Thrombospondin and a 140 kd Fragment Promote Adhesion and Neurite Outgrowth from Embryonic Central and Peripheral Neurons and from PC12 Cells

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Summary

The ability of thrombospondin (TSP), an extracellular matrix glycoprotein, and two proteolytic fragments to support adhesion and neurite outgrowth from embryonic dorsal root ganglia, spinal cord neurons, and PC12 cells was examined. Anti-TSP antibodies or a synthetic peptide (GRGDS) containing an RGD cell-binding region was also added to cells plated on TSP. TSP and its 140 kd fragment were more efficient than laminin controls in supporting adhesion. Neurites formed on laminin, on varying concentrations of TSP, and particularly the 140 kd fragment. The amino-terminal heparin-binding domain supported little adhesion and outgrowth. Both adhesion and process outgrowth on TSP were inhibited by addition of anti-TSP antibodies, but not GRGDS.

Introduction

During the histogenesis of the nervous system, cell–cell and cell–extracellular matrix interactions appear to regulate many aspects of neuronal differentiation (see Sanes, 1989, for review). In addition to the complex surfaces of cells, such as glia (Noble et al., 1984; Fallon, 1985) and muscle (Bixby et al., 1987), proteins present in the extracellular matrix, particularly laminin (LN) and fibronectin (Baron-van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983, 1985; Hunter et al., 1989), as well as adhesion molecules such as N-cadherin (Bixby and Zhang, 1990) appear to play important roles in attachment and process outgrowth from a variety of neuronal cell types.

Thrombospondin (TSP) is a multifunctional glycoprotein constituent of extracellular matrices (Dixit et al., 1984; Lawler et al., 1985) that has been implicated in cell attachment (Varani et al., 1986; Roberts et al., 1987), migration, and proliferation (Majack et al., 1986), as well as haptotaxis and chemotaxis (Taraboletti et al., 1987). TSP has a number of ligands, including other components of the extracellular matrix (Lahav et al., 1982, 1984; Dixit et al., 1984; Mumby et al., 1984; Lawler et al., 1985). TSP also binds the serine protease inhibitor PN I (Browne et al., 1988), plasminogen, and both tissue-type and urokinase plasminogen activators (Silverstein et al., 1984). Binding of TSP to the latter is unique in that plasmin production is increased more than 40-fold, with up to two-thirds of the generated plasmin bound to TSP (Silverstein et al., 1984, 1985, 1986). Thus, TSP may play a unique role in development by creating a local focus of controlled protease activity required for process outgrowth through a cell- and matrix-filled field, thereby providing a substrate for the repeated adhesion and de-adhesion events required for the early development of the nervous system.

TSP is densely deposited in the developing nervous system in regions of cell migrations, in association with developing fiber tracts, and in areas of axon outgrowth (O'Shea and Dixit, 1988). Because of its physico-chemical properties as well as its spatiotemporal pattern of deposition, we have examined the ability of neurons isolated from mouse embryonic day 13 spinal cord or dorsal root ganglion (DRG) explants and from the rat pheochromocytoma cell line (PC12; Greene and Tischler, 1976) to adhere to and extend neurites on varying concentrations of TSP. Two fragments—a 25 kd fragment containing the amino-terminal heparin-binding domain (HBD) and a 140 kd fragment containing the carboxy-terminal and putative cell-binding domains—were also tested. Consistent with its dense deposition in the embryo surrounding neurons and in association with process outgrowth, TSP and particularly the 140 kd fragment supported attachment and neurite outgrowth. Addition of anti-TSP antibodies to cells grown on TSP inhibited both attachment and process extension.

Results

Adhesion

When PC12 and spinal cord–derived cells were plated on coverslips coated with TSP or its 140 kd fragment, the number of adherent neurons after 2 hr in vitro was considerably greater than that on LN. For spinal cord cells there was a clear dose–response to TSP, with 10 μg/ml TSP supporting maximal attachment. Few cells attached to coverslips coated with bovine serum albumin, or the HBD. Addition of anti-TSP antibodies but not GRGDS, to neurons grown on TSP-coated coverslips inhibited adhesion (Table 1).

