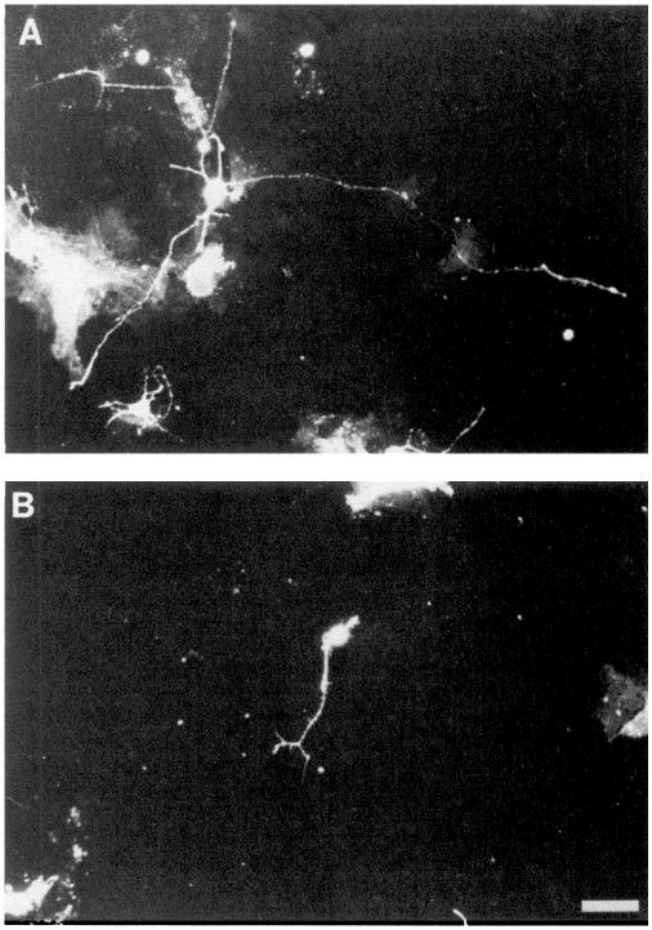

Figure 4. Representative neurite outgrowth from rat hippocampal neurons on 40% astrocytes:60% fibroblasts (*A*) or 100% fibroblasts (*B*). Rat hippocampal neurons were prelabeled with dil and cocultured for 18 hr on the astrocyte–fibroblast monolayer as described in Materials and Methods. Background fluorescence is due to particles of dil staining the monolayer. Note the reduction in neurite outgrowth between a culture containing 40% astrocytes (*A*) and one containing no astrocytes (*B*). Scale bar, 20 μm.

and perhaps *N*-cadherin (Tomaselli et al., 1988) on the aging astrocyte surface (Smith et al., 1990). Because both the 1–6-d and 1–30-d explants exhibit intense GFAP staining, it appears that this "reactive" phenotype, per se, is not a predictor of neurite outgrowth-promoting ability by astrocytes *in vitro* and probably not *in vivo.*

If mature astrocytes, unhindered by other components, can support neurite outgrowth, what factors in the scar may be contributing to the further reduction in neurite outgrowth seen between the 1–30-d explants and the glial-scar explants?

It has been shown that astrocytes sustain substantially greater neurite outgrowth when compared to fibroblasts *in vitro* (Noble et al., 1984; Fallon, 1985). To study the effect of fibroblasts on neurite outgrowth over astrocytes, we mixed astrocytes and fibroblasts together *in vitro* (30 d), resulting in a monolayer where the cells express all the components of the basal lamina such as fibronectin, laminin, and type IV collagen, though not assembled as basal lamina (data not shown; Jimenez et al., 1984; Eldridge et al., 1987). This addresses the question of whether small numbers of fibroblasts can switch off the neurite-promoting capability of the astrocyte *in vitro* with humoral effectors, or, conversely, whether astrocytes can override the inhibitory