Neurite Outgrowth

The pattern of neurite outgrowth from neurons isolated from spinal cord or from PC12 cells was similar (Table 2; Figure 1; Figure 2). Outgrowth was extensive on LN, TSP, and the 140 kd fragment. Few neurites formed on the HBD, on bovine serum albumin, or when anti-TSP antibodies were added after 24 hr to neurons grown on TSP-coated dishes. These effects are specific for TSP, as addition of anti-TSP antibodies to cells growing on LN-coated substrates had no effect on their viability or morphology. There was no effect of adding GRGDS to the culture medium of neurons growing on TSP. Unlike primary cultures of embryonic spinal cord neurons or DRG explants, addition of anti-TSP antibodies to PC12 cells "primed" with nerve growth factor (NGF) was slightly less efficient in inhibit-

Table 1. Percentage of Neurons Attached to Various Substrates

Neuron Source	Substrate											
	LN	TSP 0.1	TSP 0.5	TSP 1	TSP 5	TSP 10	TSP 25	140 kd	HBD	TSP + αTSP	TSP + GRGDS	BSA
SC	45 ± 8	26 ± 12	30 ± 7	54 ± 8	64 ± 9	76 ± 14	63 ± 9	58 ± 17	15 ± 10	13 ± 3	61 ± 19	12 ± 5
PC12	54 ± 4	–	–	–	–	69 ± 10	–	55 ± 5	–	13 ± 12	–	11 ± 2
PC12 + NGF	54 ± 11	–	–	–	–	79 ± 14	–	59 ± 11	–	15 ± 10	–	9 ± 4

LN: 25 μg/ml; TSP: 0.1, 0.5, 1, 5, 10, 25 μg/ml; 140 kd fragment: 25 μg/ml; HBD fragment: 25 μg/ml; TSP + αTSP: 10 μg/ml TSP + 25 μg/ml αTSP; TSP + GRGDS: 10 μg/ml TSP + 500 μg/ml GRGDS; BSA: 25 μg/ml.

ing neurite outgrowth from these cells, suggesting that they may synthesize additional attachment factors.

Neurite outgrowth from DRG rudiments was extensive on LN, TSP and the 140 kd fragment (Figure 3). Outgrowth on LN produced long, radial, unfasciculated processes (Figure 3A). Outgrowth on the 140 kd fragment and on TSP-coated substrates resulted in long, unbranched, highly fasciculated neurites (Figures 3B and 3D). Culture of DRG explants on the 140 kd fragment resulted in neurite outgrowth, but also in migration of cells from the explants. Migration of cell bodies on LN or TSP was not observed to precede neurite outgrowth. Process outgrowth from the explants was neuronal, as verified by staining with anti-α-melanocyte-stimulating hormone (α-MSH) antibodies.

The appearances of both processes and growth cones varied with substrate; LN consistently produced long, more often branched processes with flattened growth cones (Figures 1A and 1B; Figure 2C; Figure 3A). TSP and particularly the 140 kd fragment typically produced very long, often beaded processes (Figures 1C–1F; Figures 2D and 2F; Figures 3B and 3D–3F) that often had long microspikes (Figure 3F) and typically were less highly branched (Figures 3B and 3D–3F). As has previously been reported for LN (Wujek and Akeson, 1987), culture of PC12 cells not previously primed by exposure to NGF resulted in the formation of processes on both LN (Figure 2A) and TSP (Figure 2B). Process outgrowth was not as robust as that seen following NGF treatment; in the 12 hr

period examined, processes rarely extended farther than twice the length of the cell body.

Discussion

These results demonstrate a role for TSP in adhesion and process outgrowth from a variety of neurons. Unlike other identified matrix components that support neurite outgrowth, the distribution of TSP is unique. Like LN (Rogers et al., 1986) and HNK-1/NC-1 (Tucker et al., 1988), TSP is densely deposited in the ventral horn of the spinal cord in the region from which axons of motor neurons exit the cord, and surrounding nerve rootlets emerging from forming DRGs. Like fibronectin (Duband et al., 1986), TSP is also present in the mesenchymal matrix, through which cells and processes pass. Tenascin has recently been demonstrated in association with forming ventral roots within the sclerotome (Wehrle and Chiquet, 1990), but, unlike TSP, is not associated with initial axon outgrowth from motor neurons. This pattern suggests that TSP may play multiple roles in the early development of the region.