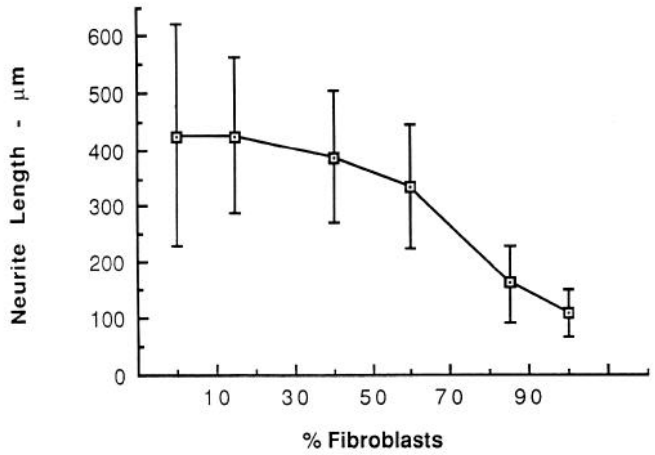

Figure 5. Mean lengths ± SD of all neurites generated by rat hippocampal neurons on mixed astrocyte–fibroblast monolayer after 18 hr in coculture. Outgrowth of neurites is virtually the same until the percentage of fibroblasts in the monolayer exceeds 60%. Neurite outgrowth is then reduced, reaching its lowest level on 100% fibroblasts.

properties of fibroblasts. It appears that, *in vitro,* fibroblasts have to be in a greater than 60:40 ratio to astrocytes before their inhibitory properties take over, which may be reflective of constraints on the ability of the growth cone to find the permissive surface of the astrocyte (data not shown). This is much the same result as seen for oligodendrocytes and astrocytes where, when given a choice, neurites will choose a permissive substrate over a nonpermissive one (Fawcett et al., 1987; Ard et al., 1988). Incubation of astrocytes and fibroblasts together for as long as 10 d *in vitro* in serum-free medium did not alter the capacity of the fibroblast to inhibit astrocyte-mediated outgrowth (data not shown).

Although oligodendrocytes and myelin debris appear to play a critical role in the inhibition of axon regeneration after traumatic injury involving white matter (Schnell and Schwab, 1990), they cannot account for the inhibition seen in gray matter where they are absent. One candidate for this inhibition present at the surface of gray-matter scars could be the basal lamina, which was abundant in scar explants, but not in the other 2 types of explant, and has been well characterized by Schultz and Pease (1959) in their studies on cerebral cicatrization. The possibility that basal lamina on the surface of scar may have inhibitory properties has been proposed for a number of years (Brown and McCouch, 1947; Clemente, 1955; Kao et al., 1977; Feringa et al., 1980; Bernstein et al., 1985), though it has been the axon-blocking mechanical properties of this membrane that have been stressed. In the present model, large amounts of aberrant basal lamina were deposited around the reactive astrocytes, whereas the majority of basal lamina on 1–6- and 1–30-d escplants was around blood vessels. This configuration of basal lamina could easily prevent growth cones from making contact with resident astrocytes in the scar. The pattern of multiple sheets or swirls of basal lamina seen in some of the escplants has also been shown *in vivo* forming near the surface of astrocyte membranes facing endothelia or pia after hemisection of the spinal cord (Bernstein et al., 1985; Reier and Houle, 1988).

The mechanism by which the basal lamina may be acting *in vitro* to inhibit axon outgrowth is an enigma, because basal lamina contains laminin, a potent neurite-promoting molecule (Manthorpe et al., 1983). It may be that the haphazard deposition of laminin in the scar basal lamina is not conducive to directed axon outgrowth, or that laminin is present in a less active form such as s-laminin (Hunter et al., 1989). Another possibility may be the polarity of presentation of the basal lamina to the growing neurite, which appears to be critical, as shown in the studies of Davis et al. (1987). They found extensive growth