TSP appears to be unique among matrix components in its ability to provide an adhesive substrate for cells and their processes, as well as a nidus of polarized protease activity for substrate release. Its deposition in association with the leading edge of migrating granule cells in the developing cerebellum (O'Shea et al., 1990), as well as in attachment sites and

Table 2. Percentage of Neurons with Neurites

Neuron Source	Substrate											
	LN	TSP 0.1	TSP 0.5	TSP 1	TSP 5	TSP 10	TSP 25	140 kd	HBD	TSP + αTSP	TSP + GRGDS	BSA
SC	69 ± 6	24 ± 7	35 ± 4	48 ± 6	54 ± 8	61 ± 4	53 ± 3	66 ± 5	18 ± 9	14 ± 5	54 ± 9	14 ± 5
PC12	51 ± 7	–	–	–	–	39 ± 6	–	40 ± 13	–	15 ± 7	–	6 ± 4
PC12 + NGF	64 ± 12	–	–	–	–	58 ± 11	–	60 ± 4	–	24 ± 3	–	8 ± 4
DRG explant	136 ± 11	–	–	–	–	121 ± 4	–	112 ± 6	31 ± 5	29 ± 8	–	–

LN: 25 μg/ml; TSP: 0.1, 0.5, 1, 5, 10, 25 μg/ml; 140 kd fragment: 25 μg/ml; HBD fragment: 25 μg/ml; TSP + αTSP: 10 μg/ml TSP + 25 μg/ml αTSP; TSP + GRGDS: 10 μg/ml TSP + 500 μg/ml GRGDS; BSA: 25 μg/ml.
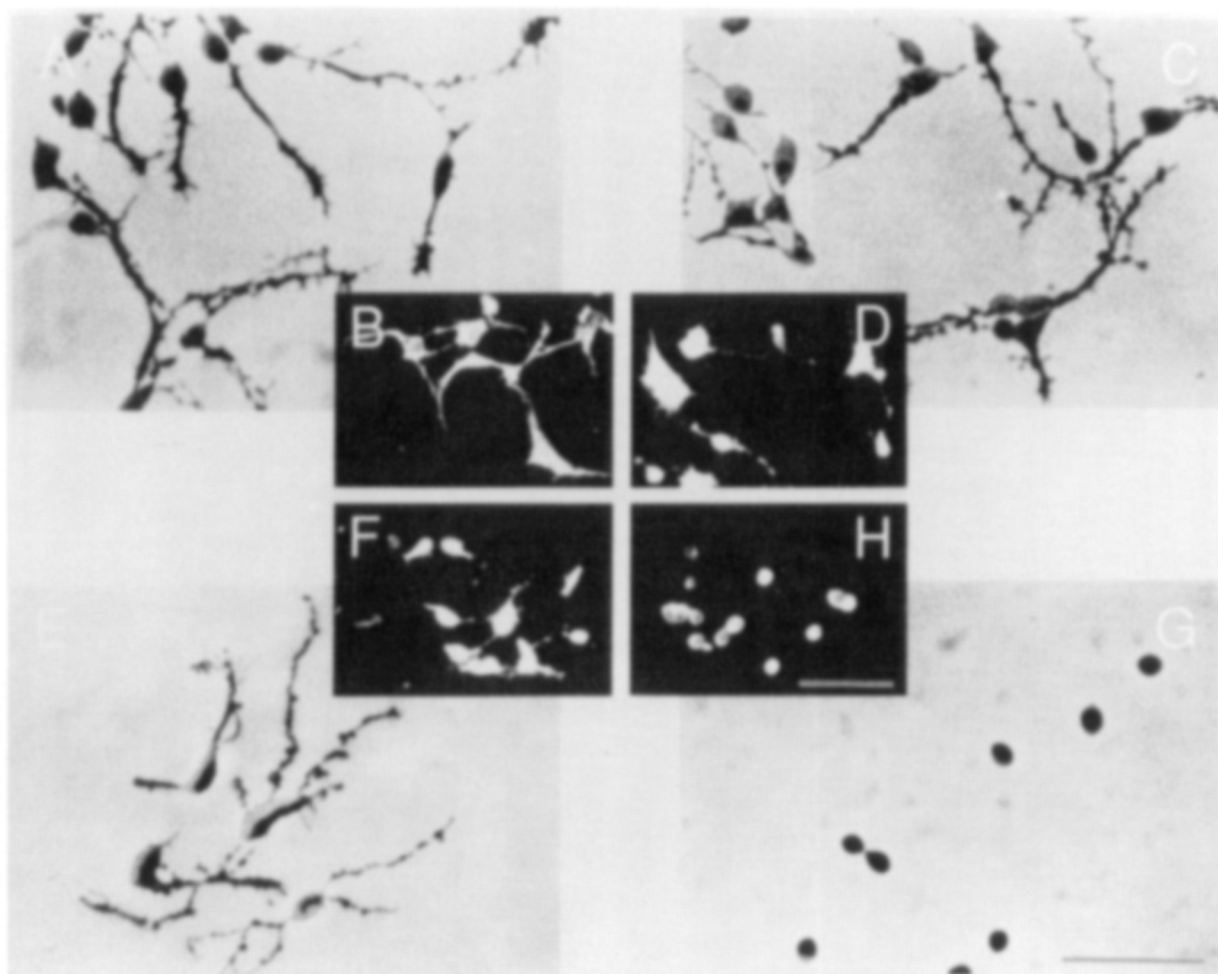For DRG explants, values reflect mean neurite length (μm ± SEM)

Figure 1. Neurite Outgrowth from Spinal Cord Neurons on Various Substrates after 12 hr In Vitro

(A, C, E, and G) Phase-contrast micrographs showing neurite outgrowth from spinal cord neurons on various substrates. (B, D, F, and H) Corresponding immunolocalization of α-MSH in neurons. LN (A and B), TSP (C and D), and the 140 kd fragment (E and F) supported outgrowth. Addition of anti-TSP antibodies inhibited neurite outgrowth (G and H). Bars, 50 μm.

"tracks" formed by migrating cells (Vischer et al., 1988), further supports the concept that TSP may play a role in the sequential adhesion and de-adhesions involved in both cell migrations and neurite outgrowth.

Each subunit of the TSP trimer is composed of a linear region connecting two unequal globular domains—the smaller globular domain is the heparin-binding amino terminus and the larger domain is the carboxyl terminus (Lawler and Hynes, 1986). TSP is rapidly incorporated into (and removed from) the extracellular matrix via its HBD (McKeown-Longo et al., 1984). This domain mediates the incorporation of TSP into the extracellular matrix (Roberts et al., 1987; Prochownik et al., 1989) and is extremely sensitive to proteolytic cleavage from the rest of the molecule, thereby generating the 140 kd fragment (Roberts et al., 1987; Prochownik et al., 1989). The increased efficiency of the 140 kd fragment compared with the intact molecule in supporting neurite outgrowth may

relate to this rapid cleavage in situ. Presence of the HBD may hinder accessibility to the cell-binding site, as addition of the HBD to cells growing on the 140 kd fragment does not alter their ability to adhere or extend neurites.