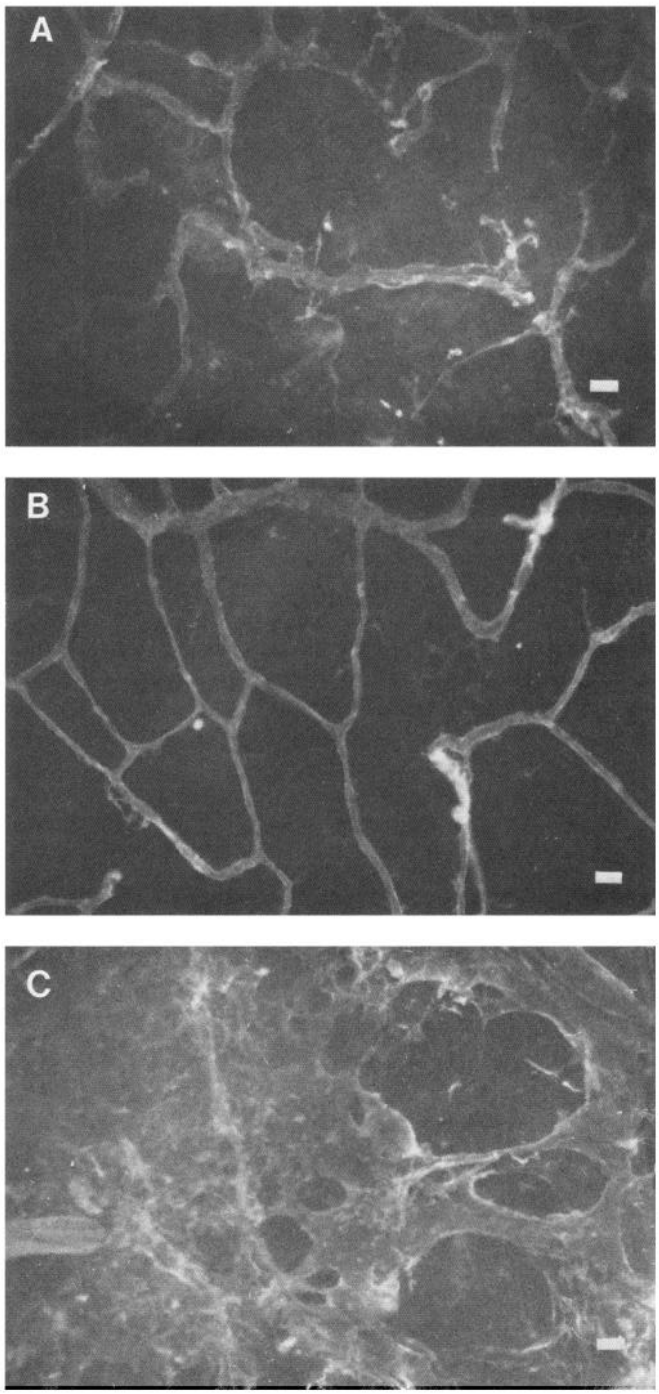

Figure 6. The surface of 1–6-d (*A*), 1–30-d (*B*), and scar explants (*C*) after labeling with antibody to laminin followed by secondary antibody linked to biotin and streptavidin fluorescein. The labeling in *A* and *B* is mainly confined to the basal lamina surrounding blood vessels, whereas the labeling on scar explants is present mostly as sheets of basal lamina across the surface. Scale bar, 20 µm.