The 140 kd fragment has previously been shown to mediate attachment of melanoma cells (Taraboletti et al., 1987) and keratinocytes (Varani et al., 1986). Even though the 140 kd fragment contains an RGD cell recognition sequence (Lawler and Hynes, 1986), this particular sequence is not involved in the attachment of these neuronal cells, as a vast molar excess of the synthetic peptide GRGDS fails to inhibit attachment, as in other cell systems (Roberts et al., 1987; Varani et al., 1988; Neugebauer et al., 1991). However, for certain cell types such as endothelial cells, attachment to TSP appears to be dependent upon the RGD sequence (Lawler et al., 1988). In the current investigation, the GRGDS peptide had no effect on attachment to TSP
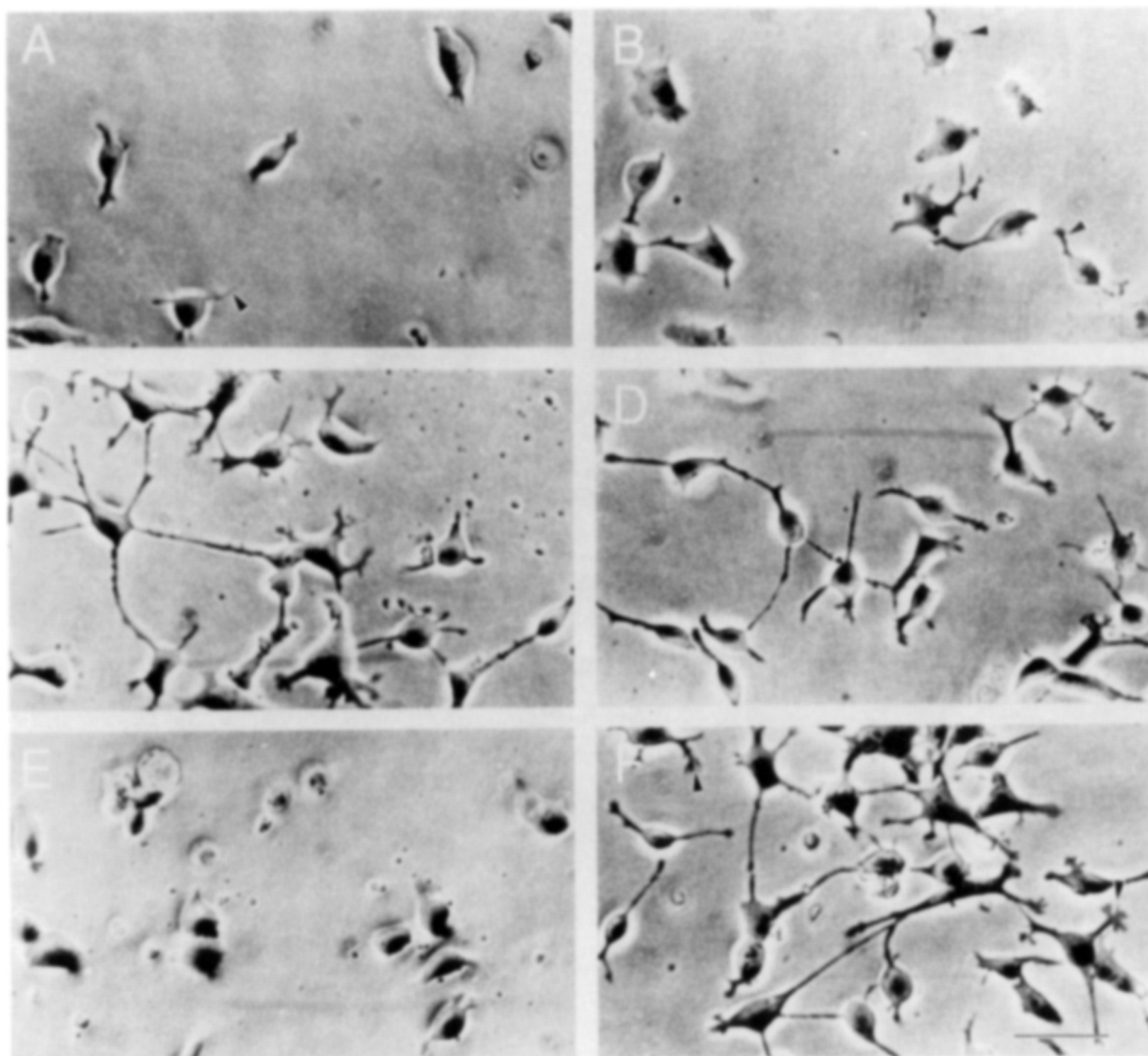
Figure 2. Neurite Outgrowth after 12 hr In Vitro from Unstimulated PC12 Cells and from PC12 Cells Previously Exposed to NGF

(A) Unstimulated PC12 cells grown on LN substrates remained largely undifferentiated, although small neurites (arrowhead) were occasionally observed.

(B) Unstimulated PC12 cells grown on TSP-coated substrates formed short processes (arrowheads) after 12 hr.

(C) On LN, NGF-treated PC12 cells formed long, sometimes branched processes, with broad growth cones.

(D) On TSP, NGF-primed PC12 cells formed an extensive outgrowth. Neurites were typically not branched.

(E) When grown on TSP substrates in medium containing anti-TSP antibodies, NGF-primed PC12 cells showed little process outgrowth; occasionally very short processes were observed.

(F) On the 140 kd fragment, neurite outgrowth from NGF-treated PC12 cells was very extensive. Very broad growth cones (arrowheads) formed on this substrate.

Bar, 50 μm.

or to the 140 kd fragment, strongly supporting the existance of an additional cell-binding domain on the 140 kd fragment. Very recently, Neugebauer et al. (1991) have demonstrated that neurite outgrowth, but not attachment, was inhibited by integrins containing α_v or β_1 subunits. These authors suggest that the β_1 integrin may recognize a site on a 70 kd fragment containing the central stalk region of the TSP molecule which has recently been shown to support at-

tachment, but limited spreading, of melanoma cells (Prater et al., 1991).

In general, RGD peptides and anti-receptor antibodies have been shown to be more efficient in blocking neurite extension on purified matrix substrates than on various cell surfaces (Tomaselli et al., 1986; Letourneau et al., 1988), indicating the likely presence on cell surfaces of binding sites for additional (unidentified?) extracellular matrix molecules
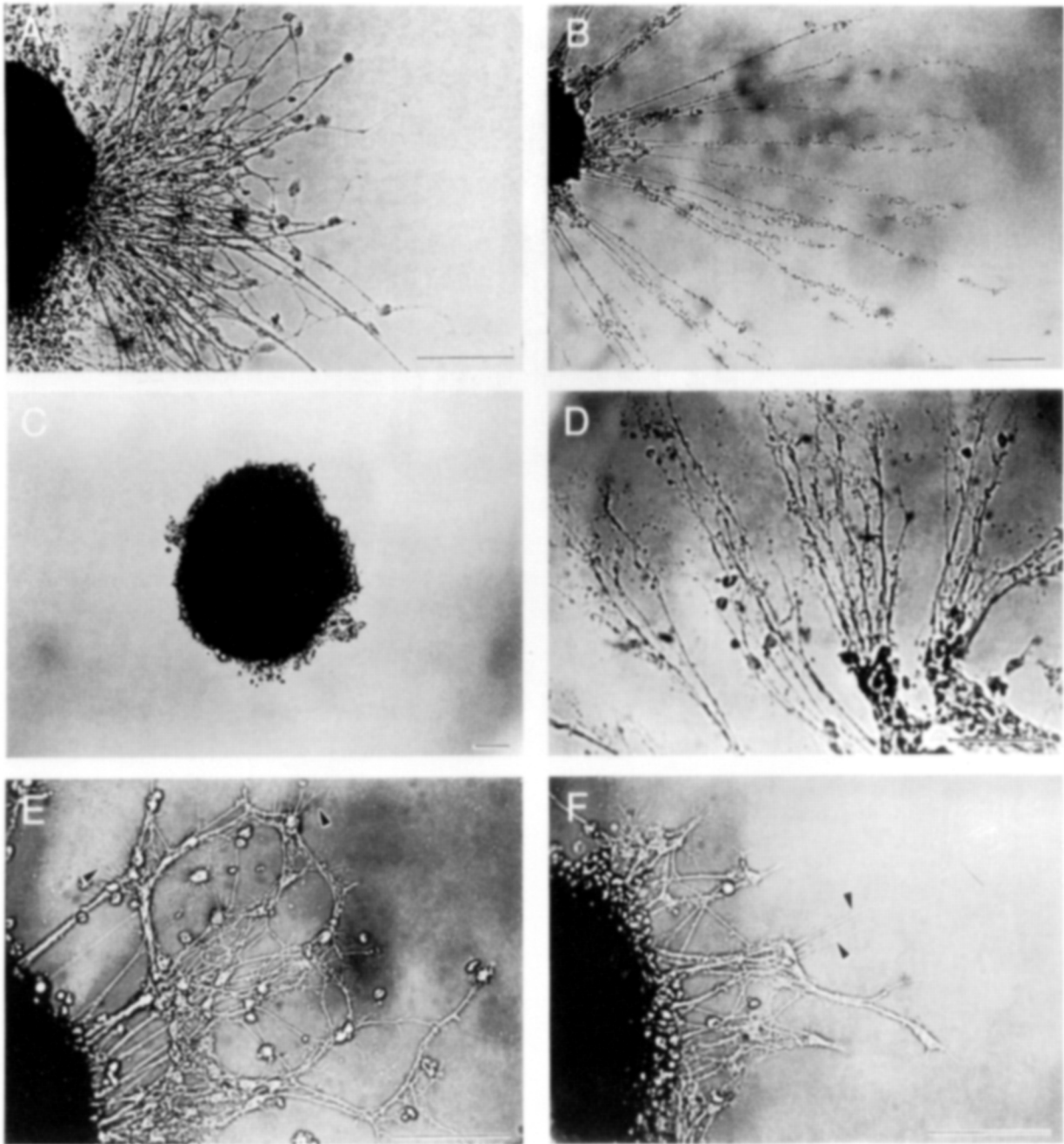
Figure 3. Neurite Outgrowth from DRG Explants after 18 hr In Vitro