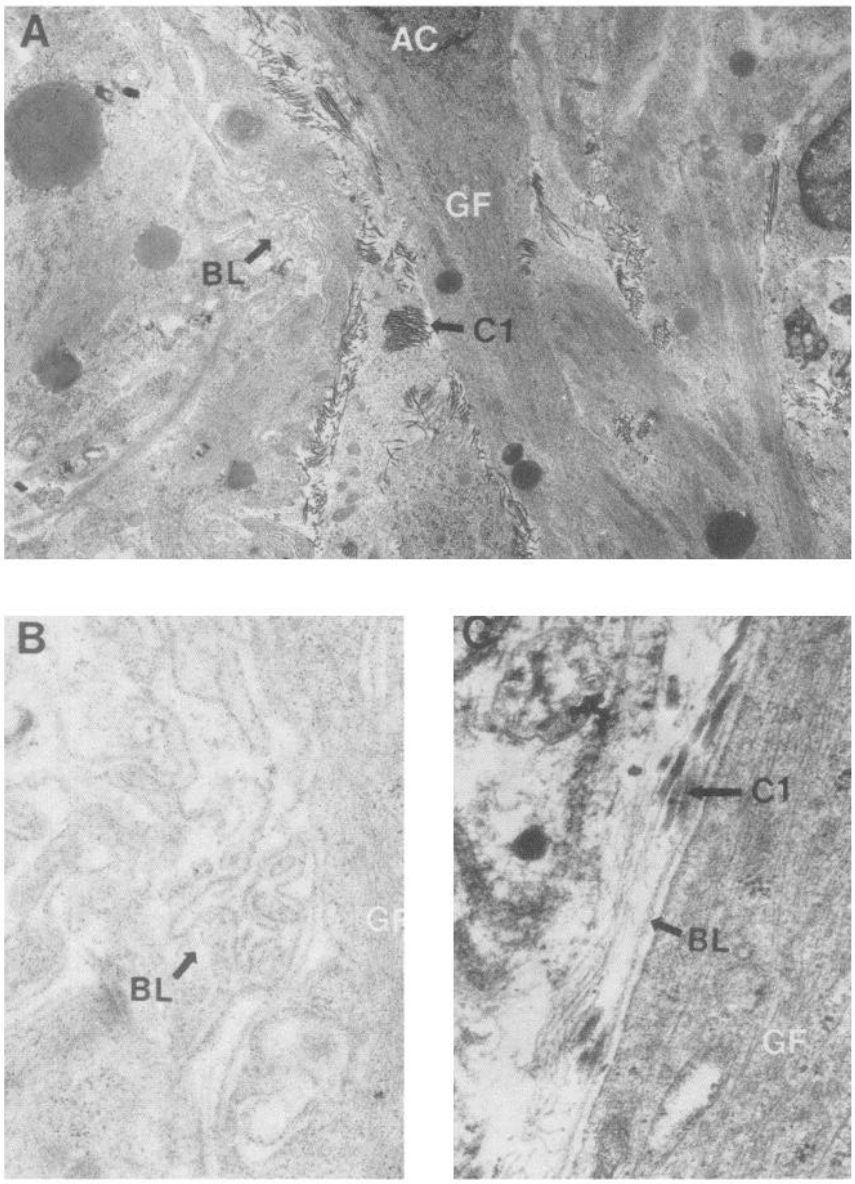

Figure 7. Transmission electron micrographs from tangential sections of surface of scar explants. The surface of the scar (*A*, 3700×) consists of astrocytes (*AC*) and their processes packed with glial filaments (*GF*) and lipid inclusions. The intercellular spaces are filled with type 1 collagen (*C1*) and sworls of basal lamina (*BL*). These sworls of basal lamina are shown at higher magnification in *B* (18,000×). Other areas express only a single layer of basal lamina coating the surface of the astrocyte (*C*, 18,000×).

over human-amnion basement membrane when the laminin-rich basement-membrane side was directed toward the growth cone, but when the membrane was reversed and the stromal side presented, no outgrowth occurred, indicating that the neurite-promoting capacity of laminin had been obscured by extracellular matrix components on the stromal side.

Apart from physically occluding axon-outgrowth-promoting factors, the possibility also exists that some molecules in the basal or reticular lamina of scar tissue may be innately inhibitory. This may account for the lack of axon outgrowth around scar tissue *in vivo* simply by utilizing growth-promoting factors present in undamaged tissue. Possible candidates for these molecules are sulfated glycosaminoglycans, which are abundant in basal lamina and appear to be inhibitory to neurite outgrowth *in vitro* (Carbonetto, 1984; Snow et al., 1990b) and *in vivo* (Snow et al., 1990a). Experiments are in progress using this model to determine whether inhibitory proteoglycans are present in the basal lamina or on astroglia in the immediate vicinity of the scar (McKeon et al., 1990).

In summary, it is the complex cellular interactions after trauma *in vivo* and their resultant chemical products that may play a critical role in the inhibition of axon outgrowth, even without the constraints of the third dimension. Therefore, promotion of axonal regeneration *in vivo* through the glial scar may require multiple strategies that not only replace the growth factors and surface molecules that stimulate axon elongation, but also remove chemical inhibitors that may be present in the scar environment. We are now in a position to examine the molecules that underlie these mechanisms, under controlled conditions *in vitro*, by selective removal of presumed inhibitory components

of the glial scar in the hope that beneficial results can be translated *in vivo*.

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