Culture on LN (A) consistently resulted in extensive radial outgrowth of long, broad, often branched processes. On TSP (B) neurites were often very long, thin, and occasionally beaded in appearance. Addition of anti-TSP antibodies to explants inhibited outgrowth (C). Like TSP, culture on the 140 kd fragment also produced fasciculated neurites (D). At higher magnification, neurites growing on TSP (E and F) often exhibited processes with many long microspikes (arrowheads). Bars, 50 μm.

that may mediate different aspects of neuronal attachment and outgrowth involved in the intricate regulation of nervous system development.

Thus, TSP must be added to the growing list of extracellular matrix proteins that play important roles in the histogenesis of the nervous system. Its precise role in producing the orderly patterns of neuronal proliferation, migrations, and target interactions remains to be elucidated.

Experimental Procedures

Tissue

CD-1 strain mice (Charles River labs, Portage, MI) were mated; the day on which a vaginal plug was found was considered the first

day of pregnancy. On day 13, females were sacrificed, and embryos were removed from the uterus and decidua and placed in PBS. The spinal cord (cervical/thoracic region) and DRG rudiments were dissected from surrounding tissue.

Cell and Explant Cultures

Spinal cord tissue was gently triturated in N2 medium (Bottenstein and Sato, 1979) using glass pipettes. Cultures were enriched for neurons by preplating on uncoated plastic dishes (Falcon 3001, Oxnard, CA) for 30 min. Unattached cells were removed, and a 1 ml volume of a cell suspension (5×10^5 cells per ml) was added to 24-well plates (Costar, Cambridge, MA) previously coated with various substrates (see below). These cultures contained 85%–90% neurons, as determined using anti-α-MSH antibodies (below).

DRG rudiments were cleaned of connective tissue and grown (five to six explants per well) in matrix-coated 24-well plates in N2 medium containing NGF (10 ng/ml; Sigma Chemical Company, St. Louis, MO) for 18 hr. NGF was added to enhance neuronal survival; control experiments in which N2 medium was not supplemented with NGF indicated that NGF was not responsible for the neurite outgrowth observed on TSP. Cells and explants were maintained in an incubator at 37°C, 5% CO_2.

To exclude possible effects of glial cells on neurite outgrowth, PC12 cells were tested for their ability to attach to and extend neurites on TSP-coated substrates. The PC12 cell line was obtained from the American Type Culture Collection and maintained in DMEM containing 10% horse serum and 5% fetal calf serum. For studies of neurite outgrowth, medium was supplemented with 50 ng/ml NGF for at least 5 days (Greene, 1977). Cells were mechanically removed from tissue culture flasks, pelleted, gently triturated, and passed through 60 μm followed by 15 μm mesh (Tetko Inc., Elmsford, NY) to remove clumps of cells. Cell suspensions were washed three times in N2 medium, and 5×10^5 cells were added to 24-well plates coated as described below. Additional PC12 cells, not previously stimulated with NGF, were also grown on these substrates.

Substrates and Reagents

For adhesion assays, glass coverslips were washed in acid alcohol, rinsed in alcohol followed by distilled water, and heat dried. Coverslips were coated with nitrocellulose by dissolving a 25 mm disk (Schleicher and Schuell, Keene, NH) in 6 ml of absolute methanol and spreading 15 μl of the nitrocellulose solution on each coverslip (Lagenaur and Lemmon, 1987). Coverslips were incubated with 15 μl of each test protein for 1 hr, then rinsed in medium also containing 10% bovine serum albumin.

For neurite outgrowth experiments, 24-well plates were first coated with 0.1% polylysine for 20 min at room temperature, rinsed five times in distilled water, and coated with extracellular matrix protein for 60 min. Nonbound protein was removed, and nonspecific adhesion was blocked by rinsing in medium containing 10% bovine serum albumin.

Substrates were as follows: LN (1–25 μg/ml; Collaborative Research, Waltham, MA), TSP (0.1–25 μg/ml; Dixit et al., 1985), a 140 kd fragment containing the carboxy-terminal and cell-binding domains of TSP (25 μg/ml; Varani et al., 1988), an additional fragment containing the amino-terminal HBD of TSP (25 μg/ml; Varani et al., 1988), and bovine serum albumin (25 μg/ml). Anti-TSP antibodies (25 μg/ml; Varani et al., 1986; O'Shea and Dixit, 1988), or a synthetic peptide GRGDS (Ruoslahti and Pierschbacher, 1987) (500 μg/ml, Penninsula Labs, Belmont, CA), was added to cultures growing on 10 μg/ml TSP.

The anti-TSP antibodies employed in the current investigation have been characterized previously (Varani et al., 1986; O'Shea and Dixit, 1988); in immunoblotting of protein extracts from 13 mouse embryos, these antibodies detected a single protein that comigrated with purified human platelet TSP (O'Shea and Dixit, 1988). Previous studies in our laboratories using [125]-I-labeled TSP and its fragments indicated that the intact molecule and the 140 kd fragment bound in equimolar amounts to tissue culture plastic. The isolated 25 kd HBD bound in 20-fold molar excess, probably reflecting the hydrophobic character of this domain (Dixit et al., 1984).

Adhesion Assay

One milliliter of cell suspension (5×10^5 cells per ml) prepared from disaggregated spinal cord or PC12 cells in N2 medium was added to each well of 24-well plates containing a matrix-coated coverslip. Cells were allowed to attach for 2 hr at 37°C, 5% CO_2. Coverslips were removed and rinsed vigorously three times, and the Hoescht dye 33342 (bisbenzimide; 0.1 mg/ml; Sigma Chemical Co.) was added. Coverslips were mounted on glass slides, and the number of adherent cells was counted from three fields (at 25× using epifluorescence) from at least four preparations. Clumps of cells were excluded from analysis.

Neurite Extension

To examine neurite outgrowth, 1 ml of spinal cord or PC12 cell suspension (5×10^5 cells per ml) or five to six DRG rudiments were added to matrix-coated, 24-well plates. Anti-TSP antibodies or GRGDS was added after 2 hr to allow attachment of cells and explants. After 12 hr, spinal cord and PC12 cultures were examined for neurite outgrowth. Neurites were scored as present if the process was longer than the cell body. Neurite outgrowth was determined from three regions of each well and from at least four replicate cultures.

To quantify outgrowth from DRG explants, rudiments were photographed after 18 hr in vitro and measurements were taken from resulting photomicrographs using a digitizing tablet interfaced with a microcomputer. Distance from the edge of the explant to the five longest neurites in each explant was measured from at least ten explants per group.

Neurons were identified using indirect immunofluorescence localization of α-MSH (which binds neurofilament protein; Drager et al., 1983). Cultures were lightly fixed using 2% paraformaldehyde in 0.1 M phosphate buffer, rinsed in PBS, lightly permeabilized with 0.5% Triton X-100, rinsed in PBS, exposed to anti-α-MSH antibodies (1:100; Incstar Corp., Stillwater, MN) for 2 hr at room temperature, rinsed again, and exposed to goat anti-rabbit IgG–FITC (1:50) for 30 min at room temperature. Cultures were examined and photographed using a Leitz Fluovert inverted fluorescence microscope.

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